Copyright © 1995 by the Genetics Society of America

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

Vincent Colot and Jean-Luc Rossignol

Institut de Génétique et Microbiologie, Centre National de la Recherche Scientifique Unité de Recherche Associeé 1354, Université Paris-sud, F-91405 Orsay Cedex, France

Manuscript received May 26, 1995
Accepted for publication August 17, 1995

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 hot-spot. 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 asci 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; Lichten *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*-transaceteylase) 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 *met2*$^-$ allele transmitted by the transformants (Faugeron *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-

Corresponding author: Vincent Colot, Bâtiment 400, Institut de Génétique et Microbiologie, Université Paris-Sud, F-91405 Orsay Cedex, France. E-mail: colot@igmors.u-psud.fr

mycin B phosphotransferase) of *Escherichia coli* (FAUGERON *et al.* 1990; RHOUNIM *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 (RHOUNIM *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 (and G to A) transitions and usually to methylation (reviewed in SELKER 1990; SINGER and SELKER 1995).

Once established, both methylation and silencing by MIP are stably transmitted vegetatively as well as sexually, independent of any duplication being present (RHOUNIM *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; ROSSIGNOL 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 (SAMBROOK *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-Cl pH 8.5, 50 mM EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/ml of freshly 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° for 1 hr with occasional swirling. Cellular debris was eliminated by brief centrifugation, and the supernatant was extracted once with 1 vol of a phenol:chloroform 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 spooled out, rinsed with 70% ethanol, and gently resuspended in 4 ml of 10 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.1 mg/ml RNase A (buffer TE + RNase). The DNA solution was incubated 30 min at 37° then centrifuged (1800 rpm, 20 min, 4°) 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° before use; CsCl centrifugation was replaced by a phenol:chloroform extraction and DNA precipitation.

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-*Δ*1230* (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 *Taq*I. The DNA mixture was heated at 100° for 5 min, cooled on ice, and added in an Eppendorf tube to 500 μl of 0.2 M Tris-Cl pH 8.5, 2.5 M $NaClO_4$, 13% redistilled phenol. The tube was fixed to a Vortex shaker, and renaturation was allowed to proceed to high C_0t 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 *Acc*I that leaves *Taq*I-compatible terminii, 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 $\times$ 10^4 colonies were recovered on one large Petri dish (22 $\times$ 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 *Bam*HI and *Hin*dIII, which flank the *Acc*I 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 *b2-Δ1230* driver DNA, corresponding to an estimated 100-fold excess (assuming a maximal *A. immersus* genome size of 10^5 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 *Bam*HI and *Hin*dIII 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 STRAUS and AUSUBEL 1990; LISITSYN *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 *b2-Δ1230* 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 *b2-Δ1230* deletion mutation. Calculation was based on the formula given by WIELAND *et al.* (1990), $Y = ERC_a/C_e$, 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_a is the complexity of target, and C_e is the complexity of haploid driver. In our case, we have $Y = (100 \times 1 \times 5)/10^5$).

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 *Sac*I fragment that is entirely missing in *b2-Δ1230* 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 *Eco*RV site of plasmid pBluescript II KS (Stratagene) and corresponds to the 3.7-kb *Hinc*II-*Sma*I fragment of the original 5.7-kb *Hin*dIII -*Hin*dIII *met2* clone (GOYON *et al.* 1988). *met2* is oriented with its 3′ end pointing toward the 3′ end of *amp*. The 18-kb λPA11 insert was cloned as a *Not*I fragment at the *Not*I site of the polylinker to give plasmids pVMB2-1 and pVMB2-2. The 18-kb λPA11 insert is orientated with its left end (see Figures 2a and 3a) pointing toward the 5′ end of *met2* in plasmid pVMB2-1 and is in the opposite 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 *b2-Δ1230* was isolated as a spontaneous mutation that recombines with none of the other known *b2* mutations (A. NICOLAS and J.-L. ROSSIGNOL unpublished data). The *b2-Δ138, b2-Δ10* and *b2-G0* mutant alleles have been described in ROSSIGNOL *et al.* (1988). The mutation *rnd1.2*, which gives round spores as opposed to long spores, is unlinked to *b2* and is used to help identify postmeiotic segregation of spore color (PAQUETTE 1978). The null *met2* allele was produced by replacement of the 5.7-kb *Hin*dIII fragment that contains *met2* (GOYON *et al.* 1988) by the acetamidase gene *amdS* of *A. nidulans* (*met2Δ::famdS*) (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 hygromycine B phosphotransferase gene *hph* of *E. coli* (RHOUNIM *et al.* 1994; S. SALAMA, G. FAUGERON and J.-L. ROSSIGNOL, unpublished data). Media 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 *b2-Δ1230* 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 *b2-Δ1230* mutant strains. To homogenize backgrounds, each of the two DNAs was isolated from 20 strains chosen at random from the progeny of a single $b2^+$ × *b2-Δ1230* cross. DNA of the pooled $b2^+$ strains (tracer DNA) was restricted to completion with the frequent cutter enzyme *Taq*I and mixed with a 100-fold excess of sonicated DNA of the pooled *b2-Δ1230* mutant strains (driver DNA). DNA was melted and reannealed to high C_0t values, using the Phenol Enhanced Reassociation Technique (PERT). Reassociated *Taq*I fragments were cloned to the compatible *Acc*I site of plasmid pUC19, released en masse from the vector using flanking restriction sites and submitted before recloning to a second round of reassociation by 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 *b2-Δ1230* 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 $b2^+$ genotype (Figure 1, left panel). Further analysis of the pool confirmed this result and led to the identification of a 180-bp *Taq*I 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 *Taq*I insert obtained by genomic subtraction was used to isolate three overlapping lambda clones (λPA11, λPA12 and λPA14) 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 *b2-Δ1230* strains, and of three additional *b2* mutants that have been genetically defined as large alterations of the gene: the two deletions *b2-Δ10*

