Isolation of the Ascobolus immersus Spore Color Gene b2 and Study in Single Cells of Gene Silencing by Methylation Induced Premeiotically

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ABSTRACT

The ascomycete Ascobolus immersus has been extensively used as a model system for the genetic study of meiotic recombination. More recently, an epigenetic process, known as methylation induced premeiotically (MIP), that acts on duplicated sequences has been discovered in A. immersus and has raised a new interest in this fungus. To try and extend these studies, we have now cloned the A. immersus spore color gene b2, a well characterized recombination hotspot. Isolation of the whole gene was verified by physical mapping of four large b2 alterations, followed by transformation and mutant rescue of a null b2 allele. Transformation was also used to duplicate b2 and subject it to MIP. As a result, we were able for the first time to observe gene silencing as early as just after meiosis and in single cells. Furthermore, we have found evidence for a modulating effect of MIP on b2 expression, depending on the region of the gene that is duplicated and hence subjected to MIP.

As is the case for many heterothallic ascomycetes, the life cycle of Ascobolus immersus is characterized by an extended haploid phase and by a sexual phase that ultimately leads to the production of asc with eight spores. The main advantage of using this fungus as a model system for genetic studies of meiotic homologous recombination lies in the existence of many mutations in different genes that control the shape or color of ascospores and in the straightforward visual analysis that can be done of their segregation at meiosis (Leblon and Rossignol 1973; Nicolas et al. 1981). Among such mutations, those affecting the spore color gene b2 show, in general, an unusually high rate of meiotic recombination (Nicolas 1979). Genetic analysis of the b2 recombination hotspot has provided some of the most compelling evidence that heteroduplex DNA (hDNA) is an essential intermediate of meiotic recombination and that recombination proceeds from preferred initiation sites (reviewed in Rossignol et al. 1988 and in Nicolas and Rossignol 1989). Work with the molecularly more tractable yeast Saccharomyces cerevisiae has since demonstrated the physical existence of recombination initiation sites at meiosis (Nicolas et al. 1989; Sun et al. 1989; CaO et al. 1990; de Massy and Nicolas 1993; NAg and Petes 1993; Fan et al. 1995) and of heteroduplex DNA (NAG et al. 1989; Lichtien et al. 1990; Haber et al. 1993; Nag and Petes 1993). Despite this, comparison of the genetic data obtained with A. immersus and S. cerevisiae indicates several apparent differences in the recombination process between these two model organisms (discussed in Nicolas and Petes 1994). For example, although the formation of hDNA simultaneously on the two interacting DNA duplexes (i.e., of symmetric hDNA) has been extensively documented in A. immersus, evidence remains elusive in S. cerevisiae (see Alani et al. 1994). Similarly, evidence for the existence of two distinct mechanisms of biased gene conversion (concerning frameshift mutants and large heterologies, respectively) comes exclusively from studies in A. immersus (Hamza et al. 1986). Thus, it is expected that molecular studies based on b2 will provide new insights into the mechanisms of meiotic recombination.

To make such studies possible, several DNA transformation procedures have been developed for A. immersus (Faugeron et al. 1989; Goyon and Faugeron 1989). Initially, these were all based on the use of Met- protoplasts and of the previously cloned met2 gene (encoding homoserine O-transaccteylase) of A. immersus (Goyon et al. 1988). Unexpectedly, it was found that many of the Met+ transformants would not transmit the transformed phenotype through sexual reproduction. Investigation of this observation revealed that inactivation was associated with extensive cytosine methylation of the transgene and of the resident metZ allele transmitted by the transformants (Faucon et al. 1989; Goyon and Faugeron 1989). This led to the discovery of a process known as methylation induced premeiotically (MIP) in A. immersus, which raised a novel interest in this fungus (Rhounim et al. 1992; reviewed in Rossignol and Faugeron 1994). MIP has also since been shown to operate on two exogenous genes that were introduced into A. immersus by DNA transformation, the amdS gene (encoding acetamidase) of Aspergillus nidulans and a chimeric construct containing the hph gene (encoding hygro-
mycin B phosphotransferase) of Escherichia coli (FAUGERON et al. 1990; RHOUNIX et al. 1994).

Work with these three genes has amply demonstrated that MIP acts only on sequences that are present in two or more copies in the same haploid nucleus (indicating that transformation per se does not trigger MIP), and that it is exclusively established during the sexual phase of the life cycle at some stage (as yet undefined) between fertilization and karyogamy. Furthermore, MIP is an epimutation process only, since neither methylation nor silencing resulting from MIP are associated with mutations (RHOUNIX et al. 1992). This distinguishes it from the related process of repeat-induced point mutation (RIP) of Neurospora crassa, which also detects repeats, but subjects them to multiple C to T transitions and usually to methylation (reviewed in SELKER 1990; SINGER and SELKER 1993).

Once established, both methylation and silencing by MIP are stably transmitted vegetatively as well as sexually, independent of any duplication being present (RHOUNIX et al. 1992). Although the mechanism of inactivation by MIP remains elusive, as does the exact nature of the original premeiotic imprint, results obtained to date suggest a role for methylation in blocking transcript elongation (BARRY et al. 1993). In this context, it should be noted that unlike the methylation commonly found in mammals and plants, methylation established by MIP (and, for that matter, by RIP) is remarkably dense and is not restricted to symmetrical sites, since it can concern nearly every cytosine residue that is contained within the repeats, as indicated by genomic sequencing (SELKER et al. 1993; GOYON et al. 1994). Genomic sequencing has also confirmed that methylation established by MIP is roughly coextensive with the length of the repeat. This latter result and the observation that both RIP and MIP never affect one, but always two or all three, elements of sequence triplications strongly suggest that both processes involve direct DNA-DNA interactions (FINCHAM et al. 1989; FAUGERON et al. 1990). This would imply therefore the participation of some kind of homology sensing machinery in RIP and MIP and raises the intriguing possibility that this machinery is the same as that involved in meiotic pairing (FOSS and SELKER 1991; ROSIGNOL and FAUGERON 1994; WEINER and KLECKNER 1994).

Here, we report the cloning of the spore color gene b2 using a genomic subtractive approach and the physical characterization of four mutant alleles corresponding to large alterations of the gene. This analysis, combined with transformation experiments, were used to delineate the gene within an 18-kb DNA fragment. We also present the results of detailed genetic and molecular analyses that demonstrate that b2 can be subjected to MIP following its duplication by transformation and that confirm previous results obtained with the endogenous gene met2 and the two exogenous genes amdS and hph. In addition, we show that gene silencing by MIP, which can be visualized in single cells thanks to b2, takes place at the latest after the first postmeiotic cell division, and that it is frequently partial when associated with methylation of only given parts of the gene.

Theoretical and practical implications of these and other results are discussed.

MATERIALS AND METHODS

DNA isolation and manipulation: DNA manipulations were performed according to standard techniques (SAM BROOK et al. 1989).

