DNA Methylation Affects Meiotic \textit{trans}-sensing, Not Meiotic Silencing, in \textit{Neurospora}

Robert J. Pratt, Dong W. Lee and Rodolfo Aramayo

Department of Biology, College of Science, Texas A&M University, College Station, Texas 77843-3258

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ABSTRACT

During the early stages of meiosis in \textit{Neurospora}, the symmetry of homologous chromosomal regions is carefully evaluated by actively \textit{trans}-sensing their identity. If a DNA region cannot be detected on the opposite homologous chromosome, then this lack of "sensing" activates meiotic silencing, a post-transcriptional gene silencing-like mechanism that silences all genes in the genome with homology to the loop of unpaired DNA, whether they are paired or unpaired. In this work, we genetically dissected the meiotic \textit{trans}-sensing step from meiotic silencing by demonstrating that DNA methylation affects sensing without interfering with silencing. We also determined that DNA sequence is an important parameter considered during meiotic \textit{trans}-sensing. Altogether, these observations assign a previously undescribed role for DNA methylation in meiosis and, on the basis of studies in other systems, we speculate the existence of an intimate connection among meiotic \textit{trans}-sensing, meiotic silencing, and meiotic recombination.

HOMOLOGY-sensing mechanisms are at center stage in biology (Wu and Morris 1999). Complex genomes have evolved sophisticated ways to sense the presence and to control the behavior of repeated DNA sequences. At risk is their chromosomal integrity and, with it, the very existence of the organism. The situation is more critical in meiosis, a developmental stage that requires cells to activate a series of sophisticated molecular mechanisms that will ensure precise chromosome duplication, repair, and recombination (Kleckner 1996; Zickler and Kleckner 1998, 1999; Roeder and Bailis 2001). Here, at least two things are critical. First, chromosomal integrity must be maintained. Even a small increase in the frequency of ectopic recombination between dispersed repeats would have catastrophic consequences for the genome. Second, the genetic information of homologous chromosomes must be compared to determine whether the chromosomes participating in meiosis belong to the same species.

The destructive potential of dispersed repeats has been thoroughly studied (Rouyer et al. 1987; Montgomerie et al. 1991; Small et al. 1997) as have been the factors responsible for limiting recombination between repeats (Shen and Huang 1986; Rayssiguier et al. 1989; Jinks-Robertson et al. 1993; Radman and Wagner 1993; Maloisel and Rossignol 1998). As a result of these studies, it is increasingly clear that DNA methylation plays a major role among the many molecular mechanisms involved in genome stability (Colot and Rossignol 1999). For example, DNA methylation is often associated with repeat-rich intergenic regions in plants and is present in DNA repeats in mammals (Bennetzen et al. 1994; Yoder et al. 1997).

It is therefore not surprising to find that the same molecular mechanisms used by cells to maintain their genome stability have been recruited to counteract the invasion of a genome by viruses, retrotransposons, and insertion sequences, which, if unchecked, can have deadly consequences to the organism. Arguably, filamentous fungal genomes are at a greater risk than those of plants and animals because a single cytoplasm is shared by many nuclei in these organisms. Genomes like that of \textit{Neurospora crassa} have developed a number of complex molecular mechanisms to preserve their integrity (Galagan et al. 2003; Borkovich et al. 2004). At least four distinct but potentially interrelated mechanisms are known: DNA methylation, quelling, repeat-induced point mutation (RIP), and meiotic silencing.

Imagine that, during haploid development (\textit{i.e.}, vegetative growth), a series of repeated elements invade the genome; if their sequence composition is distinct from that of native \textit{Neurospora} sequences, they will be detected and densely methylated (Miao et al. 2000), a process that can interfere with transcription elongation (Rountree and Selker 1997). Alternatively, their presence can activate quelling, a vegetative RNA-silencing mechanism (Cogoni and Macino 1999, 2000; Cogoni 2001; Pickford et al. 2002). Once activated, quelling produces a diffusible signal (\textit{i.e.}, small interfering RNAs) that interferes with the propagation of the repeated element within and between sibling nuclei, thereby protecting the integrity of the genomes present in the same cytoplasm (Catalanotto et al. 2002). If some of the...
nuclei containing these repeated DNA elements enter the sexual phase of their life cycle, the duplicated sequences are then subjected to another haploid-specific silencing mechanism, RIP (Selker 1990, 1997; Freitag et al. 2002). In this process, a series of GC-to-AT transition mutations are introduced into the duplicated sequences. Many of the remaining nonmutated cytosine bases are methylated by DIM-2, a DNA methyltransferase responsible for all the known cytosine methylation in Neurospora (Kouzminova and Selker 2001). Additionally, these sequences are frequently blocked in transcription elongation and associated with trimethylated histone-H3, lysine-9 (H3-K9) chromatin (or heterochromatin; Rountree and Selker 1997; Tamaru et al. 2003).

