

Suppression of crossing-over by DNA methylation in *Ascobolus*

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Homologous recombination between dispersed DNA repeats creates chromosomal rearrangements that are deleterious to the genome. The methylation associated with DNA repeats in many eukaryotes might serve to inhibit homologous recombination and play a role in preserving genome integrity. We have tested the hypothesis that DNA methylation suppresses meiotic recombination in the fungus *Ascobolus immersus*. The natural process of methylation-induced premeiotically (MIP) was used to methylate the *b2* spore color gene, a 7.5-kb chromosomal recombination hot spot. The frequency of crossing-over between two markers flanking *b2* was reduced several hundredfold when *b2* was methylated on the two homologs. This demonstrates that DNA methylation strongly inhibits homologous recombination. When *b2* was methylated on one homolog only, crossing-over was still reduced 50-fold, indicating that the effect of methylation cannot be limited to the blocking of initiation of recombination on the methylated homolog. On the basis of these and other observations, we propose that DNA methylation perturbs pairing between the two intact homologs before recombination initiation and/or impairs the normal processing of recombination intermediates.

[Key Words: *Ascobolus immersus*; chromosome rearrangements; crossing-over; DNA methylation; meiotic recombination; repeat DNA sequences]

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Crossing-over between dispersed DNA repeats results in chromosomal rearrangements. In eukaryotes, the destructive potential of dispersed repeats through ectopic homologous recombination is well documented (Rouyer et al. 1987; Montgomery et al. 1991; Small et al. 1997). In yeast, artificial duplications placed in ectopic position can interact and generate chromosomal rearrangements through homologous recombination at high frequency during meiosis (Lichten et al. 1987). In higher eukaryotes, the number of repeats per genome is often so high that no single cell would escape genomic rearrangements if ectopic recombination were to occur at high frequencies. Therefore, factors must exist that limit recombination between dispersed repeats. Thuriaux (1977) pointed out that the frequency of crossing-over per unit of physical DNA length decreases with increasing genome size, and proposed that recombination is confined to genes. According to this hypothesis, satellite DNA sequences and interspersed DNA repeats, which constitute the bulk of the intergenic regions, must be poor substrates for meiotic recombination even when in allelic positions. Nucleotidic divergence (Rayssiguier et al. 1989; Radman and Wagner 1993) and an insufficient length of

sequence identity (Shen and Huang 1986; Jinks-Robertson et al. 1993) are two factors known to reduce drastically homologous recombination. Nevertheless, other factors that suppress homologous recombination are required to protect genomes against the threat generated by families of long DNA repeats that have diverged little or not at all (e.g., after recent duplication events).

One way to identify such factors would be to determine whether DNA repeats share any feature that might suggest a role in preventing recombination. There is increasing evidence that cytosine methylation is one such common property in the eukaryotic genome that displays this form of DNA modification. In plants, the repeat-rich intergenic regions are often methylated (Benetzen et al. 1994). In mammals the bulk of DNA methylation involves DNA repeats (Yoder et al. 1997). In the fungus *Ascobolus immersus*, DNA methylation is typically restricted to DNA repeats (Goyon et al. 1996), most likely as a result of a process called methylation induced premeiotically (MIP) which detects DNA repeats efficiently before meiosis and leads to their methylation (Rhounim et al. 1992).

Some observations can be explained by the hypothesis that methylation serves as a genome stabilizer through the suppression of homologous recombination between dispersed sequences (Rossignol and Faugeron 1994; Yoder et al. 1997). In plants with large genomes, such as maize, recombination appears restricted to hypomethylated gene-containing regions (Whitkus et al. 1992). In

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humans, genomic regions subjected to parental imprinting, a sex-specific process in which methylation is likely to play a causal role (Barlow 1993; Razin and Cedar 1994), display significant differences in their frequencies of recombination during male and female meioses (Paldi et al. 1995). This last observation is in line with the hypothesis of Thomas and Rothstein (1991), who proposed that differences between male and female meiotic recombination frequencies would result from imprinting. Chromosomal translocations resulting from exchanges between decondensed and demethylated satellites are frequently associated with the immunodeficiency, centromeric heterochromatin instability and facial abnormalities (ICF) syndrome, and are also observed in culture cells treated by the demethylating agent 5-azacytidine (Miniou et al. 1994; Ji et al. 1997).

These observations, however, do not actually prove that DNA methylation suppresses homologous recombination. The only clear demonstration of an effect of methylation on recombination concerns V(D)J joining, which is decreased >100 times when tested on a methylated minichromosome substrate (Hsieh and Lieber 1992). Although abnormal V(D)J joining may generate chromosome rearrangements (Lieber 1992), it is not likely to play a generalized role in such events as it is a specialized recombination process restricted to very specific DNA motifs, and only occurs in specialized somatic cells of vertebrates. As for homologous recombination, a general process that takes place in any living organism, no direct evidence for an inhibitory effect of methylation has been established so far. Attempts to demonstrate such an effect, using extrachromosomal plasmids, have been unsuccessful (Puchta et al. 1992; Liang and Jasin 1995).