Genomic DNA used for making a library in the lambda replacement vector DASH II (Stratagene) and for subtractive hybridization was isolated as follows: fresh mycelium (4 g) was frozen in liquid nitrogen and ground to a fine powder before being transferred to a polypropylene tube containing 22.5 ml of DNA extraction buffer without SDS (final concentration: 100 mM Tris-C1 pH 8.5, 30 mM EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/ml of Teseamy added proteinase K). The powder was resuspended by gentle mixing before adding 2.5 ml of a 20% SDS solution to lyse cells. The resulting mixture was incubated at 42°C for 1 h with occasional swirling. Cellular debris was eliminated by brief centrifugation, and the supernatant was extracted once with 1 vol of a phenolchloroform mix (50:50) previously equilibrated with the extraction buffer (without proteinase K and without SDS). The aqueous phase was transferred to a fresh tube, and DNA was precipitated by adding 0.1 vol of 3 M sodium acetate pH 5.2 and 1 vol of 100% isopropanol. Precipitated DNA was spooked out, rinsed with 70% ethanol, and gently resuspended in 4 ml of 10 mM Tris-C1 pH 8.0, 1 mM EDTA, 0.1 mg/ml RNase A (buffer TE + RNase). The DNA solution was incubated 30 min at 37°C then centrifugated (1800 rpm, 20 min, 4°C) to pellet polysaccharides. DNA was finally purified by CsCl ultracentrifugation. Yields varied between 300 and 500 μg of DNA.

A scaled-down version of this protocol was used to isolate all of the other genomic DNAs with few modifications: mycelium was lyophilized and kept at −20°C before use; CsCl centrifugation was performed according to standard techniques (SAM BROOK et al. 1989).

Genomic subtraction and cloning of DNA fragments from the b2 region: Subtraction was achieved using The Phenol Emulsion Reassociation Technique (PERT) of KOHNE et al. (1977), adapted by KUNKEL et al. (1985) for the construction of genomic libraries enriched in DNA fragments present in one individual but not in another. Driver DNA (70 μg) carrying the deletion mutation b2-A1230 (see RESULTS) was sheared by sonication to an average size of 600 bp and mixed in 500 μl of water to 0.7 μg of wild-type DNA (tracer DNA) that was previously restricted to completion with the four-base cutter enzyme TaqI. The DNA mixture was heated at 100°C for 5 min, cooled on ice, and added in an Eppendorf tube to 500 μl of 0.2 M Tris-C1 pH 8.5, 2.5 M NaClO4, 1.3% redissolved phenol. The tube was fixed to a Vortex shaker, and renaturation was allowed to proceed to high Cot values by intermittently shaking the tube at room temperature over a period of 85 hr in total. DNA was extracted once with chloroform, precipitated by adding 0.1 vol of 3 M sodium acetate pH 5.2 and 1 vol of 100% isopropanol, rinsed with 70% ethanol and finally resuspended in 30 μl TE.

PERT-reassociated DNA was cloned and submitted to a second subtraction, as follows. Vector DNA (pUC19, 0.5 μg) was linearized with the enzyme AscI that leaves TaqI-compatible termini, dephosphorylated, and mixed with 3 μl (~6 μg) of PERT-DNA in a ligation reaction volume of 50 μl. Following a 4-hr ligation at room temperature, DNA was precipitated,
resuspended in 12 μl TE, and 8 μl were used to transform competent bacteria (K803, New England Biolabs) by electroporation. About 2–5 × 10⁴ colonies were recovered on one large Petri dish (22 × 22 cm) and eluted en masse to isolate plasmid DNA. (Analysis of plasmids of 17 individual colonies indicated that 13 (>75%) carried an identifiable insert). Inserts were released from vector DNA with the enzymes BamHI and HindIII, which flank the Acl cloning site, and were purified on a gel as a single fraction (fraction 0–1.6 kb). Inserts (1.5 ng) were submitted to a second round of subtractive hybridization with 5 μg of b2Δ1230 driver DNA, corresponding to an estimated 100-fold excess (assuming a maximal A. immersus genome size of 10⁹ kb and an average insert size of 300 bp). Half of the PERT-reassociated DNA was ligated to plasmid pUC19 previously cut with the enzymes BamHI and HindIII and used to transform competent bacteria. Over a thousand transformed colonies were obtained, and 384 were picked and grown individually into microtiter plates.

The maximal rate of enrichment attainable by the subtractive approach just described is fixed by the ratio of driver DNA to tracer DNA. However, previous evidence (discussed by STRAUSS and AUSUBEL 1990; LITISYN et al. 1993) suggests that the enrichment actually obtained by subtractive hybridization is usually far below that of theoretical estimates. Assuming a 10-fold instead of a 100-fold enrichment for each reassociation step and a hypothetical size of ≥5 kb for the b2Δ1230 deletion, we estimated that ~200 clones had to be examined after the second subtractive hybridization to obtain at least one clone containing sequences present in the wild type but not in the b2Δ1230 deletion mutation. Calculation was based on the formula given by WIELAND et al. (1990), Y = ERF/C, where Y is the number of clones with insert from target/number of clones examined, E is the rate of enrichment, R is the number of copies of target per haploid tester, C is the complexity of target, and C is the complexity of haploid driver. In our case, we have Y = (100 × 1 × 5)/10².

The rate of enrichment actually achieved was estimated a posteriori, by hybridizing replica filters of the 384 colonies picked in total with the cloned 8.5-kb Sau3A fragment that is entirely missing in b2Δ1230 DNA (see Figure 3). Three colonies (including the one originally identified) hybridized unambiguously to the probe, which gives an estimated 90-fold enrichment at best.

Plasmids used for transformation: The transformation marker met2 was cloned into the EcoRV site of plasmid pBluescript II KS (Stratagene) and corresponds to the 3.7-kb HindII-Smal fragment of the original 5.7-kb HindIII–HindIII met2 clone (GOVON et al. 1988). met2 is oriented with its 3' end pointing toward the 5' end of amp. The 18-kb APA11 insert was cloned as a NotI fragment at the NotI site of the polylinker KS (Stratagene) and corresponds to the 3.7-kb HincII–Smal fragment of the original 5.7-kb HindIII–HindIII met2 clone (GOVON et al. 1988). met2 is oriented with its 3' end pointing toward the 5' end of met2 in plasmid pVMB2-1. met2 and the 18-kb APA11 insert are in the same orientation in plasmid pVMB2-2.

Strains of A. immersus, transformation procedure, genetic analysis and media: Strains belonged to stock 28 (RIZET et al. 1969). The mutant allele b2Δ1230 was isolated as a spontaneous recombination with none of the other known b2 mutations (A. NICOLAS and J.-L. ROSSIGNOL unpublished data). The b2Δ138, b2Δ10 and b2Δ60 mutant alleles have been described in ROSSIGNOL et al. (1988). The mutation mrd1.2, which gives round spores as opposed to long spores, is unlinked to b2 and is used to help identify postmeiotic segregation of spore color (PAUQUETTE 1978). The null met2 allele was produced by replacement of the 5.7-kb HindIII fragment that contains met2 (GOVON et al. 1988) by the acetamidase gene amdS of A. nidulans (met2Δ::amdS) (C. BARRY unpublished data). The hygromycin resistance marker that is closely linked to b2 was obtained by random integration of a plasmid-borne construct containing the hygromycin B phosphotransferase gene hph of E. coli (RHOUNIM et al. 1994; S. SALAMA, G. FAUGERON and J.-L. ROSSIGNOL, unpublished data). Medial and transformation procedures have been previously described (RHOUNIM et al. 1994).