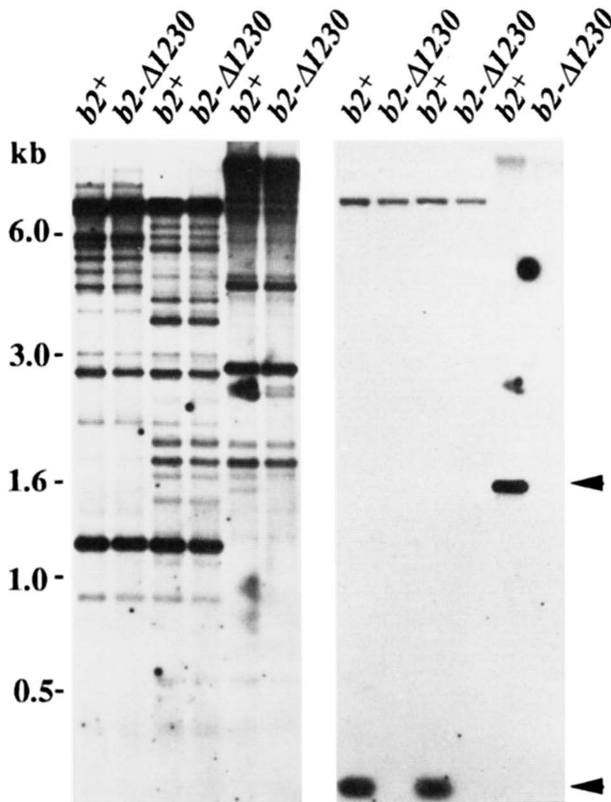

FIGURE 1.—Identification of DNA of the *b2* region following subtractive cloning. DNA corresponding to the *b2*$^+$ and *b2-Δ1230* genotypes were digested with the enzymes *Hin*dIII, *Hin*dIII + *Eco*RV and *Eco*RV (from left to right in the two panels), transferred onto a nylon membrane and sequentially hybridized with pools of inserts derived from the subtractive cloning procedure. Left panel: results obtained with the fifth pool of inserts and that reveal an ~1.6-kb *Eco*RV band unique to the *b2*$^+$ genotype. Right panel: result of hybridization of the same blot with the 180-bp purified insert of that pool. The insert reveals also an ~300-bp *Hin*dIII fragment unique to the *b2*$^+$ genotype, not visible after the first hybridization due to the weaker signals generated by the composite probe. The >8-kb bands seen in the right panel are due to weak cross-hybridization to the >100 copies of the tandemly arranged rRNA genes.

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 *Nde*II and its cytosine methylation-sensitive isoschizomer *Sau*3A and analysis using λPA11 and λPA14 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 *Msp*I and *Hpa*II 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 λPA11 (or λPA12) 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 λPA11 18-kb insert in opposite orientations and the selectable marker *met2* of *A. immmersus*. 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, Hyg and, when necessary, Amd 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

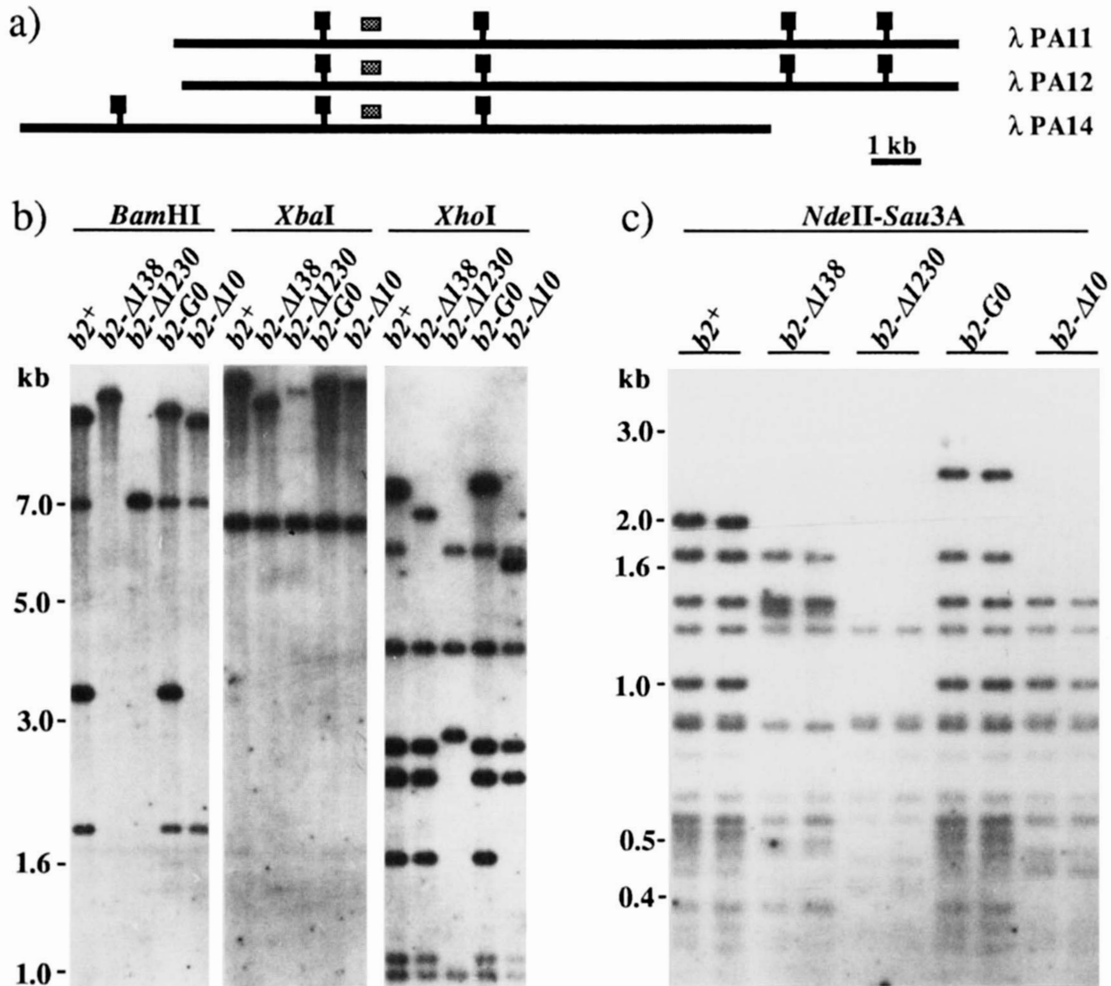

FIGURE 2.—Southern blot analysis of DNA corresponding to the *b2*$^{+}$ genotype and to four large alterations of *b2*. (a) *Eco*RI map of the inserts of the three lambda clones λPA11, λPA12 and λPA14 isolated by probing an *A. immersus* genomic library with the 180-bp fragment obtained by subtractive cloning (shown as a gray box). (b and c) Typical hybridization results produced by probing blots of restricted DNA of five different *b2* genotypes with a mixture of λPA11 and λPA14 DNA. For each genotype in c, the *Nde*II and *Sau*3A digests correspond to the left and right lanes, respectively.