By using MIP to methylate a 7.5-kb chromosomal interval encompassing a meiotic recombination hot spot of *A. immersus*, we show here that DNA methylation reduces by several hundredfold the frequency of crossing-over within this interval. We provide additional experimental evidence indicating that this effect cannot be simply accounted for by an increased resistance of methylated DNA to meiotic endonucleases.

Results

Introduction of markers flanking the *b2* recombination hot spot and *in vivo* methylation of this hot spot

The possible inhibitory role of DNA methylation on crossing-over could be tested in *Ascobolus* thanks to the MIP process, which enables the *in vivo* methylation at will of gene-size sequences, and to the existence of a well-characterized recombination hot spot, the *b2* spore color gene (Nicolas and Rossignol 1989). The *b2* gene used in this study was flanked on a plasmid by an upstream marker, the *met2* gene from *Ascobolus*, and by a downstream marker that confers hygromycin resistance and is based on the *hph* gene of *Escherichia coli*. This plasmid was used to transform a haploid *Ascobolus*

strain deleted for the resident copies of the *met2* and *b2* genes. Correspondingly, this strain exhibited methionine auxotrophy (Met^-) and gave white ascospores in crosses. We selected one transgenic strain that was hygromycin resistant (Hyg^R), restored methionine prototrophy (Met^+), and displayed the wild-type brown ascospore phenotype. Southern blot analysis (data not shown) showed that the selected transgenic strain resulted from the integration of a single unrearranged copy of the plasmid construct (strain MBH: Met^+ , Brown spores, Hyg^R ; Fig. 1). The shortened designation of strains, in the following paragraphs, will use M and m for Met^+ and Met^- , respectively, B and b from brown spores and white spores, respectively, and H and h for Hyg^R and Hyg^S , respectively.

To detect crossing-over in the *b2* interval, an epimutated allele of each of the two markers flanking *b2* was

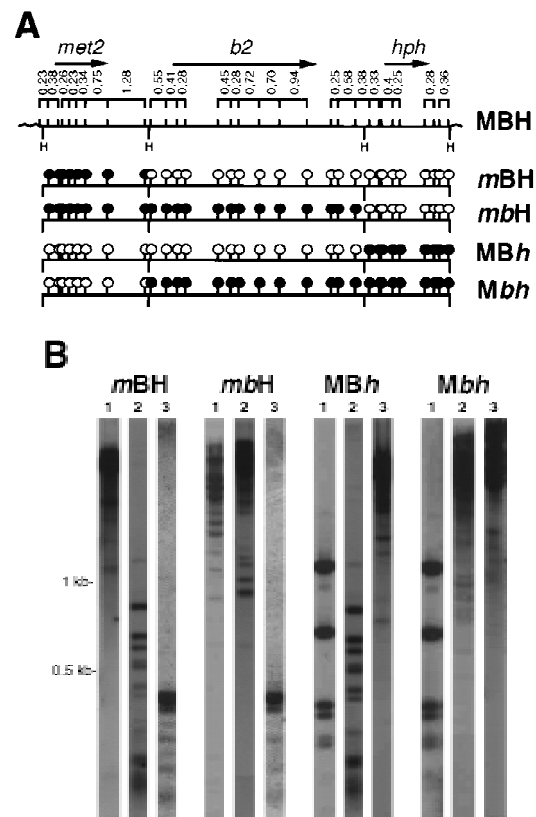


Figure 1. *HpaII* restriction map of the *met2*-*b2*-*hph* transgenic locus and DNA methylation profiles. (A) The MBH strain is the initial transformant carrying the transgenic locus. The three marker genes are each contained within a *HindIII* fragment (H). ORFs are indicated by arrows. Only *HpaII* sites bordering restriction fragments >200 bp are indicated (bars). The methylation status of the *HpaII* sites (sequence CCGG) present in the transgene is indicated [○ (unmethylated) or ● (methylated)] for the four different strains derived from the MBH initial strain. (B) *HpaII* restriction profiles revealed by Southern blot analyses using a *met2* probe (lane 1), a *b2* probe (lane 2), and a *hph* probe (lane 3) are shown for the four progeny strains. *HpaII* sites are not cut by the enzyme when methylated.

created using MIP and its gene silencing effect (Rhounim et al. 1992). Methylation and silencing of the entire *b2* interval separating the two markers was also achieved by MIP (see Materials and Methods). Strains in which *met2* alone, or both *met2* and *b2*, had been methylated and silenced by MIP were termed *mBH* and *mbH*, respectively (Fig. 1). Strains in which either *hph* alone or both *hph* and *b2* had been methylated and silenced were termed *MBh* and *Mbh*, respectively.