Standard genetic procedures were used (RIZET et al. 1960). Analysis of spore color segregation was performed under a dissection microscope after collecting ascI on agar lids. The Met, Amd and Hyg phenotypes were tested as described in RHOUNIM et al. (1994).

RESULTS

Cloning of DNA from the b2 region by genomic subtraction: The choice of this approach was prompted by the lack of any precise information concerning the product of b2 and by the fact that among the many known b2 mutations, several were genetically defined as deletions of part of b2 (ROSSIGNOL et al. 1988), and one, the b2Δ1230 mutation, as a deletion of the entire gene (A. NICOLAS and J.-L. ROSSIGNOL, unpublished data). Genomic subtraction of wild-type genomic DNA was arbitrarily performed with that of b2Δ1230 mutant strains. To homogenize backgrounds, each of the two DNAs was isolated from 20 strains chosen at random from the progeny of a single b2×b2Δ1230 cross. DNA of the pooled b2 strains (tracer DNA) was restricted to completion with the frequent cutter enzyme TaqI and mixed with a 100-fold excess of sonicated DNA of the pooled b2Δ1230 mutant strains (driver DNA). DNA was melted and reannealed to high Cot values, using the Phenol Enhanced Reassociation Technique (PERT). Reassociated TaqI fragments were cloned to the compatible Accl site of plasmid pUC19, released en masse from the vector using flanking restriction sites and submitted before recloning to a second round of reassociation with PERT in the presence of a 100-fold excess of driver DNA. Based on calculations detailed in MATERIALS AND METHODS, examination of a maximum of 200 new clones was deemed sufficient to isolate DNA corresponding to the b2Δ1230 deletion. Inserts were purified in pools of 12 and used to probe Southern blots of restricted tracer and driver DNA. Hybridization with the fifth pool of inserts produced one band specific to the b2 region (Figure 1, left panel). Further analysis of the pool confirmed this result and led to the identification of a 180-bp TaqI insert that hybridized to b2 DNA alone (Figure 1, right panel), and that was therefore likely to correspond to DNA from the b2 region.

Physical mapping of the b2 region: The 180-bp TaqI insert obtained by genomic subtraction was used to isolate three overlapping lambda clones (APA11, APA12 and APA14) from a phage library made of wild-type genomic DNA (Figure 2a). Probes were derived from the phage inserts and used on Southern blots of restricted DNA of the b2 and b2Δ1230 strains, and of three additional b2 mutants that have been genetically defined as large alterations of the gene: the two deletions b2Δ10...
and b2-Δ138 and the unstable insertion b2-G0. Typical results are shown in Figure 2, b and c, indicating loss of fragments and/or fragment size polymorphisms between the five DNA analyzed. In marked contrast, no polymorphism was detected with probes derived from several randomly cloned A. immersus DNA fragments (data not shown). Taken together, these results confirmed that we had cloned DNA of the b2 region and were used to derive a physical map of that region for each of the five genotypes that were analyzed (Figure 3a). According to these maps, b2-Δ1230 and b2-Δ10 correspond to deletions of ~13 and 5 kb, while b2-G0 represents an insertion of ~400 bp with respect to wild type and b2-Δ138, a 5- to 6-kb deletion flanked on its right by a complex rearrangement (see legend of Figure 3a). With the exception of the rearrangement exhibited by the mutant allele b2-Δ138, these data therefore confirm and extend the genetic data, as does the physical mapping of the four mutations relative to each other (see Figure 3b).

Digestions with the restriction enzyme NdeI and its cytosine methylation-sensitive isoschizomer SmaI and analysis using APA11 and APA14 DNA probes did not reveal any significant methylation of the b2 gene region for any of the five genotypes tested (Figure 2c). Similarly, no differences were detected between restrictions digests performed using the MspI and HpaII isoschizomers, which exhibit different sensitivity to DNA methylation (data not shown). Taken together, these results indicate that no detectable cytosine methylation is associated with the b2 region or with any of the four mutant genotypes, including the insertion mutant b2-G0 and the complex rearrangement b2-Δ138.

Mutant rescue of b2 and inactivation by MIP: Physical mapping of the b2 region strongly suggested that b2 is entirely contained within the APA11 (or APA12) 18-kb insert, since this insert extends well outside the breakpoints of the b2-Δ1230 deletion. To verify this by mutant rescue, DNA transformations were performed with each of the two nonlinearized plasmids pVMB2-1 and pVMB2-2, which contain the APA11 18-kb insert in opposite orientations and the selectable marker met2 of A. immersus. These transformations were also used to test whether MIP operates on b2.

Experimental approach: The recipient haploid strain carries a resident b2" allele that is closely linked to the hygromycin-resistance marker hph to facilitate subsequent analysis, and a deletion of the resident met2 gene obtained by replacement with amds (met2Δ::amds). The use of this strain allows MIP and mutant rescue of b2 to be tested directly and simultaneously, simply by crossing primary transformants with a b2 mutant strain. Indeed, most transgenes are likely to segregate at meiosis independently of their resident counterparts since transformation of A. immersus with circular plasmids harboring sequences already present in the genome mainly takes place by integration at sites unlinked to the native sequences (GOYON et al. 1988). The tester strains of opposite mating type used in crosses with the primary transformants carries the resident b2-Δ1230 allele and lacks the hph marker, so as to permit identification of progeny strains that had inherited the resident b2" allele and/or the transgene(s) solely on the basis of the Met, Hgy and, when necessary, Amr phenotypes, and thus independent of spore color. Lastly, given that in A. immersus, transformation often results in integration of only part of the transforming DNA (GOYON et al. 1988), a large number of transformants have to be analyzed for mutant rescue to be tested adequately.

Transformation and test crosses: Three separate transformation experiments were performed, two with plasmid pVMB2-1 and one with plasmid pVMB2-2. Transformation efficiencies were similar to each other (5–10 transformants/μg plasmid DNA). Among over 200 transformants obtained, 60 pVMB2-1 transformants and
30 PVMB2-2 transformants were crossed with a *b2*Δ1230, *met2Δ::fads* tester strain of the opposite mating type. While 21 out of the 60 PVMB2-1 transformants produced fertile crosses, a proportion that is within the norm for *met2Δ* transformants (Fauqeron et al. 1989; G. Fauqeron and J.-L. Rossignol, unpublished results), test-crosses with the 30 PVMB2-2 transformants were all sterile, being in this respect undistinguishable from crosses between *A. immersus* *met2Δ* strains. This suggests that the expression of the *met2* gene provided by plasmid pvMB2-2, although phenotypically indistinguishable before fertilization from that provided by plasmid pvMB2-1, is insufficient to allow sexual reproduction to proceed normally. Although this was not investigated further, this is in keeping with the fact that six of the 30 transformants obtained with PVMB2-2 were found to be fertile in crosses with a *b2*Δ1230 tester strain that carried a resident *met2Δ* allele.