Finally, any remaining element will be scrutinized by two highly interrelated mechanisms that rely on chromosome pairing to control the integrity of the genomes participating in meiosis. These processes are called meiotic transvection (Aramayo and Metzenberg 1996) and meiotic silencing (Shiu et al. 2001). Meiotic transvection (or trans-sensing) is initiated in the diploid zygotic cell of Neurospora immediately after karyogamy, in the narrow window of time at the onset of meiosis during which homologous chromosomes align and “sense” each other, but before the first meiotic division occurs (Aramayo and Metzenberg 1996). The zygote is the only known diploid cell of Neurospora and thus the only cell in Neurospora in which trans-sensing, first seen in Drosophila (Lewis 1954), occurs. When a gene fails to “sense” its partner in the homologous chromosome, the resulting unpaired DNA triggers meiotic silencing, an RNA-mediated post-transcriptional gene-silencing mechanism that, once activated, persists during the subsequent meiotic divisions but is reset at some point prior to germination.

Meiotic trans-sensing was discovered through a combination of design and serendipity during our studies of the complex Ascospore maturation-1 (Asm-1) gene in Neurospora (Aramayo and Metzenberg 1996; Aramayo et al. 1996). Asm-1 (the product of asm-1+) is an abundant nuclear protein essential for the formation of aerial hyphae, the development of protoperithecia (the haploid female sexual structures), and the maturation of the ascospores (the haploid sexual spores; Aramayo and Metzenberg 1996; Aramayo et al. 1996). Spores carrying recessive, loss-of-function mutations in Asm-1 fail to develop, whereas spores with the asm-1+ allele mature normally. Surprisingly, we discovered that in Asm-1 deletion vs. wild-type crosses (i.e., Asm-1^2 × asm-1^+), the Asm-1^2 deletion allele is ascus dominant; all spores within the ascus fail to develop, including the ones carrying the asm-1^+ allele (clearly defying any Mendelian notion of segregation). Further work established that pairing of the Asm-1 gene, regardless of where it occurs, is essential for normal development.

The paired alleles could “sense” (i.e., trans-sense) the presence of their partners in homologous chromosomes (Aramayo and Metzenberg 1996).

But, how could genes be dominant by being absent in homologous chromosomes? This apparent genetic contradiction was resolved by Shiu et al. (2001), who demonstrated that the mechanism behind the ascus dominance observed was probably post-transcriptional, on the basis of the observation that mutations in Suppressor of ascus dominance-1 (Sad-1), coding for an RNA-dependent RNA polymerase (RdRP), suppress the ascus dominance exerted by unpaired copies of Asm-1 or other equivalent reporter genes (Shiu et al. 2001; Lee et al. 2003b). The production of a hypothetical RNA-based diffusible signal could explain why the presence of a single unpaired copy of Asm-1 could silence all paired and unpaired copies of the gene. The presence of a meiotic-specific RNA-silencing pathway in Neurospora has now been well established by our demonstration of an Argonaute-like protein, Suppressor of meiotic silencing-2 (Sms-2; Lee et al. 2003b), and a Dicer-like protein, Suppressor of meiotic silencing-3 (Sms-3; M. McLaughlin and R. Aramayo, unpublished results), in meiotic silencing.

This study was prompted by the need to genetically dissect meiotic trans-sensing from meiotic RNA silencing. This is not trivial, given the intimate relationship between these two processes. Intensive studies on the unpaired behavior of different DNA regions of the Asm-1 reporter gene confirmed a post-transcriptional gene-silencing model for meiotic silencing (Kutil et al. 2003; Lee et al. 2003b, 2004), but they did not dissociate trans-sensing from meiotic silencing. Here, we tested the hypothesis that meiotic trans-sensing and meiotic silencing could be genetically dissected by studying the genetic behavior of homologous (i.e., partially homologous) DNA regions. We observed that DNA methylation affects meiotic trans-sensing but not meiotic silencing. We further determined that in the absence of DNA methylation, the dominance of the alleles tested moderately correlates with DNA identity, suggesting that DNA sequence is also an important parameter during meiotic trans-sensing. Together, these experiments assign a previously undescribed role for DNA methylation in meiosis and, on the basis of studies in other systems, we speculate the existence of an intimate connection among meiotic trans-sensing, silencing, and recombination.