Methylation of the *b2* recombination hot spot leads to a several hundredfold decrease of crossing-over frequency

Crossing-over frequency between the *met2* and *hph* markers in the presence or absence of methylation of the *b2* interval was measured by performing two crosses (Fig. 2A). The two parental *b2* alleles were unmethylated in cross I (*MBh* × *mBH*), and methylated in cross II (*Mbh* × *mbH*). The frequency of crossing-over in these crosses was first assessed by ascus analysis. A total of 13 crossovers (tetratype asci) were detected in 200 asci analyzed in cross I. In the six tetratype asci that were analyzed at the molecular level by Southern blot analysis, crossing-over was confirmed with full methylation of both flanking markers in one recombinant product and no methylation of these in the reciprocal product (data not shown). In marked contrast, no crossover was detected in 200 asci from cross II. To estimate precisely the decrease in crossing-over frequency between crosses I and II, a random spore analysis was conducted. The fre-

quency of *Met*⁺, *Hyg*^R progeny was 15×10^{-3} among 26,500 germinated spores scored in cross I, and 0.4×10^{-3} among 114,000 germinated spores scored in cross II. Although the incidence of *Met*⁺, *Hyg*^R progeny should reflect crossing-over in the *b2* interval (Fig. 2A), it is also possible to obtain such progeny by reversion of the *met2* flanking marker. Indeed, unlike the silencing of *hph*, which never reverts spontaneously, the *met2* silencing undergoes reversion at a low frequency. Because the reversion of *met* silencing is never accompanied by a complete loss of methylation (Rhounim et al. 1992), it is possible to distinguish between reversion and crossing-over by analyzing the methylation status of the *met2* flanking marker. This was assessed using the methylation-sensitive restriction enzyme *Hpa*II on a fraction of the *Met*⁺, *Hyg*^R strains recovered from random spore analysis from crosses I and II (Fig. 3). Whereas true recombinants are expected to show no methylation of the flanking markers, revertant strains should show instead some residual methylation of the *met2* marker at all restriction sites tested. All 32 progeny strains analyzed from cross I showed methylation patterns indicative of crossing-over (Fig. 3). This indicates that the 15×10^{-3} *Met*⁺, *Hyg*^R progeny does reflect the frequency of true recombinants in cross I. Therefore, we can calculate the frequency of tetratype asci resulting from meiotic crossing-over (see Fig. 2A) in this cross. In these tetratype asci, only one meiotic product out of four is *Met*⁺, *Hyg*^R. Therefore, the frequency of crossing-over per meiosis in cross I is equal to four times the frequency of *Met*⁺, *Hyg*^R ($15 \times 10^{-3} \times 4$). The estimated value (60×10^{-3}) is close to


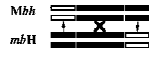


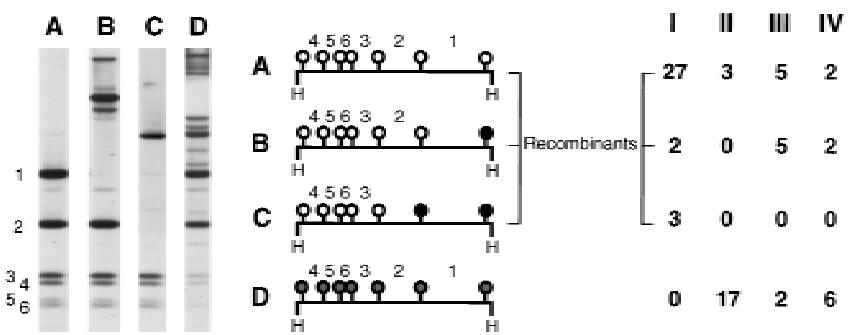
Cross	Ascus type	Total number	Analyzed sample	PD	TT	Tr.Met		Tr.Hph		Tr.Met+Hph				
						+	-	+	-	+	-			
A		I	8B:0W	-	200	164	13	4	3	8	5	2	1	
														
B		III	4B:4W	3929	106	104	0	1	0	1	0	0	0	
			2B:6W	39	26	14	1	7	0	2	1	0	0	1
			0B:8W	18	14	6	1	3	2	0	0	1	1	
			Others	14	12	12	0	0	0	0	0	0	0	
			4B:4W	3878	101	98	0	1	1	1	0	0	0	
IV		IV	2B:6W	72	15	12	1	1	0	1	0	0	0	
			0B:8W	26	6	4	2	0	0	0	0	0	0	
			Others	24	6	5	0	1	0	0	0	0	0	

Figure 2. Ascus analysis in crosses I to IV. (A) Crosses I (*MBh* × *mBH*) and II (*Mbh* × *mbH*). For each cross, the three marker genes are symbolized on the four chromatids at meiosis I by white rectangles when unmodified or black rectangles when methylated and silenced. Ascus of the progeny are indicated by their spore color phenotype (B, brown; W, white). In crosses I and II, they were composed exclusively (several thousand asci scored) of 8B:0W and 0B:8W asci, respectively. Because meiosis is followed by one cell division before ascospore formation, asci contain four pairs of spores that correspond to the four meiotic products. Segregation of the two flanking markers *met2* and *hph* in the different types of asci is shown (+: *Met*⁺; -: *Met*⁻; S: *Hyg*^S; R: *Hyg*^R). Parental ditype asci (PD), which reflect an absence of events, are the most frequently observed. Crossing-over, symbolized by crosses (left), are detected because they lead to tetratype

asci (TT, shaded rectangle). Three additional classes of asci were observed as the result of methylation transfer (↑, ↓ at left) from the methylated parental allele to the previously active and unmethylated parental *met2* allele (Tr.Met) and/or *hph* allele (Tr.Hph) and Tr.Met+Hph). (B) Crosses III (*mbH* × *MBh*) and IV (*Mbh* × *mBH*). These crosses, which involve one methylated allele and one unmethylated allele of *b2*, can give rise to asci showing a transfer of methylation at *b2* as well as at the two flanking markers. Methylation transfer at *b2* leads to three classes of asci with less than four brown spores (2B:6W, 0B:8W, and Others). (Others) Ascus with pink spores indicative of partial inactivation (Colot et al. 1996). A total of 4000 asci were scored in each cross.