**Visual inspection of progeny:** As indicated in Table 1, progeny produced by the 27 fertile transformants fell into five distinct categories. Progeny of categories 1 and 2 contained asci with six or eight brown spores all truly wild type in color and thus demonstrated rescue of the mutant phenotype associated with the *b2*Δ1230 allele. In addition, progeny of categories 1 and 2 were produced by transformants obtained with plasmids pvMB2-1 and PVMB2-2 and hence were not dependent on the orientation of the *XPAl1* 18-kb insert relative to the flanking plasmid sequences. This indicates therefore that the *XPAl1* 18-kb insert contains all the information necessary for the proper expression of *b2* in spores.

Progeny of categories 1 and 2 also contained asci with eight white spores, in proportions ranging from 50 to 90% of all asci. This suggests that 0B:8W asci originated from inactivation by MIP of both the *b2* resident allele and the functional transgene(s) transmitted by the transformants. Likewise, many 0B:8W asci were present in the progeny of categories 3 and 4, in proportions ranging from 10 to 90% of all asci. However, progeny of these two categories contained no asci indicative of...
Figure 3.—Physical and genetical maps of the b2 region and of four mutant b2 alleles. (a) Physical maps. Question mark over a restriction site indicates that the presence of this site could not be determined unambiguously. For each enzyme, the leftmost and rightmost sites that are indicated were identified by hybridization of Southern blots of genomic DNA with APA14 and λPA11 DNA, respectively, and do not therefore exclude the possible presence of further sites beyond these boundaries. The rightmost XbaI site is −6 kb away from the rightmost EcoRI site. The four mutations b2-GO, b2-Δ1230, b2-Δ138 and b2-Δ10 correspond respectively to an insertion (■) and three deletions (□). The mutation b2-Δ138 also contains a rearrangement of sequences to the right of the deletion (---). (b) Genetic maps. The leftmost and rightmost b2 markers belong to the intragenic suppression groups F and A, respectively, and are −4.5 cM apart (Leblon and Paquette 1978; Rossignol et al. 1988). The complete deletion b2-Δ1230 and the two partial deletions b2-Δ138 and b2-Δ10 were genetically defined on the basis of their nonrecombination with either all or some of the other known b2 mutant alleles. The extent of the three deletions could not be determined genetically outside of b2 since no known gene marker flanks b2. The mutation b2-GO was genetically defined as a transposon insertion on the basis of its instability and of the nature of the revertants generated (Nicolas et al. 1987).

Table 1

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<th>Categories of progeny produced in crosses of primary transformants with b2-Δ1230 tester strains</th>
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<td>Category number</td>
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*M Mostly asci with one to four pink spores.

* Ascii with pink spores together with at least four white spores.
with four brown spores. Genetic analysis of one such progeny (that produced by transformant T2, see below) indicates that in the latter case, pink spores originated exclusively from partially inactivated transgenic copies, independent of any inactivation of the resident \( b2^+ \) allele (this result is discussed below).

Finally, progeny of category 5 were exclusively composed of 4B:4W asci and did not differ in that respect from that of untransformed \( b2^+ \), met2+ controls. Thus, none of the transgenes harbored by the transformants that produced such progeny were able to inactivate the resident \( b2^+ \) allele to any noticeable degree nor to rescue the \( b2^\Delta 1230 \) mutation. Although this was not investigated further, this result therefore suggests that none of these transgenes contain extensive parts of \( b2 \) in addition to the transformation marker \( met2 \).

**Genetic analysis of transformants:** Six transformants (called hereafter T1–T6) corresponding to the four categories of mixed progeny (Table 1, categories 1–4) observed in crosses with \( b2^\Delta 1230 \) tester strains were chosen for further analysis. As primary transformants often contain a mixture of transformed and untransformed nuclei, this can lead to the production of untransformed progeny and hence to an overrepresentation of 4B:4W asci in crosses with \( b2^\Delta 1230 \) tester strains. To permit the proper analysis of spore color segregation and to test whether the primary transformants contained one or several transgenic loci, transgenic strains harboring the active resident \( b2^+ \) allele (i.e. strains that were Met+, Amd+ and Hyg+) were isolated from the progeny of these primary transformants and back-crossed with \( b2^\Delta 1230 \) strains. All of the transgenic strains tested in this manner produced the same types of asci as those produced by the primary transformants from which they derived. Notably, derivatives of T2 and T5 produced pink spores and exhibited again postmeiotic segregations of spore color. Taken together, these results confirmed the original distinction of four categories of mixed progeny in addition to that composed exclusively of 4B:4W asci in crosses with \( b2^\Delta 1230 \) strains (Table 2, and Figure 4 for examples of the four categories of mixed progeny). Furthermore, the relative frequency of asci of the different types did not differ significantly in the progeny of transgenic strains derived from the same primary transformant (data not shown). Analysis of back-crosses also indicated a ratio of 4B:4W, 6B:2W and 8B:0W asci close to 1:4:1 in the progeny of the derivatives of transformants T1 and T3, indicating that they contain a single functional \( b2 \) transgenic locus, unlinked to the resident \( b2^+ \) allele (Table 2 and data not shown). The same conclusion was reached for T2 and its derivatives, although in this case the analysis of the ratio of the different types of colored asci was complicated by the frequent postmeiotic segregation of spore color and the frequent inactivation of the \( b2 \) transgene independent of the resident \( b2^+ \) allele (see section on partial inactivation). In turn, segregation analysis of the Hyg, Met and, when necessary, Amd phenotypes in the progeny of these three transformants and of their derivatives was indicative in each case of the presence of a single \( met2 \) transgenic locus, linked to the \( b2 \) transgene (data not shown). A similar analysis revealed that transformants T4, T5 and T6 each contained and transmitted a single \( met2 \)

### Table 2

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<th>Name of primary transformant (and category of progeny produced)</th>
<th>Asci' (500 scored per progeny)</th>
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<td>4B:4W</td>
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<td>T1 (category 1)</td>
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<td></td>
<td>(5.0)</td>
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<td>T2 (category 2)</td>
<td>101</td>
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<td></td>
<td>(20.2)</td>
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<td>T3 (category 1)</td>
<td>37</td>
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<td>(7.4)</td>
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<td>T4 (category 3)</td>
<td>181</td>
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<td>(36.2)</td>
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<td>T5 (category 4)</td>
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<td>T6 (category 3)</td>
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*From two to five independent backcrosses were performed in total for each of the six primary transformants T1–T6. Results are shown for just one backcross in each case. Values in parentheses are percentages.

**Primary transformants T1, T4 and T5 were obtained with plasmid pWB2.1, the other three transformants T2, T3 and T6 with plasmid pVM2.2.

≤4B:4W ascigers that composed exclusively of 4B:4W asci and did not differ in that respect from that of untransformed \( b2^+ \), met2+ controls. Thus, none of the transgenes harbored by the transformants that produced such progeny were able to inactivate the resident \( b2^+ \) allele to any noticeable degree nor to rescue the \( b2^\Delta 1230 \) mutation. Although this was not investigated further, this result therefore suggests that none of these transgenes contain extensive parts of \( b2 \) in addition to the transformation marker \( met2 \).