MATERIALS AND METHODS

Procedures for Southern blot analysis and other nucleic acid manipulations were performed as described (Pratt and Aramayo 2002). Similarly, Neurospora crassa growth conditions, conidial spheroplast preparation, and fungal transformation were performed as described (Pratt and Aramayo 2002). Homokaryon purification was performed as described (Pratt and Aramayo 2002; Lee et al. 2003a). The formulas for the
Vogel’s medium N. Westergaard’s medium, and the sugar mixture of Brockman and de Serres have been described by Davis and de Serres (1970).

Genetic crosses were set up and scored as described in the supplementary materials and methods at http://www.gene.tics.org/supplemental/. Similarly, the construction of the Roundspore (Rsp) deletion allele (Rsp\(^b\)), the isolation and sequencing of Roundspore RIP alleles (Rsp\(^{RIP}\)), and the procedures used for construction of other strains for growing cultures and for DNA dot blots are all described in detail in the supplementary materials and methods at http://www.gene.tics.org/supplemental/. The GenBank accession numbers corresponding to the Rsp\(^+\), Rsp\(^{RIP}\), Rsp\(^{RIP102}\), Rsp\(^{RIP104}\), Rsp\(^{RIP}\), and Rsp\(^{RIP105}\) alleles are cited in Table 1.

RESULTS

Experimental rationale: To overcome the experimental limitations associated with silencing Asm-1 (dead ascospores), we cloned the Rsp gene of Neurospora, a gene that, when mutant, affects the morphology of the sexual spores, but not their viability (D. W. Lee and R. Aramayo, unpublished results). In wild-type crosses, all ascospores produced have a spindle-shaped or American football-shaped morphology. In contrast, in crosses between Roundspore deletion mutants and wild type (i.e., Rsp\(^b\) x Rsp\(^+\)), all spores produced have a round or ovoid morphology. A similar ascus-dominant spore morphology is observed when a duplication-containing strain is crossed to wild-type strains (i.e., Rsp\(^+\) (ect) x Rsp\(^+\)). In these last two situations the presence of an unpaired copy of the gene triggers the silencing of all copies of the gene, whether paired or unpaired.

We postulated that meiotic trans-sensing could be readily detected and quantified if we were to study the “sensing” behavior of partially homologous (i.e., homologous) alleles of the Rsp gene in meiosis. This is because the partial homology present in a particular DNA region (in this case Rsp) would allow some degree of pairing, generating a more sensitive assay. Given that the degree of unpairing correlates with the level of meiotic silencing, which is, in turn, directly reflected in the progeny of a cross (i.e., phenotypic output), we reasoned that by quantifying the percentage of round vs. spindle-shaped ascospores in crosses of strains carrying homologous regions (e.g., Rsp\(^{RIP} \times Rsp\(^+\)\)), we could indirectly determine the level of meiotic trans-sensing.

The DNA methyltransferase gene (dim-2\(^+\)) affects meiotic trans-sensing, not meiotic silencing: To genetically dissect trans-sensing from silencing, we hypothesized that DNA methylation might affect trans-sensing but not silencing. If this were the case, among a series of lightly RIP alleles, we should obtain Rsp\(^{RIP}\) alleles that would be dominant in the presence, but recessive in the absence, of DNA methylation. This prediction was confirmed. We started by isolating two Rsp\(^{RIP}\) alleles that we called Rsp\(^{RIP102}\) and Rsp\(^{RIP104}\) (Figure 1A). DNA sequence