Figure 3. Methylation pattern of the *met2* marker of Met⁺, Hyg^R strains. DNA was digested with the enzyme *Hpa*II. Southern blots were hybridized with the *met2* *Hind*III fragment. The *Hpa*II sites are indicated by solid circles when fully methylated, shaded circles when partially methylated, and an open circle when unmethylated. *Hpa*II fragments are numbered according to their size. The Roman numerals (I–IV) heading the columns (right), refer to the four genetic crosses in Fig. 2. Four distinct classes of methylation patterns (A,B,C,D) were observed. Within each class, all the strains analyzed exhibited a methylation pattern identical to that exemplified in the figure, except for class B. Classes A, B, and C are all indicative of crossing-over. In class A, there was no methylation at any of the *met2* restriction sites tested. In class B, there was also no methylation, except at the rightmost *Hpa*II site. Methylation of this site leads to the loss of the *Hpa*II fragment 1. In cross I, where *b2* is unmethylated, this fragment was replaced by one fragment 120 bp longer (not shown). In crosses III and IV, it was replaced by various fragments of higher molecular mass, depending on the methylation status of the neighboring *Hpa*II sites within *b2* (one example is shown). In class C, the two rightmost *Hpa*II sites were methylated. This led to the loss of *Hpa*II fragments 1 and 2 and to the appearance of a unique fragment of higher molecular mass. Methylation in classes B and C is restricted to the region closest to *b2*, as expected if they result from events initiated in the *b2* interval and propagating in the direction of *met2* (see paragraph on methylation transfers; Colot et al. 1996). Class D shows partial methylation of every *met2* *Hpa*II site corresponding to the reversion of silencing of the *met2* marker.



that obtained after analysis of individual asci (65×10^{-3}). Conversely, only 3 of 20 progeny strains analyzed from cross II showed no methylation of *met2* and therefore, were true recombinants. The 17 other progeny strains showed partial methylation of every site indicating the reversion of silencing of the *met2* marker (profile D in Fig. 3). As expected from the absence of spontaneous reversion of *hph* silencing, no methylation was ever found at the *hph* marker in all Met⁺, Hyg^R progeny tested (data not shown). On the basis of these results, the frequency of true recombinant Met⁺, Hyg^R strains was estimated to 0.06×10^{-3} (i.e., $0.4 \times 10^{-3} \times 3/20$) corresponding to a frequency of crossing-over per meiosis in cross II of 0.24×10^{-3} (i.e., $0.06 \times 10^{-3} \times 4$). Thus, in these experiments, methylation of *b2* reduced crossing-over 250-fold within that interval.

Methylation of only one parental *b2* allele is sufficient to decrease markedly the frequency of crossing-over

Crosses in which only one of the two parental *b2* alleles was methylated were also performed. In cross III (MBh \times mbH) the two methylated alleles *b2* and *met2* were associated in the same parent. In the reciprocal cross IV (MbH \times MBh), the methylated *b2* allele was associated with the methylated allele of *hph* (Fig. 2B). The frequencies of Met⁺, Hyg^R progeny were 0.5×10^{-3} and 0.6×10^{-3} , in crosses III and IV, respectively, among ~94,000 ascospores issued from each of these crosses. The number of true recombinants among these strains was estimated after Southern blot analysis, as before (Fig. 3). The deduced frequencies of crossing-over per meiosis were 1.5×10^{-3} and 1×10^{-3} in crosses III and IV, respectively. These values, which are ~50-fold lower than those obtained for cross I (60×10^{-3}), therefore, indicate that methylation of one parental *b2* allele is sufficient to

strongly decrease crossing-over. The samples studied do not allow us to decide whether this reduction is really smaller than that found in cross II.