**Genetic analysis of transformants:** Six transformants (called hereafter T1–T6) corresponding to the four categories of mixed progeny (Table 1, categories 1–4) observed in crosses with \( b2^\Delta 1230 \) tester strains were chosen for further analysis. As primary transformants often contain a mixture of transformed and untransformed nuclei, this can lead to the production of untransformed progeny and hence to an overrepresentation of 4B:4W asci in crosses with \( b2^\Delta 1230 \) tester strains. To permit the proper analysis of spore color segregation and to test whether the primary transformants contained one or several transgenic loci, transgenic strains harboring the active resident \( b2^+ \) allele (i.e. strains that were Met+, Amd+ and Hyg+) were isolated from the progeny of these primary transformants and back-crossed with \( b2^\Delta 1230 \) strains. All of the transgenic strains tested in this manner produced the same types of asci as those produced by the primary transformants from which they derived. Notably, derivatives of T2 and T5 produced pink spores and exhibited again postmeiotic segregations of spore color. Taken together, these results confirmed the original distinction of four categories of mixed progeny in addition to that composed exclusively of 4B:4W asci in crosses with \( b2^\Delta 1230 \) strains (Table 2, and Figure 4 for examples of the four categories of mixed progeny). Furthermore, the relative frequency of asci of the different types did not differ significantly in the progeny of transgenic strains derived from the same primary transformant (data not shown). Analysis of back-crosses also indicated a ratio of 4B:4W, 6B:2W and 8B:0W asci close to 1:4:1 in the progeny of the derivatives of transformants T1 and T3, indicating that they contain a single functional \( b2 \) transgenic locus, unlinked to the resident \( b2^+ \) allele (Table 2 and data not shown). The same conclusion was reached for T2 and its derivatives, although in this case the analysis of the ratio of the different types of colored asci was complicated by the frequent postmeiotic segregation of spore color and the frequent inactivation of the \( b2 \) transgene independent of the resident \( b2^+ \) allele (see section on partial inactivation). In turn, segregation analysis of the Hyg, Met and, when necessary, Amd phenotypes in the progeny of these three transformants and of their derivatives was indicative in each case of the presence of a single \( met2 \) transgenic locus, linked to the \( b2 \) transgene (data not shown). A similar analysis revealed that transformants T4, T5 and T6 each contained and transmitted a single \( met2 \)
FIGURE 4.—Examples of the four categories of mixed progeny observed in test-crosses of selected transformants with \( h2\Delta1230 \) strains. Transformants contained the resident \( b2^+ \) allele in addition to one transgenic locus. To help detect postmeiotic segregations of spore color, the spore shape marker \( rnd1.2 \) was also used in these crosses (see MATERIALS AND METHODS). (a) Control progeny composed exclusively of 4B:4W asci and produced in a cross between an untransformed wild-type strain and a \( h2\Delta1230 \) strain. (b) Mixed progeny composed of a majority of 0B:8W asci together with 4B:4W, 6B:2W and 8B:0W asci (category 1). (c) Mixed progeny composed of 0B:8W and 4B:4W asci only (category 3). (d) Mixed progeny composed of asci with pink spores in addition to 0B:8W, 4B:4W, 6B:2W and (not shown) 8B:0W asci (category 2). (e) Mixed progeny composed of asci with pink spores and at least four white spores in addition to 0B:8W and 4B:4W asci (category 4). Note in d and e that many asci show postmeiotic segregation of spore color (see enlarged examples).

Transgenic locus, which cosegregated with the capacity to inactivate the resident \( b2^+ \) allele in crosses and which was unlinked to that allele (data not shown).

Molecular characterization of transgenic loci: As described, genetic analysis of the six transformants T1–T6 indicated that each harbored and transmitted to its progeny a single transgenic locus. To determine the content of these six loci (designated hereafter \( t1–t6 \)) with respect to the 18-kb \( \lambdaPA11 \) insert used for transformation, Southern blots of restricted DNA of transformed progeny strains also harboring the \( h2\Delta1230 \) resident allele were probed successively with the entire 18-kb \( \lambdaPA11 \) insert and with various subfragments. Examples of this analysis are shown in Figures 5a and 6, b and c. Results indicate that the \( t2 \) locus contains the entire \( \lambdaPA11 \) insert together with a duplication of between 4 and 5 kb of its right end (Figures 5 and 6a) and that the \( t1 \) and \( t6 \) loci carry respectively, one and at least three copies of a left end truncated version of that insert. The remaining three transgenic loci (\( t3–\)
t5) possess a complex structure, characterized by the presence of two or three distinct deletions of parts of the 18-kb λPA11 insert. Although no attempts were made to define further the internal organization of any of the transgenic sequences derived from the 18-kb λPA11 insert nor to identify flanking sequences, it can be concluded that none of the six transformants analyzed had integrated a single intact copy of the insert. Furthermore, comparison of the nonfunctional transgenic loci t4 and t5 to the two deletion mutants b2-Δ138 and b2-Δ10 indicates that these two transgenic loci contain only part of b2 (Figures 5b and 6a). On the other hand, comparison of transgenic loci t3 and t6 suggests that the nonfunctionality of the latter is not caused by a truncation of b2 (Figures 5b and 6a, and see below).

Methylation analysis: To ascertain whether MIP was responsible for the inactivation of b2 seen in the progeny of transformants chosen for analysis, DNA was isolated from strains derived from two asci per transformant: one 0B:8W ascus, indicating inactivation of the resident b2' allele and of the transgenic locus, if functional; and one control ascus, i.e., showing no visible sign of inactivation for either of these. In each case, the two asci were taken from the same progeny (either that of the original cross or that of a back-cross). To facilitate the methylation analysis, asci were also considered so that the resident b2' allele and the transgenic locus had segregated. Two strains were therefore considered for each ascus, one carrying the resident b2' allele alone (lanes a and c in Figure 6), the other carrying the transgenic locus together with the resident b2-Δ1230 allele (lanes b and d in Figure 6).

Methylation of DNA was assessed primarily by using the restriction enzyme NdeII and its methylation-sensitive isoschizomer Sau3A. After transfer, digests were hybridized to the 13-kb XbaI fragment of clone λPA14, which corresponds almost exactly to the region absent in the b2-Δ1230 mutant. Although this probe excluded from analysis five kilobases of sequence that was potentially duplicated between the resident b2' allele and the different transgenic loci, it ensured that no hybridization could take place with the resident b2-Δ1230 allele present in the transgenic strains (data not shown).

Methylation analysis was further facilitated by only considering the 16 larger NdeII restriction fragments of the b2' resident allele that hybridize to the 13-kb probe and their exact transgenic counterparts (Figure 6a). Due to the interspersion of nine of the 16 larger fragments with smaller ones (indicated in Figure 6a as small vertical bars), this analysis therefore concerned 23 NdeII/Sau3A sites in total.