30 PVMB2-2 transformants were crossed with a *b2*-Δ*1230, met2*Δ*::famds* 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 *met2*$^{+}$ transformants (FAUGERON *et al.* 1989; G. FAUGERON 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 met2*$^{-}$ strains. This suggests that the expression of the *met2* gene provided by plasmid pVMB2-2, although phenotypically undistinguishable 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*-Δ*1230* tester strain that carried a resident *met2*$^{+}$ 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*-Δ*1230* 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 λPA11 18-kb insert relative to the flanking plasmid sequences. This indicates therefore that the λPA11 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

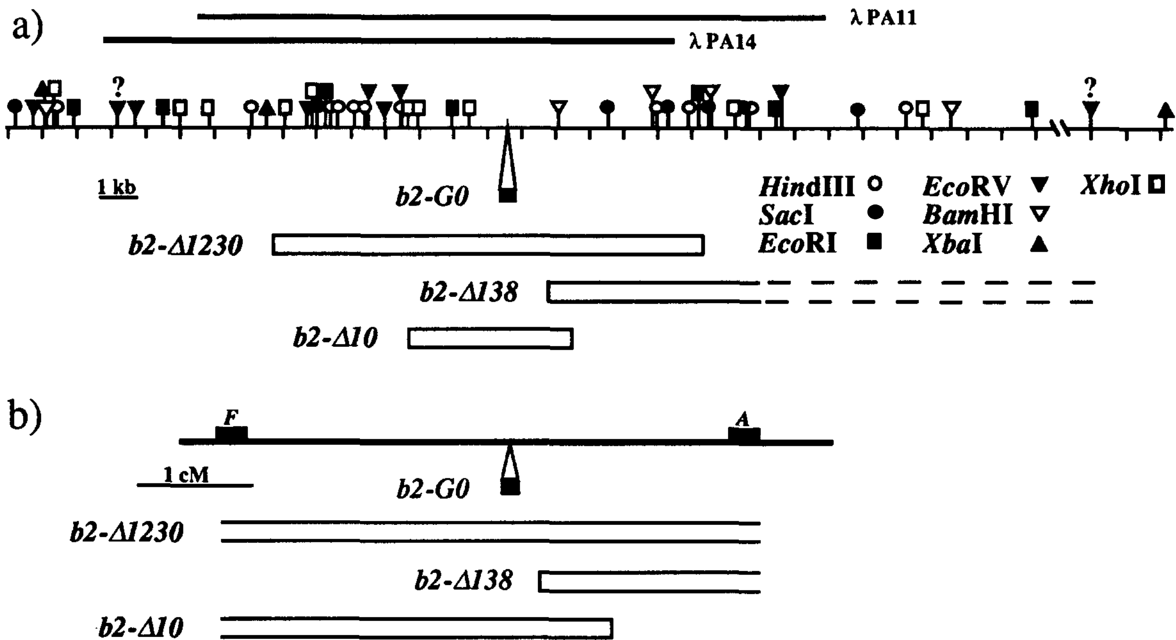

FIGURE 3.—Physical and genetical maps of the *b2* region and of four mutant *b2* alleles. (a) Physical maps. Question mark over a restriction site indicates that the presence of this site could not be determined unambiguously. For each enzyme, the leftmost and rightmost sites that are indicated were identified by hybridization of Southern blots of genomic DNA with λPA14 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-G0, 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-G0* 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).

mutant rescue. This therefore suggests that they were produced by transformants harboring defective *b2* transgenes that are nevertheless capable of inactivating the resident $b2^+$ allele by MIP.

In marked contrast to progeny of categories 1 and 3, those of categories 2 and 4 also contained asci with pale brown-reddish spores (described hereafter as pink spores) and thus showed partial inactivation in addition to complete inactivation of *b2.* Moreover, progeny of categories 2 and 4 were unique in exhibiting postmeiotic segregations of spore color (white/pink, brown/pink and, more rarely, brown/white) and hence in showing that spores produced from a given meiotic product could display different levels of *b2* activity. Pink spores varied in number from one to four per ascus in progeny of category 4 and reflected the partial inactivation of the resident $b2^+$ allele. As for the pink spores present in progeny of category 2, they occasionnally varied in number from five to eight per ascus, indicating in these rare instances partial inactivation of the resident and transgenic *b2* alleles, but mostly varied in number from one to four, and were then always associated

TABLE 1

Categories of progeny produced in crosses of primary transformants with *b2-Δ1230* tester strains

Category number	Transformants obtained with plasmids pVMB2-1 + pVMB2-2	Asci				
		4B:4W	0B:8W	6B:2W	8B:0W	Others
1	1 + 1	+	+	+	+	—
2	1 + 1	+	+	+	+	+[a]
3	11 + 1	+	+	—	—	—
4	1 + 0	+	+	—	—	+[b]
5	7 + 3	+	—	—	—	—

[a] Mostly asci with one to four pink spores.
[b] Asci with pink spores together with at least four white spores.

TABLE 2

Progeny produced in backcrosses of selected transformants[a] with *b2-Δ1230* tester strains

Name of primary transformant[b] (and category of progeny produced)	Asci[c] (500 scored per progeny)						
	4B:4W	≤4B/P:≥4W	6B:2W	≤6B/P:≥2W	8B:0W	≤8B/P:≥0W	0B:8W
T1 (category 1)	25 (5.0)	—	101 (20.2)	—	30 (6.0)	—	344 (68.8)
T2 (category 2)	41 (8.2)	16 (3.2)	19 (3.8)	108 (21.6)	2 (0.4)	20 (4)	294 (58.8)
T3 (category 1)	9 (1.8)	—	37 (7.4)	—	4 (0.8)	—	450 (90.0)
T4 (category 3)	146 (29.2)	—	—	—	—	—	354 (70.8)
T5 (category 4)	85 (17.0)	181 (36.2)	—	—	—	—	234 (46.8)
T6 (category 3)	219 (43.8)	—	—	—	—	—	281 (56.2)

[a] 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.

[b] Primary transformants T1, T4 and T5 were obtained with plasmid pVMB2.1, the other three transformants T2, T3 and T6 with plasmid pVMB2.2.

[c] ≤4B/P:≥4W: asci with four or less colored spores (brown or pink), other than 4B:4W asci. ≤6B/P:≥2W: asci with six or five colored spores (brown or pink), other than 6B:2W asci. ≤8B/P:≥0W: asci with eight or seven colored spores (brown or pink), other than 8B:0W asci.

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-Δ1230* 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-Δ1230* 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-Δ1230* 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^r) were isolated from the progeny of these primary transformants and back-crossed with *b2-Δ1230* 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-Δ1230* 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*