Association of crossing-over with transfers of methylation at *b2*

Ascus analyses were also performed on the progeny of crosses III and IV. In both cases, a majority of the progeny was composed of asci with four brown and four white spores (4B:4W), indicative of the Mendelian segregation of the active and silenced parental *b2* alleles. Nevertheless, a fraction of asci displayed aberrant segregation patterns of spore color, distinguished by a deficit of brown-colored spores (Fig. 2B). These aberrant patterns reflect transfers of methylation and silencing during meiosis from the methylated parental *b2* allele to the formerly unmethylated parental *b2* allele (Colot et al. 1996; Fig. 4). On the basis of the analysis of a sample of each class of asci (4B:4W, 2B:6W, 0B:8W, and others), the frequency of crossing-over was estimated to be 0.7×10^{-3} in cross III and 3.3×10^{-3} in cross IV (Fig. 2B). Remarkably, all five crossing-over events detected arose in 2B:6W and 0B:8W asci and thus, were associated with a transfer of methylation. To investigate this further, the methylation status of the entire *met2*-*b2*-*hph* transgenic locus was assessed for each of the four meiotic products recovered from the five crossover asci (Fig. 5). Crossing-over was confirmed at the molecular level, with methylation of both flanking markers in one recombinant product and no methylation of these in the reciprocal product. All the crossing-over events were associated with methylation transfers extending to various lengths along the gene. More significant, the methylation transfer tracts always included the site of exchange. Indeed, the two recombinant products always showed a reciprocal exchange be-

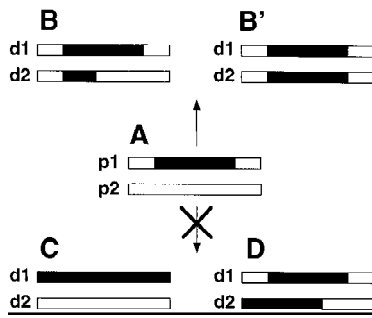


Figure 4. Properties of methylation transfer. Each pair of rectangles represents two allelic regions. Parental DNAs are named p1 and p2. (A) The p1 parental DNA displays methylation (black portion), the p2 parental DNA is unmethylated. The corresponding descendant DNAs after meiosis are named d1 and d2. Methylation transfers are characterized by the appearance of methylated segments of variable length in the d2 DNA issued from p2. This methylated segments always extends within a region allelic to that which was methylated in p1 (B,B'). Methylation never spreads in *cis* (C), nor is transferred to nonallelic regions (D) (from Colot et al. 1996; this study).

tween the unmethylated and the methylated parental molecules on either side of the methylation transfer tract. This indicates that crossing-over never occurred outside the region involved in transfer. In asci 1 and 5, methylation transfer observed in the Met⁺, Hyg^R product included the rightmost *met2* *Hpa*II site. This situation accounts for profile B in Figure 3.

Methylation transfers initiated in *b2* show a bidirectional polarity

Previously, we had shown that methylation transfer at the resident *b2* locus is polarized 5' to 3' (Colot et al. 1996). The same polarity was found in the present study as shown in Figure 6. Eleven of 12 transfer events analyzed in *b2* extend rightward from a region located in the left 5' part. Transfer tracts starting from this same origin extend toward the left and reach the *met2* fragment in four cases. Altogether, these transfers display a bidirectional polarity with only one exception.

The flanking markers *met2* and *hph* also exhibited inactivation transfers in crosses I and II (see Fig. 2A) and in crosses III and IV (see Fig. 2B). As expected, these events correspond to methylation transfers (data not shown). As for *b2*, inactivation transfers involved either one or the two meiotic products that would otherwise harbor the active allele, leading to asci with either 2Met⁺:6Met⁻ or 0Met⁺:8Met⁻, or either 2Hyg^R:6Hyg^S or 0Hyg^R:8Hyg^S. In crosses III and IV, transfers at *met2* and *hph* were associated frequently with a transfer at *b2* (18/20 and 3/6, respectively) (Fig. 2B). Because the process driving the methylation transfer seems to be bidirectional (Fig. 6), we expect that its propagation over longer distances may inactivate *b2* together with *met2* or *hph*.

Methylation of *b2* leads to a decrease in the frequency of methylation transfers

Remarkably, in crosses I–IV, the frequency of transfers at one or the other of the two flanking markers decreased when the *b2* interval was methylated, from 11.5% in cross I to 3% in crosses III and IV and to 1% in cross II. This result can be best explained if most of the methylation transfer events at *met2* and *hph* are initiated within the *b2* recombination hot spot. Therefore, DNA methylation affects negatively both homologous recombination and methylation transfer.