Hybridization results obtained with the 13-kb probe are shown in Figure 6, b and c for the 24 progeny strains that were originally tested. For five progeny strains (see lanes a and b that relate to the progeny of the b2', t1 and b2', t3 transformants, and lane a that relate to the progeny of the b2', t2 transformant), no noticeable difference can be seen in the hybridization pattern generated after digestion with either of the enzymes NdeII and Sau3A, indicating that none of the probed NdeII/Sau3A sites are methylated in these strains. In marked

![Figure 5](image-url)
FIGURE 6.—Methylation analysis of the b2+ resident allele and of the six transgenic loci t1–t6 in the progeny of test crosses with b2-Δ1230 strains. (a) NdeI/Sau3A restriction maps of the b2+ resident allele, of the the two mutants b2-Δ138 and b2-Δ10 and of the six transgenic loci t1–t6. These maps are limited to the ~13-kb region that is completely absent in the mutant b2-Δ1230. Only the NdeI/Sau3A fragments that are apparently nontruncated with respect to the wild type are considered in the other maps. The lines at the top of the figure indicate the extent of the eight different probes used for mapping and for subsequent methylation analysis. NdeI/Sau3A fragments of < ~250 bp are indicated as vertical bars, and only for the b2+ allele. The 16 larger fragments are numbered by order of decreasing size (see b). Doublets are indicated as fragments 10 and 10' and fragments 13 and 13' (and as bands 10* and 13* in b). Regions that are duplicated in t2 and t6 are indicated by gray lines, as in Figure 5. (b and c) Examples of the methylation analysis performed on progeny strains of transformants containing the resident b2+ allele and one or the other of the six transgenic loci t1–t6, as well as on the primary transformants T2, T5 and T6. Hybridization was with the ~13-kb fragment that extends from the Xbal site of the APA14 insert to the Xbal site present in the polylinker of the lambda DASH II vector. For each pair of lanes a, b, c and d, the NdeI digest is on the left and the Sau3A digest on the right. Lanes a and c relate to active resident b2+ alleles and to inactivated alleles, respectively, and lanes b and d to the transgenic loci that segregated from these resident b2+ alleles. Methylation of NdeI/Sau3A sites is indicated by the replacement of one or several of the 16 NdeI fragments by fragments of higher molecular weight in the Sau3A restriction digests. A faint hybridizing band of 3 kb is always detected in the Sau3A digests. Analysis of cloned DNA indicates that it results...
contrast, clear signs of partial or complete methylation of many or all of the probed NdeI/Sau3A sites are visible for the remaining 19 progeny strains tested (see legend of Figure 6).

Smaller probes were then used to determine precisely the methylation profile of the resident b2' allele or of the transgenic locus carried by the 19 progeny strains that showed signs of methylation. Results are summarized in Figure 7. Perusal of profiles reveals that methylation is restricted to sites corresponding to DNA fragments that are repeated between the resident b2' allele and the different transgenic loci or else to DNA fragments repeated within a given transgenic locus (see also legend of Figure 7). Furthermore, methylation never involved only one copy of the repeated sequences [in the case of profiles 2b, 6b and 6d, this is documented by the strong methylation (>50%) of the internally duplicated fragments, which rules out that only one copy of these fragments is methylated in each case] and was either undetectable or at most very limited in the three primary transformants that were analyzed alongside their progeny (Figure 6c, lanes T2, T5 and T6). Our data also show that in most cases methylation profiles are similar if not identical between the resident b2' allele and the transgenic locus when both are derived from the same ascus. Where this is not the case (see Figure 7, profiles 2a and 2b, 6a and 6b, and 6c and 6d), the transgenic loci (t2 and t6) contain internal repeats (see Figures 5a and 6a), and the patterns observed correspond to the extensive methylation of these repeats, independent of any equivalent methylation of the resident b2' allele. The three cases of dissimilar methylation profiles between resident and transgenic copies derived from the same ascus can therefore be interpreted as resulting from the preferential methylation of repeats that are tightly linked over that of repeats that are dispersed in the genome. Hence, they do not constitute exceptions to the observation that methylation profiles are similar or identical between the two members of a duplication when both are derived from the same ascus.

Taken together, these results demonstrate unambiguously that MIP is responsible for the methylation detected in the progeny of the different crosses, since it is established at the earliest after fertilization, concerns exclusively sequences that were repeated in the transformed parent, and results from their interaction before meiotic DNA synthesis. However, contrary to all previous results (reviewed in ROSSIGNOL and FAUGERON 1995), the methylation that we observed did not always extend along the entire length of the region that was repeated between a given transgenic locus and the resident b2' allele (see, for example, profiles 3c and 3d, and 4a and 4b of Figure 7). To confirm this, the two DNA samples corresponding to profiles 3c and 3d were digested with another methylation-sensitive restriction enzyme, HhaI, that cuts frequently within the b2 region of wild-type DNA (data not shown), and that cleaves the CpG-containing sequence 5'GCGC3' only when both C's are nonmethylated. Methylation profiles derived from the HhaI digests were in keeping with those obtained with the enzyme Sau3A (data not shown).

Since methylation by MIP is best maintained at CpG dinucleotides (GOYON et al. 1994), this latter result strongly suggests that part of the repeat length is indeed devoid of any methylation in the two DNA samples corresponding to profiles 3c and 3d. Furthermore, the observation that profiles 3c and 3d, like most other profiles, are similar if not identical between the resident b2' allele and the transgenic locus when both derive from the same ascus, indicates that lack of methylation probably results from the nonestablishment of methylation during MIP rather than from defects in methylation maintenance. Similar results were obtained for an additional pair of strains that was isolated from the same progeny. Indeed, the two additional profiles 3e and 3f show a methylation gap as profiles 3c and 3d, and apparently only differ from these two profiles by the length of the gap (Figure 7).

As would be expected if MIP were solely responsible for the inactivation of the b2 copy(ies) transmitted to the progeny of test crosses, the resident b2' alleles and functional transgenes that were examined after inactivation (i.e., that derived from white spores) are all methylated and suffer no apparent DNA rearrangement (see Figures 6 and 7). However, our data also indicate that methylation is not always associated with the inactivation of the resident b2' allele or of the functional transgenes (see profiles 2b, 4a, 5a, 5c and 6a of Figure 7). Examination of profiles 2b and 6a suggests, for example, that methylation starting from the NdeI/Sau3A site located between fragments 4 and 19' (site 4/19') and extending only to its right is not associated with any visible inactivation. Likewise, examination of profile 4a suggests that methylation starting at site 7/10' and extending only to its left is not associated with any visible inactivation. This indicates that b2 must be contained, at least in part, within the interval defined by sites 7/10' and 4/13', in agreement with the physical map of the three deletions b2-Δ1230, b2-Δ138 and b2-Δ10. This last result suggests in turn that the transgenic locus t6 contains in fact several functional copies of b2. This is also in agreement with the fact that, apart from its repeated nature, t6 apparently differs little in structure from the functional
locus \(l_3\), and raises the possibility that the nonfunctionality of transgenic locus \(l_6\) resides in its systematic methylation and inactivation by MIP.