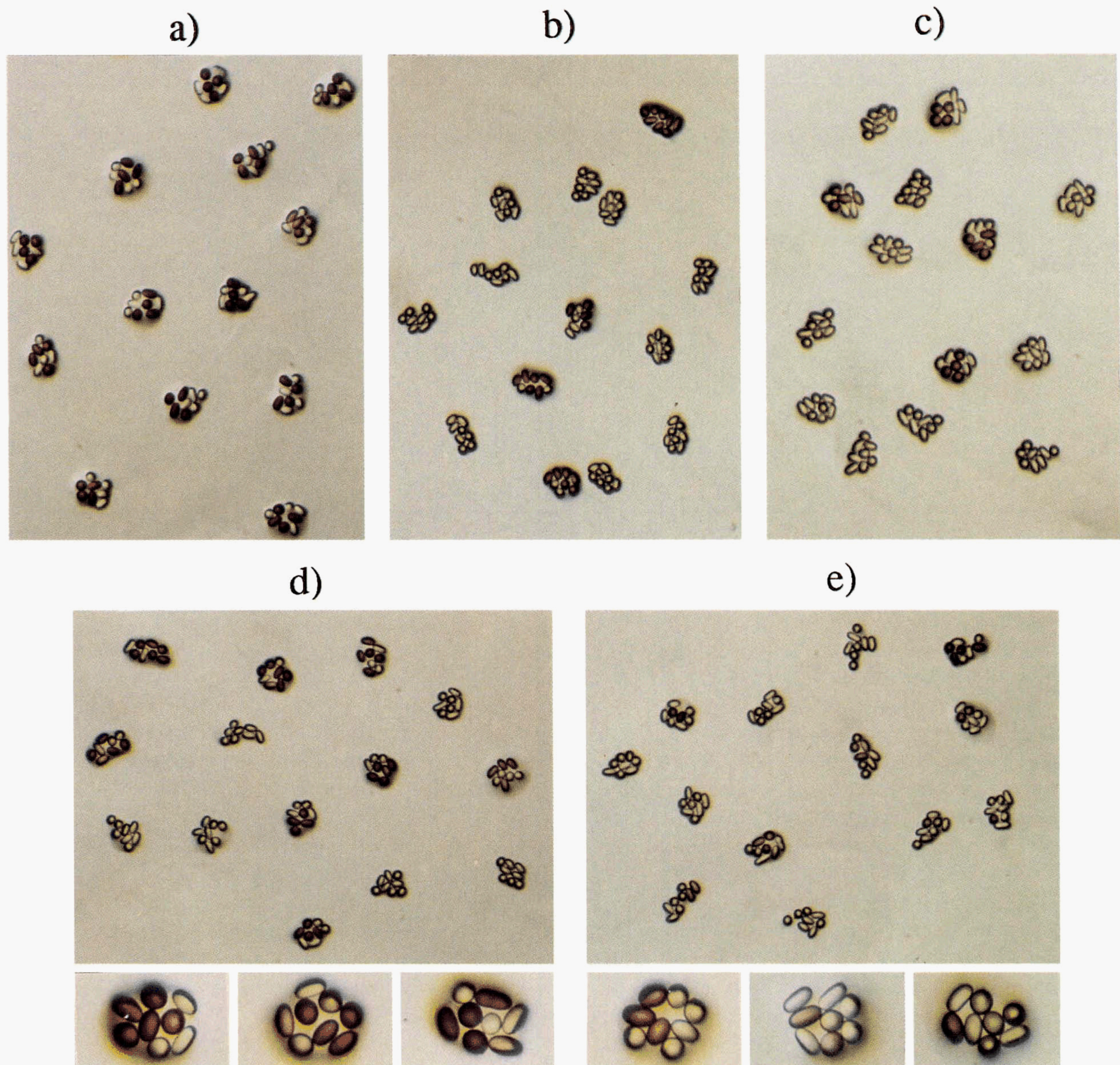

FIGURE 4.—Examples of the four categories of mixed progeny observed in test-crosses of selected transformants with *b2-Δ1230* strains. Transformants contained the resident $b2^+$ allele in addition to one transgenic locus. To help detect postmeiotic segregations of spore color, the spore shape marker *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 *b2-Δ1230* 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 λPA11 insert used for transformation, Southern blots of restricted DNA of transformed progeny strains also harboring the *b2-Δ1230* resident allele were probed successively with the entire 18-kb λPA11 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 λPA11 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 trangenic 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 chosen 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 *Nde*II and its methylation-sensitive isoschizomer *Sau*3A. After transfer, digests were hybridized to the 13-kb *Xba*I 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 *Nde*II 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 *Nde*II/*Sau*3A 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 *Nde*II and *Sau*3A, indicating that none of the probed *Nde*II/*Sau*3A sites are methylated in these strains. In marked

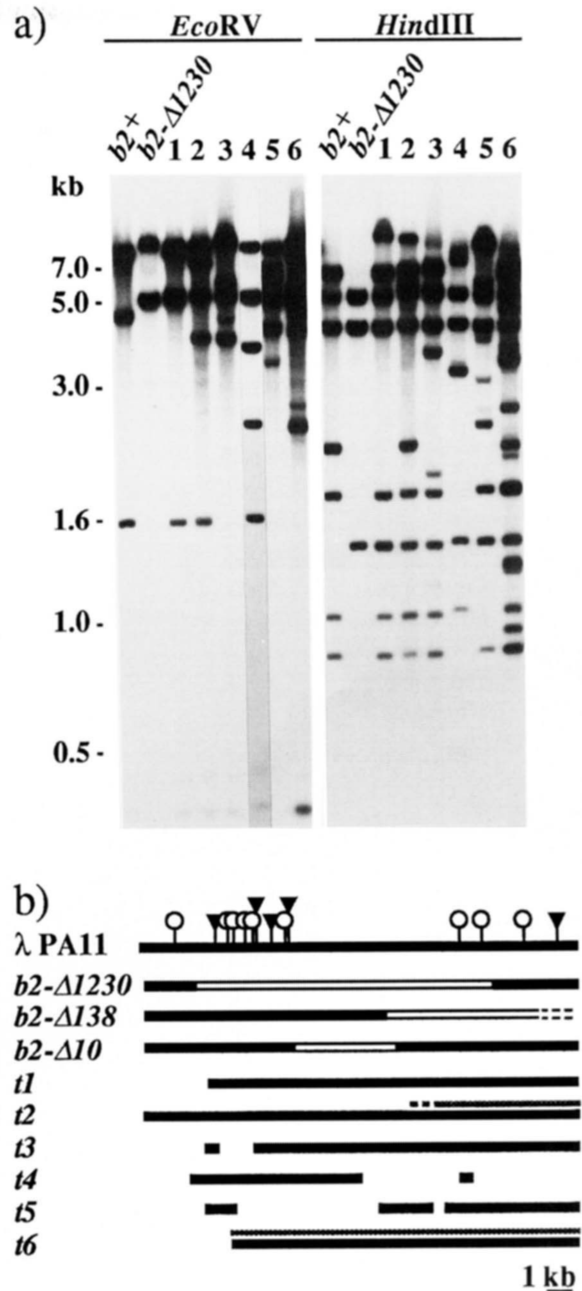

FIGURE 5.—Molecular characterization of six transgenic loci. (a) Examples of the Southern blot analysis of transgenes *t1–t6* (lanes 1–6) in the *b2-Δ1230* mutant background. Results were obtained after hybridization with the entire 18-kb λPA11 insert. (b) Extent of sequences derived from the 18-kb λPA11 insert that are present in the six transgenes *t1–t6*. *Hin*dIII sites (○) and *Eco*RV sites (▼) are only indicated for the λPA11 insert. Regions that are duplicated within transgenic loci are designated by a gray line above the corresponding map. The left end of the duplication present in *t2* was not determined precisely (dashed grey line).