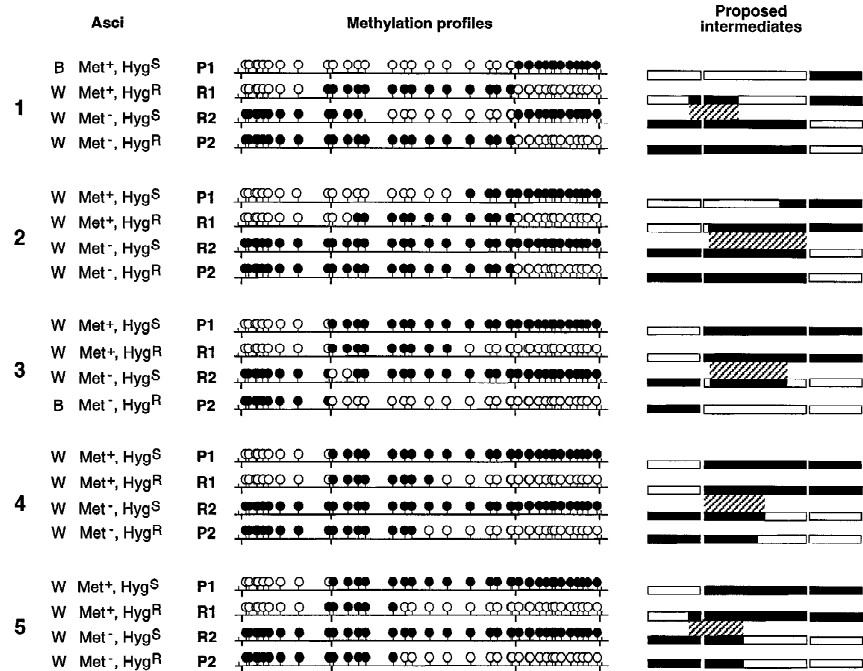
Discussion

By using a meiotic recombination hot spot, the *b2* gene, that could be methylated in vivo, we have performed a direct test on the effect of methylation on homologous recombination. Our results indicate that methylation of this hot spot in both parents suppresses allelic crossing-over efficiently between flanking markers. Methylation of one parental allele also affects drastically homologous recombination, resulting in a 50-fold reduction. In such crosses, methylation transfers to the unmethylated *b2* parental allele were observed frequently and the rare crossing-over events that were detected were always physically associated with these transfers. This latter result reinforces the conclusion that meiotic recombination in *b2* is mechanistically associated with methylation transfer (Colot et al. 1996) and must be considered in the discussion of the suppressing effect of DNA methylation on recombination.

Molecular insights into meiotic recombination come chiefly from studies in yeast. DNA–DNA pairing interactions are thought to occur at the sites of recombination initiation between the intact duplexes of homologous chromosomes (Weiner and Kleckner 1994). These early interactions have been proposed to play a role in the sensing of DNA homology and in the decision to make double strand breaks (DSBs) (Xu and Kleckner 1995; Rocco and Nicolas 1996). DSBs occur at specific sites that lie close to the high end of gene conversion gradients and are the first chemical disruptions of DNA in meiotic recombination (for review, see Lichten and Goldman 1995). DSBs are followed by the exonucleolytic degradation of their 5' ends to yield 3' single strand tails (Sun et al. 1991; Bishop et al. 1992). Invasion of the homologous chromatid by the broken strands occurs subsequently and leads to the formation of joint molecules stabilized by heteroduplex DNA and bordered by double Holliday junctions (Schwacha and Kleckner 1995). DNA repair synthesis from the 3' ends, together with mismatch correction of the heteroduplex DNA generated by the double Holliday junctions, leads to gene conversion, and resolution of these junctions leads to recombinant products that are either noncrossover or crossover (for other possibilities, see Schwacha and Kleckner 1995). The various steps after the break were predicted by the DSB model of meiotic recombination (Szostak et al. 1983; Sun et al. 1991). Most of the extensive genetic data

Maloisel and Rossignol

Figure 5. Methylation transfer events associated with crossing-over. The five asci from cross III (asci 1 and 2) and cross IV (asci 3, 4, and 5) showing a crossing-over event (Fig. 2B) are represented (left) with the phenotypes of their four meiotic products. The two parental products are designated P1 and P2; the two recombinant products are designated R1 and R2. The methylation profiles of the four meiotic products of each ascus were determined using the *HpaII* restriction enzyme. (●) Methylated *HpaII* sites; (○) unmethylated. The *HindIII* sites separating the three genes are indicated below the horizontal bar. (Right) Proposed intermediates after methylation transfer. *met2*, *b2*, and *hph* are represented by rectangles; methylated regions are in black and unmethylated regions are in white. For the sake of simplicity, crossing-over was dissociated from transfer of methylation in these drawings. The final products are obtained by performing a reciprocal exchange within the hatched region between the two homologs, which will recombine. This hatched region defines the length of the methylation transfer together with the region where crossing-over must have occurred. Crossing-over outside of this region would have given patched methylation profiles. Methylation transfer involves one (asci 1 and 3) or both (asci 2, 4, and 5) homologous chromatids.



accumulated on recombination within *b2* (Nicolas and Rossignol 1989) and the striking parallel between the bipolarity of gene conversion events in yeast and of methylation transfers in *Asco-bolus* justify the use of the DSB model as a legitimate framework to discuss at which step the recombination process is inhibited by methylation.

In the present study, the methylation of the *b2* gene results in its transcriptional inactivation. Several examples show a link between recombination and transcription (Stewart and Roeder 1989; Nickoloff and Reynolds 1990; Thomas and Rothstein 1992). Meiotic recombination is mainly initiated in promoter regions (Lichten and Goldman 1995). The deletion of these regions prevents the initiation of recombination on the deleted parental strand and leads to a twofold reduction in the frequency of recombination (Nicolas et al. 1989; Schultes and Szostak 1991). Nevertheless, it has been shown that the transcription process itself is not required for recombination. Indeed, although the promoter regions of *ARG4* and *HIS4* are necessary for homologous recombination in these genes, small deletions in their promoter resulting in a strong decrease of transcription, leave unaltered the rate of recombination (Schultes and Szostak 1991; White et al. 1992). These observations make unlikely the hypothesis that the decrease of recombination when *b2* is methylated results simply from a transcriptional effect unless the recombination features in *Asco-bolus* and in yeast are completely different. Independent studies in *Saccharomyces cerevisiae* (White et al. 1993) and *Schizosaccharomyces pombe* (Kon et al. 1997) indi-