Finally, it should be noted that among the 24 methylation profiles that were originally established, profiles 5a and 5c gave paradoxical results with respect to \(b_2\) inactivation, since although identical, they relate to an active and an inactivated resident \(b_2^+\) allele, respectively. This prompted us to analyze another two pairs of strains, one pair derived from a \(4B:4W\) ascus and another pair derived from a \(0B:8W\) ascus. Results (profiles 5e to 5h of Figure 7) indicated a less extended methylation of the active allele (profile 5c) compared to the inactivated resident \(b_2^+\) allele (profile 5g). Incidentally, this latter allele showed a profile identical to profiles 5a (\(b_2^+\) active) and 5c (\(b_2^+\) inactive) (Figure 7). Thus, while apparent methylation differences can exist between active and inactivated resident \(b_2^+\) alleles subjected to MIP as a consequence of the duplication generated by the transgenic locus \(l_5\), this is not always the case (see next section).

**Partial inactivation of \(b_2\) by MIP as a result of partial duplications:** As described previously, the mixed progeny obtained in the original test-crosses with 17 transformants always contained \(0B:8W\) asc. Furthermore, in three cases the mixed progeny also contained asc with at least one pink spore and either brown or white spores (see Table 1). In light of the results presented above, the appearance of pink spores in the mixed progeny of only some transformants indicates therefore that duplications capable of inactivating \(b_2\) by MIP are of two types, one of which leads to complete inactivation only, while the other leads to both partial and complete inactivation, often in the same ascus.

Comparison of the structures of the transgenic loci \(l_1, l_3, l_4\) and \(l_6\), which lead to complete inactivation only, to those of transgenic loci \(l_2\) and \(l_5\), which can also give rise to partial inactivation, shows that these two transgenes are unique in creating partial duplications of \(b_2\) that are limited to sequences right of fragment 2 (see Figures 5b and 6a) (even though transgenic locus \(l_2\) also contains a functional copy of \(b_2\)). Therefore, it is highly likely that these partial duplications are the cause of both complete and partial inactivation. In keeping with this, genetic analysis of asc with pink spores that were produced in test-crosses of strains car-

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**Figure 7.—** Methylation profiles of the \(b_2^+\) resident allele and of the six transgenic loci \(l_1\) to \(l_6\) in the progeny of test crosses with \(b_2\Delta1230\) strains. The color of the spores from which they derive is indicated on the left of each profile (filled ovals, brown spores; open ovals, white spores). Three levels of methylation are distinguished. Each pair of profiles relates to a resident \(b_2^+\) allele (top) and a transgenic locus (bottom) derived from a single \(IISCIIS\). Profiles a–d refer to the identically marked lanes of Figure 6. Note that methylation of fragments that are solely present on the resident locus (indicated on the profiles) in the relevant transgenic loci, and hence from their partial duplication and MIP. Probing of Southern blots with the entire 18-kb \(H\)PA11 insert indicated a less extended methylation of the active allele (profile 5e) compared to the inactivated resident \(b_2^+\) allele (profile 5g). Incidentally, this latter allele showed a profile identical to profiles 5a (\(b_2^+\) active) and 5c (\(b_2^+\) inactive) (Figure 7). Thus, while apparent methylation differences can exist between active and inactivated resident \(b_2^+\) alleles subjected to MIP as a consequence of the duplication generated by the transgenic locus \(l_5\), this is not always the case (see next section).
The resident $b_2^+$ allele and $t_2$ indicates that in the majority of these ascis, inactivation (either partial or complete) affects $t_2$ alone, and thus that it occurs through an interaction that necessarily involves the partial copy of $b_2$ carried by the transgenic locus (Table 2 and data not shown). Remarkably, this latter result, coupled to the fact that MIP affects 100% of premeioses in the case of tightly linked duplications (Rhounim et al. 1992; our results), strongly suggests that partially inactivated copies and active copies of $b_2$ carried by the transgenic locus $t_2$ will all exhibit very similar methylation profiles. Indeed, both types of copies are expected to be systematically and exclusively methylated along the internal duplication present at this locus and thus independent of the resident $b_2^+$ allele. (Applying the same reasoning, it is also expected that fully inactivated transgenic copies of $b_2$ derived from ascis with four active copies of the resident $b_2^+$ allele will be systematically and exclusively methylated along the internal duplication carried by $t_2$ and would therefore show methylation profiles similar to that of active or partially inactivated copies of $t_2$). As described earlier, direct evidence of similar profiles being associated with differences of gene activity is provided by the comparison of profiles 5a, 5c and 5g, that relate to one active and two fully inactivated resident $b_2^+$ alleles issued from the progeny of $t_5$ transformants (Figure 7). Finally, the observation of frequent meiotic and postmeiotic segregation of spore color (reflecting differences of $b_2$ activity in sister spores) in the progeny of both $t_2$ and $t_5$ transformants also indicates that probably few if any visible differences in methylation are associated with active, fully inactivated or partially inactivated copies of $b_2$ that are only methylated to the right of the NdeI/Sau3A site 1/6. Possible implications of these results are discussed below. However, it can be stated that the appearance of pink spores in addition to white spores provides direct evidence at the single cell level of a possible modulating effect of MIP on $b_2$ expression, as opposed to a simple all or none effect.

Effects of the three deletions $b_2$-$\Delta_{1230}$, $b_2$-$\Delta_{138}$ and $b_2$-$\Delta_{10}$ on transgene expression following MIP: As indicated by their structure (see Figures 5b and 6a), these three deletions provide a unique opportunity to reinforce the observations concerning the consequences on the expression of $b_2$ of subjecting different parts of the gene to the MIP process. To this effect, strains carrying the structurally simple and functional transgenic locus $t_1$ were first crossed with $b_2$-$\Delta_{138}$ and $b_2$-$\Delta_{10}$ mutant strains, just as had previously been done with $b_2$-$\Delta_{1230}$ mutant strains. In this manner, progeny strains could be isolated that harbored an active $b_2$ transgene in one or the other of the three $b_2$ deletion backgrounds. Several self-crosses were then performed for each background between transgenic strains of opposite mating type. Results of the self-crosses were consistent with each other for a given background but differed markedly between the three mutant backgrounds (see Figure 8 for typical results obtained for each mutant background as well as for the control wild-type background). Namely, no inactivation of the $b_2$ transgene was ever observed in the progeny of crosses involving the $b_2$-$\Delta_{1230}$ mutant background, as expected from the genetic analysis of this mutation, which suggests that it corresponds to a null allele, and from the methylation analysis described earlier, which indicates that methylation of the region that is duplicated between $b_2$-$\Delta_{1230}$ and $t_1$ has no effect on $b_2$ expression. In marked contrast, self-crosses involving the $b_2$-$\Delta_{138}$ mutant background produced frequent and complete inactivation of the $b_2$ copy harbored by $t_1$, in full agreement with results obtained earlier with $b_2^+$, $t_4$ transformants, which generate a duplication similar to that found between $t_1$ and mutant $b_2$-$\Delta_{138}$. Finally, both partial and complete inactivation were observed in the progeny of crosses involving the $b_2$-$\Delta_{10}$ mutant background. In this case, this is almost certainly as a result of the duplication of the fragments that extend rightward from fragment 1, since duplication and methylation of fragments extending only leftward from fragment 7 has no effect on $b_2$ activity (see above). This therefore confirms results obtained previously with $b_2^+$, $t_2$ and $b_2^+$, $t_5$ transformants, which also generate duplications that are limited to the right of fragment 1.