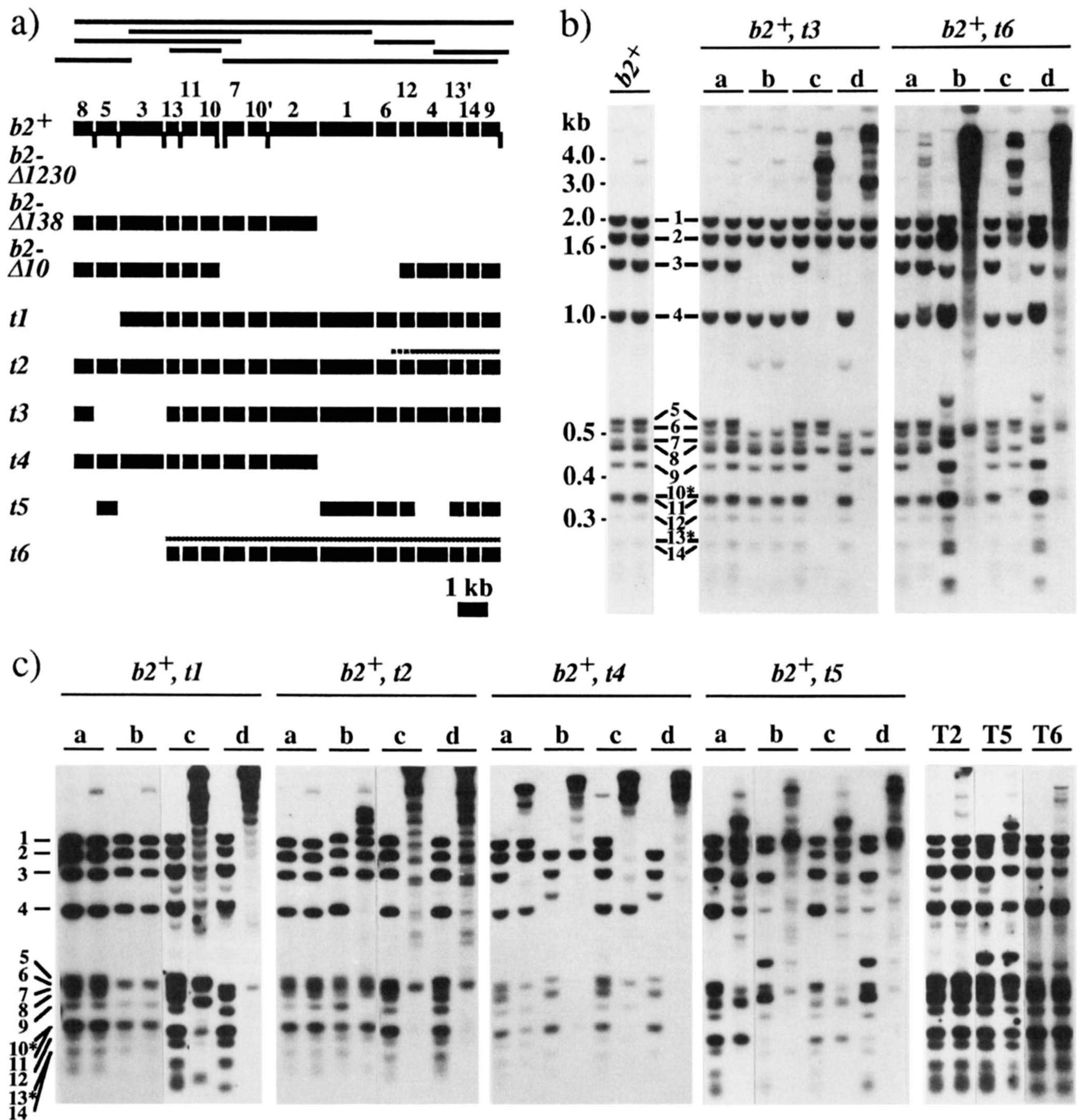

FIGURE 6.—Methylation analysis of the $b2^+$ resident allele and of the six transgenic loci *t1–t6* in the progeny of test crosses with *b2-Δ1230* strains. (a) *Nde*II/*Sau*3A restriction maps of the $b2^+$ resident allele, of the the two mutants *b2-Δ138* and *b2-Δ10* and of the six transgenic loci *t1–t6*. These maps are limited to the ~13-kb region that is completely absent in the mutant *b2-Δ1230*. Only the *Nde*II/*Sau*3A 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. *Nde*II/*Sau*3A 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 *Xba*I site of the λPA14 insert to the *Xba*I site present in the polylinker of the lambda DASH II vector. For each pair of lanes a, b, c and d, the *Nde*II digest is on the left and the *Sau*3A 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 *Nde*II/*Sau*3A sites is indicated by the replacement of one or several of the 16 *Nde*II fragments by fragments of higher molecular weight in the *Sau*3A restriction digests. A faint hybridizing band of 3 kb is always detected in the *Sau*3A digests. Analysis of cloned DNA indicates that it results

contrast, clear signs of partial or complete methylation of many or all of the probed *Nde*II/*Sau*3A 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 premeiotic 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, *Hha*I, 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 *Hha*I digests were in keeping with those obtained with the enzyme *Sau*3A (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 nonestablishement 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, 5e and 6a of Figure 7). Examination of profiles 2b and 6a suggests, for example, that methylation starting from the *Nde*II/*Sau*3A site located between fragments 4 and 13′ (site 4/13′) 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*-Δ*1230*, *b2*-Δ*138* and *b2*-Δ*10*. 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

from site 2/1 being partially resistant to digestion, independent of any methylation. Completeness of digestion was verified by hybridizing the Southern blots with the 2.3-kb fragment that extends from the left end of the λPA14 insert to the first *Xho*I site of that insert. This was also used to verify that methylation never extended outside of the region duplicated in the various transformants. The origin of the extra bands in the *Sau*3A digests of T5 and T6 DNA was not investigated.