cate that transcription factors play a role in inducing recombination, but that this is likely to be a consequence of an alteration of chromatin structure necessary for the initial break rather than an effect of these factors on transcription itself. In the present study, methylation preventing or altering the binding of transcription factors necessary for both transcription and the triggering of the initial break could account for the almost total suppression of crossing-over when the two parents are methylated. However, this hypothesis cannot explain the 50-fold reduction of crossing-over observed when only one parent is methylated. Indeed, in this case, the other parent is still transcribed, as attested by the segregation of spore color in meiotic products. This parent should undergo normal recombination initiation, which would result in a twofold reduction of crossing-over only. Therefore, a masking by methylation of the initiation site cannot account for the observed effect. To explain the data, we need to assume either that methylation also inhibits the production of the initial break in *trans*, on the unmethylated homologous chromatids, or that it acts at a further step.

A way to inhibit the break in *trans* would be by impairing the process of sensing of homology (Xu and Kleckner 1995; Rocco and Nicolas 1996), which must be the earliest step of recombination and methylation transfer (Colot et al. 1996). If methylation were to inhibit recombination by acting at this step only, then the methylation of one parent would make this step 50-fold less efficient, although allowing a frequency of 1.5%–3% methylation transfers. Therefore, this hypothesis im-

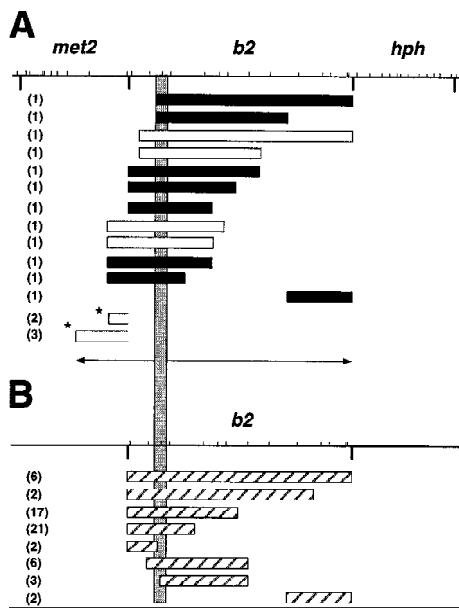


Figure 6. (A) Bidirectional polarity of methylation transfer events. The shaded vertical rectangle defines the region of *b2* in which 73 of 76 methylation transfers shown could have been initiated. The bidirectional polarity is suggested by the two opposite arrows. The *HpaII* restriction map of the *met2*-*b2*-*hph* transgene is at the top (bars). The *HindIII* sites separating the three genes are indicated below the horizontal bar. Rectangles show the different lengths of methylation transfer observed. Solid rectangles correspond to methylation transfers shown in Fig. 5; open rectangles indicate methylation transfers observed in randomly selected *Met*⁺, *Hyg*^R recombinants (Fig. 3) and correspond to profiles A and B from cross IV. [The extent of the methylation transfer cannot be deduced from the *Met*⁺, *Hyg*^R recombinants in cross III (Fig. 5).] Transfers indicated by asterisks correspond to profiles B and C from cross I and are limited to *met2* because *b2* was not methylated in this cross. However, they can result from molecular events initiated in *b2* and propagating leftward (see Discussion). This is suggested by the right opening of the rectangles. (B) Hatched rectangles show the methylation transfers previously observed at the resident *b2* gene, which is devoid of the *met2* and *hph* flanking markers (Colot et al. 1996). For each event, the number of occurrence is given in parenthesis.

plies that every meiosis undergoes an efficient sensing of homology at *b2* in the unmethylated control cross. It also implies that the sensing of homology events that would escape the inhibitory effect of methylation in crosses with one methylated parent, allow methylation transfer every time. At present, there is no known molecular mechanism that could simply explain directed transfers of methylation in the hypothetical DNA-DNA structure, which is thought to be formed between the two intact DNA duplexes at this step.

Another way to account for the effect of methylation is to assume that it acts at a step after the initial break. Methylation would not prevent the formation of recombination intermediates, but would impair their stability and correspondingly their maturation into recombinant

products. This could account for the severely reduced crossing-over frequencies associated with methylation. In this hypothesis, methylation transfer likely occurs through the formation of hemimethylated recombination intermediates (Colot et al. 1996). It is noteworthy, however, that the frequency of methylation transfer at *b2* observed in crosses III and IV ranges from 1.5% to 3%, which is between two and four times less than the 6% of crossing-over observed in cross I. This implies in the present hypothesis that 50% or more of the recombination intermediates that are formed in any case are destabilized by methylation before methylation transfer can occur. This conclusion is in agreement with the observation that methylation transfer at the markers flanking *b2* is reduced when the *b2* interval is methylated. Nevertheless, the latter reduction is approximately fourfold in crosses III and IV, and thus is 10-fold lower than the reduction in crossover frequency observed in the same crosses. Therefore, this would suggest that methylation affects the stability of recombination intermediates in a second step, at least after they become substrates for methylation transfer but before their maturation into crossover products.