![Figure 1](image_url).—The DNA methyltransferase gene (dim-2\(^+\)) can affect meiotic trans-sensing, not meiotic silencing. (A) The Rsp region. (Top) A diagram of the Rsp locus indicating the positions of the transcription start site (coordinate 1796), predicted translation start and stop sites (coordinates 2736 and 6027, respectively), and the polyadenylation site (coordinate 6327) are presented, along with the relative positions of the four exons (open rectangles) and three introns (solid rectangles) in the region. The arrow spans the region corresponding to the Rsp transcript. (Bottom) Diagrams of the molecular structures of the different Rsp alleles are presented. Both Rsp\(^{RIP102}\) and Rsp\(^{RIP104}\) alleles were constructed by RIP of the Rsp\(^+\) allele by duplicating the 4391-bp region (coordinates 1962–6353) at the histidine-3 (his-3) locus. The percentage of identity indicated for the region is relative to the Rsp\(^+\) wild-type allele. Each vertical bar represents one point mutation. Alleles Rsp\(^{RIP102}\) and Rsp\(^{RIP104}\) have 129 and 254 mutations present along the duplicated region, respectively (Table 1). Allele Rsp\(^{RIP102}\) is a large natural deletion estimated to be between 20 and 30 kbp in length. Allele Rsp\(^{RIP104}\) was constructed by replacing a 3.8-kbp region corresponding to the coding region of Rsp\(^+\) with the hygromycin B phosphotransferase (hph\(^+\)) gene selection marker. Strains carrying the Rsp\(^{RIP102}\) (ectopic) allele contain the 5252-bp fragment corresponding to the minimal promoter, leader, and coding region of Rsp\(^+\) (coordinates 1123–6375) integrated at the his-3 locus in a Rsp\(^+\) (wild-type) background. (B) dim-2\(^+\) is an allele-specific enhancer of meiotic trans-sensing. The column plot presents the percentage of spindle-shaped ascospores observed for the different crosses in the presence (dim-2\(^+\)) or absence (dim-2) of DNA methylation. The higher the number of spindle-shaped ascospores, the lower the degree of meiotic silencing. Each percentage number indicated is the average of at least three crosses with a mean of 971 ascospores counted per cross. The Rsp genotype of each parent in the cross is indicated below.
analysis of the RIP regions revealed that these alleles are 94 and 97% identical to the wild-type region, respectively (Figure 1A). Surprisingly, when crossed to wild type, \textit{Rsp}^{RIP93} was dominant and \textit{rspb}^{RIP4} was recessive (Figure 1B), indicating that only a 6% divergence between \textit{Rsp}^{RIP93} and \textit{rspb}^{+} and only an 8–9% divergence between \textit{Rsp}^{RIP93} and \textit{rspb}^{RIP4} might determine their dominant behavior in meiosis. To test for the effects of DNA methylation on these alleles, we constructed double mutants between each one of these RIP alleles and \textit{dim-2}. For this we used \textit{dim-2(1)} allele containing a nonsense mutation (Kouzminova and Selker 2001). When crossed to \textit{rspb}^{+} \textit{dim-2} strains, the genetic behavior of \textit{rspb}^{RIP93} allele remained unchanged. In contrast, we observed a dramatic change for \textit{Rsp}^{RIP93}, from dominant to semirecessive (Figure 1B).

It was formally possible that the removal DNA methylation was relieving the transcriptional repression of a highly mutant but ultimately functional \textit{Rsp}^{RIP93} allele. This possibility was formally discarded on the basis of two observations: First, mutations introduced by RIP obliterated the translational start signal of the \textit{Rsp}^{RIP93} DNA region and introduced a series of in-frame stop codons (data not shown). Second, \textit{Rsp}^{RIP93} homozygous crosses produced all round ascospores, regardless of their methylation state (Figure 1B). Together these observations demonstrate the nonfunctional nature of the \textit{Rsp}^{RIP93} allele.

To demonstrate that DNA methylation was affecting only \textit{trans}-sensing but not meiotic silencing, we compared the dominance of deletion or insertion alleles of \textit{Rsp} when crossed to wild type in either the presence or the absence of DNA methylation. As expected, crosses between \textit{Rsp}^{A} alleles and \textit{rspb}^{+} \textit{dim-2} strains, the genetic behavior of \textit{rspb}^{RIP93} allele remained unchanged. In contrast, we observed a dramatic change for \textit{Rsp}^{RIP93}, from dominant to semirecessive (Figure 1B).

In summary, we conclude that the dominance of the \textit{Rsp}^{RIP93} allele is mostly due to DNA methylation. These data are consistent with a model in which DNA methylation, directly or indirectly, interferes with \textit{trans}-sensing but not with meiotic silencing.