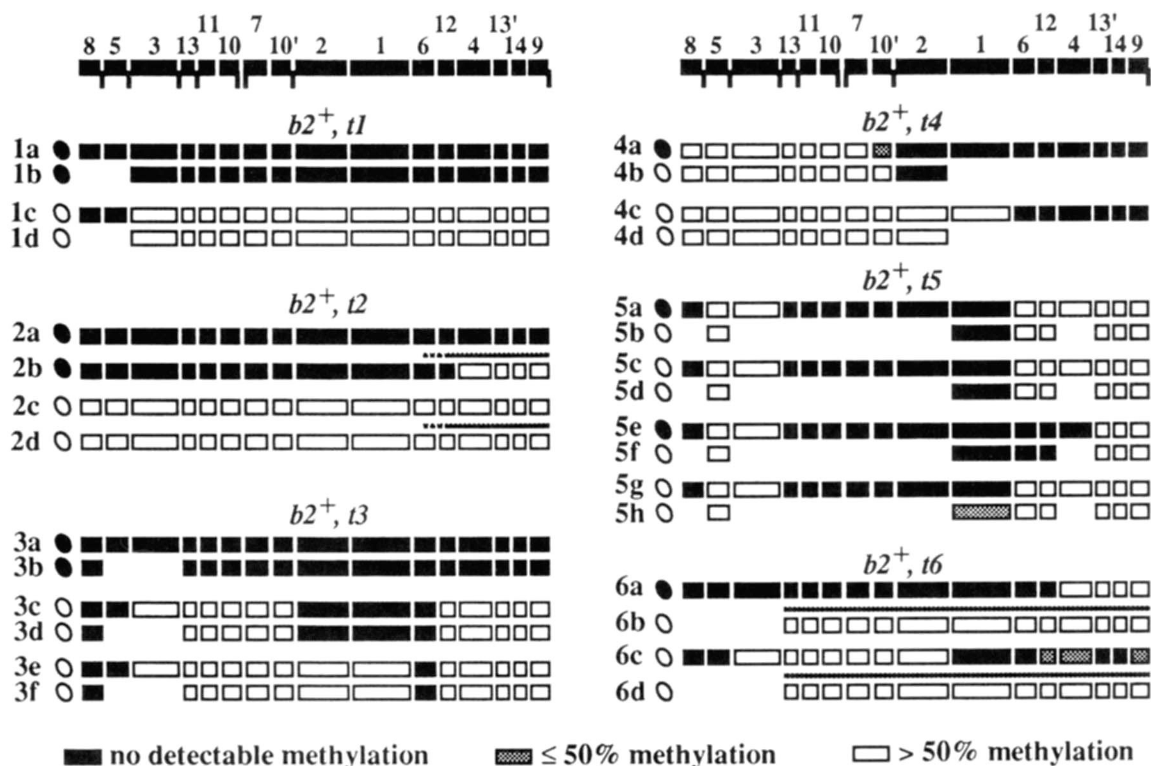

FIGURE 7.—Methylation profiles of the *b2*⁺ resident allele and of the six transgenic loci *t1–t6* in the progeny of test crosses with *b2-Δ1230* strains. The color of the spores from which they derive is indicated on the left of each profile (filled ovals, brown spores; open ovals, white spores). Three levels of methylation are distinguished. Each pair of profiles relates to a resident *b2*⁺ allele (top) and a transgenic locus (bottom) derived from a single ascus. Profiles a–d refer to the identically marked lanes of Figure 6. Note that methylation of fragments that are solely present on the resident *b2*⁺ allele (fragments 3 of 3c, 3e, 5a, 5c, 5e, 5g and 6c; fragment 1 of 4c; fragment 4 of 5a, 5c and 5g) most certainly results from the presence of part of these fragments (not 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 λPA11 insert indicated that methylation always extends left of fragment 8 when it is itself methylated, never otherwise. Similarly, methylation to the right of fragment 9 was always observed when this fragment was methylated, never otherwise.

locus *t3*, and raises the possibility that the nonfunctionality of transgenic locus *t6* 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 *b2* inactivation, since although identical, they relate to an active and an inactivated resident *b2*⁺ 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 5e) compared to the inactivated resident *b2*⁺ allele (profile 5g). Incidentally, this latter allele showed a profile identical to profiles 5a (*b2* active) and 5c (*b2* inactive) (Figure 7). Thus, while apparent methylation differences can exist between active and inactivated resident *b2*⁺ alleles subjected to MIP as a consequence of the duplication generated by the transgenic locus *t5*, this is not always the case (see next section).

Partial inactivation of *b2* 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 asci. Furthermore, in three cases the mixed progeny also contained asci 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 *b2* 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 *t1*, *t3*, *t4* and *t6*, which lead to complete inactivation only, to those of transgenic loci *t2* and *t5*, which can also give rise to partial inactivation, shows that these two transgenes are unique in creating partial duplications of *b2* that are limited to sequences right of fragment 2 (see Figures 5b and 6a) (even though transgenic locus *t2* also contains a functional copy of *b2*). 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 asci with pink spores that were produced in test-crosses of strains car-

rying the resident $b2^+$ allele and *t2* indicates that in the majority of these asci, inactivation (either partial or complete) affects *t2* alone, and thus that it occurs through an interaction that necessarily involves the partial copy of *b2* 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 *b2* carried by the transgenic locus *t2* 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 $b2^+$ allele. (Applying the same reasoning, it is also expected that fully inactivated transgenic copies of *b2* derived from asci with four active copies of the resident $b2^+$ allele will be systematically and exclusively methylated along the internal duplication carried by *t2* and would therefore show methylation profiles similar to that of active or partially inactivated copies of *t2*). 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 $b2^+$ alleles issued from the progeny of *t5* transformants (Figure 7). Finally, the observation of frequent meiotic and postmeiotic segregation of spore color (reflecting differences of *b2* activity in sister spores) in the progeny of both *t2* and *t5* transformants also indicates that probably few if any visible differences in methylation are associated with active, fully inactivated or partially inactivated copies of *b2* that are only methylated to the right of the *Nde*II/*Sau*3A 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 *b2* expression, as opposed to a simple all or none effect.

Effects of the three deletions *b2-Δ1230*, *b2-Δ138* and *b2-Δ10* 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 *b2* of subjecting different parts of the gene to the MIP process. To this effect, strains carrying the structurally simple and functional transgenic locus *t1* were first crossed with *b2-Δ138* and *b2-Δ10* mutant strains, just as had previously been done with *b2-Δ1230* mutant strains. In this manner, progeny strains could be isolated that harbored an active *b2* transgene in one or the other of the three *b2* 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 *b2* transgene was ever observed in the progeny of crosses involving the *b2-Δ1230* 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 *b2-Δ1230* and *t1* has no effect on *b2* expression. In marked contrast, self-crosses involving the *b2-Δ138* mutant background produced frequent and complete inactivation of the *b2* copy harbored by *t1*, in full agreement with results obtained earlier with $b2^+$, *t4* transformants, which generate a duplication similar to that found between *t1* and mutant *b2-Δ138*. Finally, both partial and complete inactivation were observed in the progeny of crosses involving the *b2-Δ10* 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 *b2* activity (see above). This therefore confirms results obtained previously with $b2^+$,*t2* and $b2^+$,*t5* 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 *b2* region. A detailed physical map of that region was obtained, and four genetically defined large alterations of *b2* were molecularly characterized. This opens up the prospect of molecular studies on genetic recombination based on *b2*, notably on the precise nature of the difference of recombination properties exhibited by different *b2* mutations.