DISCUSSION

We have used the PERT technique to clone by genomic subtraction DNA of the $b_2$ region. A detailed physical map of that region was obtained, and four genetically defined large alterations of $b_2$ were molecularly characterized. This opens up the prospect of molecular studies on genetic recombination based on $b_2$, notably on the precise nature of the difference of recombination properties exhibited by different $b_2$ mutations.

We have also shown by transformation of A. immersus that all of the sequences necessary and sufficient for the proper expression of $b_2$ are contained within at most 18 kb and that like met2, $b_2$ can be subjected to MIP. This further suggests that any A. immersus sequence that is present as a single copy in the genome will become a target for MIP after its duplication via DNA transformation and therefore that MIP can be used as a tool to inactivate and study genes of unknown function.

Punctuated methylation by MIP: To date, the 18-kb $b_2$ region is the largest sequence to have been artificially duplicated in A. immersus and subjected to MIP. Our results indicate that methylation can be established and maintained over the whole $b_2$ region, just as it is with shorter regions, and that the frequency of methylation at a given site, which reflects the efficiency of methylation maintenance, is neither reduced nor increased noticeably for methylated regions that are about three
times larger than those previously tested. Furthermore, no apparent methylation was found outside the repeated regions studied, indicating that the boundaries of methylation coincide with the boundaries of the repeated regions, irrespective of the total length of the methylated sequence (i.e., there is no apparent spread of methylation into flanking DNA when the repeated regions exceed a particular size). Finally, the almost systematic identical methylation profiles observed between the resident b2’ allele and the transgene when both are derived from the same ascus, but not when they are derived from different asci, provide new evidence that MIP involves a direct DNA-DNA recognition step.

In addition, we have documented for the first time several cases of apparent absence of methylation for part of a repeat unit (including two cases where this lack of methylation is centrally located, see profiles 3c to 3f of Figure 7) and have shown evidence that indicates that this apparent absence is probably a result of escape from MIP rather than of defects in methylation maintenance. No escape of this sort has been observed for RIP (E. Selker, personal communication), and thus more data are needed concerning large duplications before any conclusion can be drawn as to its cause. Indeed, they could simply reflect some unusual structural features of certain large repeats that might locally hamper methylation. Alternatively, they could indicate that MIP acts in a processive manner, from one or both extremities of the repeat unit toward the center, or that it is discontinuous in the case of large repeat units.

**Developmental timing of gene inactivation by MIP:**

So far, gene inactivation by MIP could only be detected at the earliest after the first filamentous protrusions that form around germinating spores, at a stage estimated to represent a minimum of six cell divisions after meiosis. As a result, we have documented for the first time partial as well as complete inactivation of gene expression by MIP, in single cells, in the form of pink spores in addition to white spores. This demonstrates that the effect of MIP on gene expression can vary in intensity, and thus that it is not always an all or none phenomenon. Also, given that the initial imprint of MIP is premeiotic, the frequent postmeiotic segregation of spore color observed in progeny containing pink spores must reflect differences in gene expression that are established after the initial imprint. Furthermore, given that none of the 27 transformants that were analyzed produced pink spores without also producing white spores, it is likely that the occurrence of pink spores reflects the existence of metastable levels of b2 expression after

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**Figure 8.**—Recapitulation of the effects of duplicating various parts of b2 on gene inactivation by MIP. Self-crosses were performed with strains containing the functional b2 transgenic locus tI, and one or the other of four distinct resident b2 alleles. (a) tI, b2': the two asci at the bottom indicate that inactivation of both tI and b2’ took place after MIP in one parent only (6B:2W ascus) or in both parents (0B:8W ascus). The 8B:0W ascus indicates either absence of MIP in both parents, or MIP in one parent and segregation of the active resident and transgenic b2 loci transmitted by the other parent. No other types of ascus were observed in the progeny of this cross. (b) tI, b2-Δ1230: only 8B:0W asci were observed in this cross, indicating the absence of any detectable inactivation of tI. (c) tI, b2-Δ138: inactivation of tI after MIP in one parent only (4B:4W ascus) or in both parents (0B:8W ascus). 8B:0W asci were also observed in the progeny of this cross (not shown). (d) tI, b2-Δ10: full and partial inactivation of tI (4B:3P:1W ascus, 4B:2P:2W ascus and 4B:1P:3W ascus). As spore color most often segregates postmeiotically in this and related crosses, in the case of asci with four to six brown spores, it cannot be determined visually whether MIP took place in one or both parents. 8B:0W asci and asci with fewer than four brown spores were also observed in the progeny of this cross (not shown).
MIP. This, however, is not an obligatory consequence of MIP but must arise from particular situations, since pink spores were only observed in three of the original 17 progenies that showed gene inactivation. Indeed, we have shown that among nine duplications that were analyzed, the three that led to the modulation of b2 expression by MIP (including that associated with the b2Δ10 allele) were unique in being restricted to the right part of the gene.

Preliminary sequence analysis (V. COLOT and O. LESPINET, unpublished results) indicates that the b2 gene encompasses the entire 2-kb NdeI/Sau3A fragment 1 (which includes the site of the insertion mutation b2-G0 see Figure 2c, 3 and 6), and that it is transcribed in the F to A direction (where F and A refer to the intragenic suppression groups of b2 that are the closest to the two extremities of the gene, see Figure 3). On this basis, it can therefore be concluded that modulation of b2 expression by MIP is associated with an imprint over its 3' part. Furthermore, it has previously been shown that strains harboring a met2 allele methylated over its 3' part produce only truncated met2 transcripts, whose size suggests that truncation results from a blocking effect of methylation (or of some other underlying imprint) on transcript elongation (BARRY et al. 1993). It is therefore reasonable to predict that a similar blocking mechanism might be responsible for the inactivation of b2 after duplication and MIP of its 3' part only. The variable intensity of gene inactivation observed in such circumstances would then result from stochastic and fine variations in the blocking of transcript elongation around a phenotypically detectable threshold. Given that the maintenance methylase of Ascomobolus immersus does not exactly reproduce at each round of DNA replication the methylation status of every cytosine within sequences initially subjected to MIP (GOYON et al. 1994), the variegated expression of b2 seen when only the 3' region of the gene is affected by MIP may also be partly due to slight imprinting differences between sister b2 copies of the same ascus.

In many organisms, DNA methylation has been associated with a variety of processes that relate to the epigenetic control of gene expression (for a recent review see MARTIENSSEN and RICHARDS 1995). The importance of DNA methylation in the normal programming of gene expression is particularly well exemplified in mammals, as indicated by the lethality observed in mice embryos homozygous for a DNA methyltransferase mutation (LI et al. 1992), or else by the association of several human cancers with alterations of genomic imprinting and abnormal methylation (see, for example, MOULTON et al. 1994; STEENMAN et al. 1994; and for reviews, LAIRD and JAENISCH 1994; RAINIER and FEINBERG 1994). In this respect, the observation that gene inactivation by MIP can lead to marked and unprogrammed phenotypic differences between daughter cells submitted to a common parental imprint could be of great significance in other organisms exhibiting genomic imprinting. Indeed, were they to occur for imprinted genes that play a central role in regulatory pathways (i.e., master genes), such differences might have dramatic consequences for the organism.

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LITERATURE CITED


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