In the absence of DNA methylation, different \textit{Rsp}^{RIP} alleles have different degrees of meiotic dominance: The loss of dominance associated with \textit{Rsp}^{RIP93} was not absolute (i.e., 71.6% as opposed to >95% spindle-shaped ascospores observed for \textit{rspb}^{RIP4} crosses; Figure 1B), which suggested that parameters other than DNA methylation itself are important and perhaps actively used during meiotic \textit{trans}-sensing. We tested if we could isolate dominant \textit{Rsp}^{RIP} alleles in a DNA methylation-independent manner by generating a new series of \textit{Rsp}^{RIP} alleles and by screening among them for those that were dominant in \textit{dim-2} homozygous crosses (see materials and methods). Thirty-five new \textit{Rsp}^{RIP} alleles were isolated. Of those, 11 (\textit{Rsp}^{RIP93}–\textit{Rsp}^{RIP105}), representing a range of DNA methylation-independent dominance were selected for further analysis (Figure 2A).

The results of crosses between each of these alleles and wild type (i.e., \textit{Rsp}^{RIP} \textit{rspb}^{+}) homozygous for \textit{dim-2} or for \textit{dim-2} are shown in Figure 2A. Similar to \textit{Rsp}^{RIP93}, alleles \textit{Rsp}^{RIP93}–\textit{Rsp}^{RIP105} showed a partial DNA methylation-dependent dominance. In contrast, unlike \textit{Rsp}^{RIP93}, alleles \textit{Rsp}^{RIP93}–\textit{Rsp}^{RIP94} were dominant in a DNA methylation-independent manner. These results suggest DNA methylation is not the only parameter determining the dominance of \textit{Rsp}^{RIP} alleles in meiosis.

Ideally, the presence or absence of DNA methylation on the different \textit{Rsp}^{RIP} alleles would be determined in cells undergoing meiosis. This is not possible because the developing meiotic cells are immersed in maternal tissue and cannot be isolated in a pure form in Neurospora. The best experiment we could do was to determine the methylation state of the alleles in vegetative tissues, which would represent at least the methylation state of the nuclei entering the cross. We therefore extracted DNA from vegetative tissues of strains carrying the different \textit{Rsp}^{RIP} alleles in a \textit{dim-2} background. DNA was digested with the methylation-sensitive enzyme \textit{Sau}3AI, fractionated by electrophoresis on a 1.5% agarose gel, transferred to nylon membranes, and probed with radiolabeled fragments corresponding to the \textit{rspb}^{+} allele. Relative to the wild-type band patterns observed in the methylated or demethylated condition, the band-shifts observed in the demethylated condition reflect the restriction enzyme sites mutated by RIP on each allele. In contrast, for each allele, relative to the band pattern observed in the demethylated condition, the band-shifts detected in the methylated condition represent those restriction sites affected by the DNA methylation in the region. Under these conditions, with exception of the \textit{rspb}^{RIP3} allele, the number of sites blocked by DNA methylation was similar for all the alleles examined (Figure 2B), suggesting that these alleles are indeed methylated and demethylated in a \textit{dim-2}^{+} and a \textit{dim-2} background, respectively. Similar results were ob-
In addition to DNA methylation, meiotic dominance: therefore of interest to test if the degree of dominance is expected to significantly decrease or eliminate the 22, 9.4, 6.6, 5.0, 4.4, 4.3, 3.5, 2.3, 2.0, 1.9, 1.6, 1.4, 0.95, 0.83, and given the absolute requirement of the SAD-1-RdRP and 0.56. for silencing, we reasoned that if, in addition to unpairing, both Hin/H9261 allele, was extracted and processed as described in the lencing induced by unpairing Rsp/Rsp. Each percentage number presented is the result of assaying strategy that the suppression of meiotic RIP alleles to wild type, the higher the degree of their sequence identity to the complementary Figure 2S). The lower the sequence identity of the different RIP alleles (identified by their numbers) or the wild-type heterozygous crosses (Figure 4). Here, the level of silencing of the rps+ wild-type allele in rps+ × Rsp/H11001 (wild type, light shading) or dim-2 (mutant, dark shading). Each percentage number presented is the result of assaying two dim-2+ or four dim-2 crosses for each allele with a mean of 1038 ascospores counted per cross. (B) The different RspRsp alleles are methylated and demethylated in a dim-2+ and a dim-2 background, respectively. DNA from dim-2+ (wild type, +) or dim-2 (mutant, −) strains, each carrying either a different RspRsp allele (identified by their numbers) or the wild-type (wt) allele, was extracted and processed as described in the text. The solid bars flanking the autoradiograms represent the relative positions of DNA fragments corresponding to the molecular weight markers consisting of the mixture of AdNA digested with HindIII only plus λDNA digested with both HindIII and EcoRI. Sizes in kilobase pairs are as follows: 22, 9.4, 6.6, 5.0, 4.4, 4.3, 3.5, 2.3, 2.0, 1.9, 1.6, 1.4, 0.95, 0.83, and 0.56.