We have also shown by transformation of *A. immersus* that all of the sequences necessary and sufficient for the proper expression of *b2* are contained within at most 18 kb and that like *met2*, *b2* 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 *b2* 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 *b2* 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

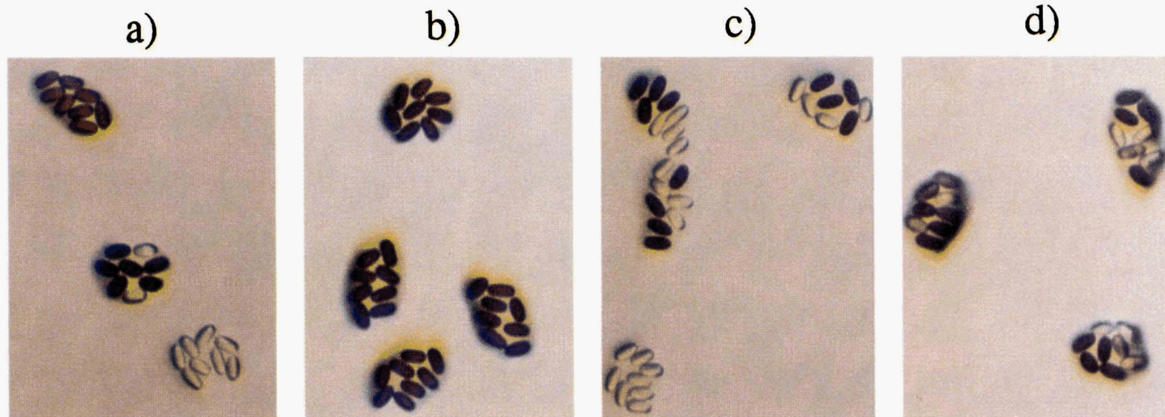

FIGURE 8.—Recapitulation of the effects of duplicating various parts of *b2* on gene inactivation by MIP. Self-crosses were performed with strains containing the functional *b2* transgenic locus *t1,* and one or the other of four distinct resident *b2* alleles. (a) *t1, b2*$^+$*:* the two asci at the bottom indicate that inactivation of both *t1* 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 asci were observed in the progeny of this cross. (b) *t1, b2-Δ1230:* only 8B:0W asci were observed in this cross, indicating the absence of any detectable inactivation of *t1.* (c) *t1, b2-Δ138:* inactivation of *t1* after MIP in one parent only (4B:4W asci) or in both parents (0B:8W ascus). 8B:0W asci were also observed in the progeny of this cross (not shown). (d) *t1, b2-Δ10:* full and partial inactivation of *t1* (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).

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 (D. ZICKLER, personal communication). Our new data, based on the spore color phenotype provided by *b2,* indicate that gene inactivation must occur at the latest after the first postmeiotic cell division after MIP. This result would suggest that the initial premeiotic imprint produced by MIP, be it methylation or some other form of DNA or chromatin modification that is later translated into methylation, leads immediately, or after only a few cell divisions, to gene inactivation.

Variegated gene expression as a result of MIP: As mentioned above, *b2* is unique among the four markers now available for the study of MIP in allowing observations to be made in spores, that is directly 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

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 *b2-Δ10* 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 *Nde*II/*Sau*3A 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 *Ascobolus 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.

The *b2-Δ1230* and *met2Δ::amdS* alleles and the *hph* marker gene were kindly provided by ALAIN NICOLAS, CAROLINE BARRY and SALAH SALAMA, respectively. We are grateful to PATRICK ZARZOV and ANNIE GRÉGOIRE for their help with the cloning of PERT-DNA and with *A. immersus* transformation, respectively, and to VICKI HAEDENS for participating in the preliminary analysis of *A. immersus* transformants. V.C. is especially indebted to ALAIN NICOLAS and members of his laboratory for providing a constant source of inspiration during the course of this work. We are grateful to GODELEINE FAUGERON, EDITH HEARD, CHRISTOPHE GOYON and LAURENT MALOISEL for helpful comments on the manuscript. This work was initiated under the tenure of an European Molecular Biology Organization postdoctoral fellowship to V.C. and was supported by grants from the Association pour la Recherche sur le Cancer (6200), the Ligue Nationale Française Contre le Cancer, the Fondation pour la Recherche Médicale and the Institut National de la Santé et de la Recherche Médicale (910114).

LITERATURE CITED

ALANI, E., R. A. G. REENAN and R. D. KOLODNER, 1994 Interaction between mismatch repair and genetic recombination in *Saccharomyces cerevisiae*. Genetics **137:** 19–39.

BARRY, C., G. FAUGERON and J.-L. ROSSIGNOL, 1993 Methylation induced premeiotically in Ascobolus: Coextension with DNA repeat lengths and effect on transcript elongation. Proc. Natl. Acad. Sci. USA **90:** 4557–4561.

CAO, L., E. ALANI and N. KLECKNER, 1990 A pathway for generation and processing of double strand breaks during meiotic recombination in *S. cerevisiae*. Cell **61:** 1089–1101.

DE MASSY, B., and A. NICOLAS, 1993 The control in *cis* of the position and the amount of the *ARG4* meiotic double-strand break of *Saccharomyces cerevesiae*. EMBO J. **12:** 1459–1466.

FAN, Q., F. XU and T. D. PETES, 1995 Meiosis-specific double strand DNA breaks at the *HIS4* recombination hot spot in the Yeast *Saccharomyces cerevisiae:* control in *cis* and *trans*. Mol. Cell. Biol. **15:** 1679–1688.

FAUGERON, G., C. GOYON and A. GRÉGOIRE, 1989 Stable allele replacement and unstable non-homologous events during transformation of *Ascobolus immersus*. Gene **76:** 109–119.

FAUGERON, G., L. RHOUNIM and J.-L. ROSSIGNOL, 1990 How does the cell count the number of ectopic copies of a gene in the premeiotic inactivation process acting in *Ascobolus immersus*. Genetics **124:** 585-591.

FINCHAM, J. R. S., I. CONNERTON, E. NOTARIANNI and K. HARRINGTON, 1989 Premeiotic disruption of duplicated and triplicated copies of the *Neurospora crassa am* (glutamate dehydrogenase) gene. Curr. Genet. **15:** 327–334.

FOSS, H. M., and E. U. SELKER, 1991 Efficient DNA pairing in a Neurospora mutant defective in chromosome pairing. Mol. Gen. Genet **231:** 49–52.

GOYON, G., and G. FAUGERON, 1989 Targeted transformation of *Ascobolus immersus* and *de novo* methylation of the resulting duplicated DNA sequences. Mol. Cell. Biol. **9:** 2818–2827.

GOYON, C., G. FAUGERON and J.-L. ROSSIGNOL, 1988 Molecular cloning and characterization of the met2 gene from *Ascobolus immersus*. Gene **63:** 297–308.

GOYON, C., T. I. V. NOGUEIRA and G. FAUGERON, 1994 Perpetuation of cytosine methylation in *Ascobolus immersus* implies a novel type of maintenance methylase. J. Mol. Biol. **240:** 42–51.

HABER, J. E., B. L. RAY, J. M. KOLB and C. I. WHITE, 1993 Rapid kinetics of mismatch repair of heteroduplex DNA that is formed during recombination in yeast. Proc. Natl. Acad. Sci. USA **90:** 3363–3367.