The hypothesis that unstable intermediates are formed can also account for the frequent occurrence of asci with transfer of methylation to both unmethylated sister chromatids (0B:8W, 0*Met*⁺:8*Met*⁻, 0*Hyg*^R:8*Hyg*^S). Accordingly, the instability of the recombination intermediates after the initial break would result in unrepaired chromatids, which could then enter a second round of interaction involving different partners. In particular, the broken chromatid may form a new recombination intermediate with the unmethylated chromatid that was not involved in the first round of interaction. This hypothesis assumes that recombination intermediates could be formed between sister chromatids. It has been shown in yeast that meiotic recombination strongly favors intermediates formed between homologous chromatids (Schwacha and Kleckner 1997). Methylation might affect this bias, by favoring the formation of recombination intermediates between sister chromatids.

The way in which methylation might impair recombination intermediates is unknown. DNA methylation is often associated with changes in chromatin conformation (Kass et al. 1997) and triggers such changes in *Ascobolus* independently of the transcriptional state (J.L. Barra, G. Almouzni, G. Faugeron, and J.-L. Rossignol, unpubl.). DNA methylation, or associated changes, may impair the progress of the recombination process, either by preventing the faithful formation of the molecular complex that constitutes the recombination machinery, or by interfering with one or more activities of this machinery.

Our finding that methylation prevents crossing-over in *Ascobolus* raises the question of its effect on recombination in other organisms displaying methylation such as plants and vertebrates. In *Ascobolus*, methylation involves every cytosine, even when they are not part of a symmetrical sequence, which is not the case of verte-

Maloisel and Rossignol

brates where methylation mostly involves CpG. An important issue is to know whether methylation in these organisms may contribute to stabilize their genomes by preventing homologous recombination between dispersed DNA repeats. This would provide a major biological significance for the observation that in many eukaryotes, methylation is triggered by DNA repeats (Assaad and Signer 1992; Rossignol and Faugeron 1994) or by foreign DNA (Doerfler 1992; Bestor and Tycko 1996).

Materials and methods

Plasmid construction

The 7.5-kb *Hind*III fragment, which contains the *b2* gene (Colot et al. 1996), was inserted downstream of the *Ascobolus met2* gene harbored in PVM1.1, a pBKS-derived plasmid (Colot and Rossignol 1995). This new plasmid was then used to insert downstream of *b2* the 3-kb *Hind*III fragment from plasmid pMP6 (M. Plamann, pers. comm.) that carries a chimeric construct of the *E. coli hph* gene. This gave plasmid pLmbh, which contains the *b2* gene flanked upstream and downstream by *met2* and *hph*, respectively, all three in the same orientation.

Media and strains

Standard *Ascobolus* genetic techniques and media were used (referred to in Colot and Rossignol 1995).

MIP triggers the silencing of duplicated genes through cytosine methylation, which is coextensive with the size of the duplications. To methylate and silence the *met2* and *b2* copies carried by the transgene of the MBH strain (Fig. 1), we first introduced this *met2-b2-hph* transgene, through an appropriate cross, in a strain that carries the resident *met2* and *b2* genes. MIP of the *met2* and *b2* duplications was triggered by crossing the resulting strain with a strain of the opposite mating type. Because this strain carries a deletion of the *b2* and *met2* resident genes, progeny strains could be isolated that had inherited no resident copy of *b2* and *met2* and in which the *met2* copy alone or both the *met2* copy and the *b2* copy carried by the *met2-b2-hph* transgenic locus were methylated and silenced (strains mBH and mbH, respectively; Fig. 1). A similar protocol (using duplications of *b2* and *hph*) was used to isolate strains devoid of *b2* and *met2* resident copies and in which the *met2-b2-hph* transgenic locus was methylated and silenced either at *hph* alone or at both *hph* and *b2* (strains MBh and Mbh, respectively; Fig. 1). These strains exhibited stable inactivated phenotypes, as expected (Rhounim et al. 1992; Colot et al. 1996).

Crosses

Five mBH strains and five mbH strains were isolated from the same cross. Five MBh strains and five Mbh strains were isolated from another cross. Crosses I-IV consisted in crossing one individual strain from the first set of strains with one individual strain from the other set. Five different crosses, all involving different strains, were performed for each type of cross I-IV. This allowed to randomize possible genetic background effects.

Ascus and random spore analyses

For ascus analysis an equal number of asci was picked from the progeny of the five crosses constituting each type of cross I-IV, whereas random spore analysis was performed on the progeny of four such crosses. The Met⁺, Hyg^R strains were detected on

selective medium (without methionine and with hygromycin) as germinated spores that showed a normal growth and were clearly distinguishable from the weak residual growth seen with Met⁻ or Hyg^S germinated spores.

DNA methylation analysis

DNA isolation and manipulation were as described in Colot and Rossignol (1995). *Hpa*II digestions were performed on 3 µg of DNA.

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