HAMZA, H., A. KALOGEROPOULOS, A. NICOLAS and J.-L. ROSSIGNOL, 1986 Two mechanisms for directional gene conversion. Proc. Natl. Acad. Sci. USA **83:** 7386–7390.

Kohne, D. E., S. A. Levison and M. J. Byers, 1977 Room temperature method for increasing the rate of DNA reassociation by many thousand fold: the phenol emulsion reassociation technique. Biochemistry **16:** 5329–5341.

Kunkel, L. M., A. P. Monaco, W. Middlesworth, H. D. Ochs and S. A. Latt, 1985 Specific cloning of DNA fragments absent from the DNA of a male patient with an X chromosome deletion. Proc. Natl. Acad. Sci. USA **82:** 4778–4782.

Laird, P. W., and R. Jaenisch, 1994 DNA methylation and cancer. Hum. Mol. Genet. **3:** 1487–1495.

Leblon, G., and J.-L. Rossignol, 1973 Mechanism of gene conversion in *Ascobolus immersus.* III. The interaction of heteroalleles in the conversion process. Mol. Gen. Genet. **122:** 165–182.

Li, E., T. H. Bestor and R. Jaenish, 1992 Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell **69:** 915-926.

Lichten, M., C. Goyon, N. P. Schultes, D. Treco, J. W. Szostak *et al.*, 1990 Detection of heteroduplex DNA molecules among the products of *Saccharomyces cerevisiae* meiosis. Proc. Natl. Acad. Sci. USA **87:** 7653–7657.

Lisitsyn, N., N. Lisitsyn and M. Wigler, 1993 Cloning the differences between two complex genomes. Science **259:** 946–951.

Martienssen, R. A., and E. J. Richards, 1995 DNA methylation in eukaryotes. Curr. Opin. Genet. Dev. **5:** 234–242.

Moulton, T., T. Crenshaw, Y. Hao, J. Moosikasuwan, N. Lin *et al.*, 1994 Epginetic lesions at the *H19* locus in Wilms' tumor patients. Nature Genet. **7:** 440–447.

Nag D. K., and T. D. Petes, 1993 Physical detection of heteroduplexes during meiotic recombination in the yeast *Saccharomyces cerevisiae.* Mol. Cell. Biol. **13:** 2324–2331.

Nag, D. K., M. A. White and T. D. Petes, 1989 Palindromic sequences in heteroduplex DNA inhibit mismatch repair in yast. Nature **340:** 318-320.

Nicolas, A., 1979 Variation of gene conversion and intragenic recombination frequencies in the genome of *Ascobolus immersus.* Mol. Gen. Genet. **170:** 129–138.

Nicolas, A., and T. D. Petes, 1994 Polarity of meiotic gene conversion in fungi: contrasting views. Experientia **50:** 242–252.

Nicolas, A., and J.-L. Rossignol, 1989 Intermediates in homologous recombination revealed by marker effects in Ascobolus. Genome **31:** 528–535.

Nicolas, A., S. Arnaise, V. Haedens and J.-L. Rossignol, 1981 Ascospore mutants and genetic map of *Ascobolus immersus* stock 28. J. Gen. Microbiol. **125:** 257–272.

Nicolas, A., D. Treco, N. P. Schultes and J. W. Szostak, 1989 An initiation site for meiotic gene conversion in the yeast *Saccharomyces cerevisiae.* Nature **338:** 35–39.

Paquette, N., 1978 Detection of aberrant 4:4 asci in *Ascobolus immersus.* Can. J. Genet. Cytol. **20:** 9–17.

Rainier, S., and A. P. Feinberg, 1994 Genomic imprinting, DNA methylation, and cancer. J. Natl. Cancer Inst. **86:** 753–759.

Rhounim, L., J.-L. Rossignol and G. Faugeron, 1992 Epimutation of repeated genes in *Ascobolus immersus.* EMBO J. **11:** 4451–4457.

Rhounim, L., A. Grégoire, S. Salama and G. Faugeron, 1994 Clustering of multiple transgene integrations in highly-unstable *Ascobolus immersus* transformants. Curr. Genet. **26:** 344–351.

Rizet, G., N. Engelman, C. Lefort, P. Lissouba and J. Mousseau, 1960 Sur un Ascomycete intéressant pour l'étude de certains aspects du problème de la structure du gène. C. R. Acad. Sci. Paris **250:** 2050–2052.

Rizet, G., J.-L. Rossignol and C. Lefort, 1969 Sur la variété et la spécificité des spectres d'anomalies de ségrégation chez *Ascobolus immersus.* C. R. Acad. Sci. Paris **269:** 1427–1430.

Rossignol, J.-L., A. Nicolas, H. Hamza and A. Kalogeropoulos, 1988 Recombination and gene conversion in Ascobolus, pp. 23–72 in *The Recombination of Genetic Material,* edited by B. Low. Academic Press, New York.

Rossignol, J.-L., and G. Faugeron, 1994 Gene inactivation triggered by recognition between DNA repeats. Experientia **50:** 307–317.

Rossignol, J.-L., and G. Faugeron, 1995 MIP: an epigenetic gene silencing process in *Ascobolus immersus,* pp. 179–191 in *Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes,* edited by P. Meyer. Springer-Verlag, Berlin.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Selker, E. U., 1990 Premeiotic instability of repeated sequences in *Neurospora crassa.* Annu. Rev. Genet. **24:** 579–613.

Selker, E. U., D. Y. Fritz and M. J. Singer, 1993 Dense nonsymetrical DNA methylation resulting from repeat-induced point mutation in Neurospora. Science **262:** 1724–1728.

Singer, M. J., and E. U. Selker, 1995 Genetic and epigenetic inactivation of repetitive sequences in *Neurospora crassa:* RIP, DNA methylation and quelling, pp. 165–177 in *Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes,* edited by P. Meyer. Springer- Verlag, Berlin.

Steenman, M. J. C., S. Rainier, C. J. Dobry, P. Grundy, I. L. Horon *et al.*, 1994 Loss of imprinting of *IGF2* is linked to reduced expression and abnormal methylation of *H19* in Wilms' tumor. Nature Genet. **7:** 433-439.

Straus, D., and F. M. Ausubel, 1990 Genomic subtraction for cloning DNA corresponding to deletion mutations. Proc. Natl. Acad. Sci. USA **87:** 1889–1893.

Sun, H., D. Treco, N. P. Schultes and J. W. Szostak, 1989 Double-strands breaks at an initiation site for meiotic gene conversion. Nature **338:** 87–90.

Weiner, B. M., and N. Kleckner, 1994 Chromosome pairing *via* multiple interstitial interactions before and during meiosis in yeast. Cell **77:** 977–991.

Wieland, I., G. Bolger, G. Asouline and M. Wigler, 1990 A method for difference cloning: gene amplification following subtractive hybridization. Proc. Natl. Acad. Sci. USA **87:** 2720–2724.

Communicating editor: P. J. Pukkila