The DNA methyltransferase gene (dim-2*) affects meiotic transsensing at other loci as well: To demonstrate that the suppression of meiotic transsensing observed applies to other loci, we studied how the behavior of Sad-1RIP64, a semidominant RIP allele of Sad-1 that we previously isolated (Lee et al. 2003b), affected the silencing of the rps+ wild-type allele in rps+ × Rsp/H11001 heterozygous crosses (Figure 4). Here, the level of silencing induced by unpairing rps+ is constant and independent of DNA methylation (compare cross 1 with 2, Figure 4). Because the inability of sad-1+ to pair with the Sad-1RIP64 allele induces partial silencing of the sad-1+ wild-type allele itself (Shiu et al. 2001; Lee et al. 2003b), and given the absolute requirement of the SAD-1-RdRP for silencing, we reasoned that if, in addition to unpairing rps+ (in a DNA methylation-independent manner), we were to unpair the sad-1+ wild-type allele as well, the level of rps+ silencing would correlate with the level of sad-1+ pairing, which in turn would be dependent on the level of DNA methylation of the Sad-1RIP64 allele. The dominance of the Sad-1RIP64 allele would then be directly proportional to its own level of DNA methylation, assuming DNA methylation does indeed affect the pairing of sad-1+ with the Sad-1RIP64 allele. Since sad-1+ silencing is expected to significantly decrease or eliminate the amount of sad-1+ transcript and SAD-1 protein below that which is predicted for a single functional gene, a direct correlation between the dominance of Sad-1RIP64.
and the level of sad-1+ silencing was expected. Lowering the amount of SAD-1 protein should, in turn, translate into both lower silencing of rsp+ and higher percentage of spindle-shaped ascospores.

This prediction was confirmed; in sad-1+ × Sad-1tep4 heterozygous crosses, the degree of rsp+ silencing was lower in the presence of DNA methylation (compare cross 3 with 4, Figure 4). Consistent with what we observed before, meiotic silencing was unaffected by the loss of genome methylation (cross 2, Figures 1B and 4). These data are consistent with the proposal that DNA methylation, directly or indirectly, interferes with meiotic trans-sensing of all methylated alleles, but not with meiotic silencing.

**DISCUSSION**

In this work, we genetically dissected meiotic trans-sensing from meiotic silencing by demonstrating that DNA methylation affects sensing without interfering with silencing. This observation assigns DNA methylated regions a previously undetected role in chromosome sensing. We also determined that DNA sequence is an important parameter considered in the process.

Our observations are consistent with the existence of two mechanisms, meiotic trans-sensing and silencing, operating side by side in meiosis. During chromosome pairing chromosomes “sense” the identity of the regions with which they are supposed to pair in the homologous chromosome. If the regions are equivalent, development proceeds normally. If not, the meiotic trans-sensing machinery recognizes the unpaired region and activates meiotic silencing. The trans-sensing step must be exquisitely sensitive, given that even the methylation status of the alleles compared is taken into account in the determination of their pairing potential (i.e., DNA methylation, directly or indirectly, inhibits this meiotic trans-sensing).

The model predicts that meiotic trans-sensing can be dissociated from meiotic silencing. It also predicts that the strength of meiotic silencing is dependent upon the degree of unpairing. Previously, we demonstrated that the larger the region of DNA that is unpaired, the greater the level of meiotic silencing of a reporter gene that is observed (Lee et al. 2004). Confirming these observations, crosses between sad-1+ and different Sad-1 alleles result in different levels of SAD-1 enzyme, which, in turn, directly affect the level of meiotic silencing.

Some time ago, Color et al. (1996), made the amazing observation that the DNA methylation state of an allele could be transferred to its homolog during meiotic chromosome pairing by a mechanism related to gene conversion. This observation suggests a mechanism by which a RIP allele could potentially transfer its state to its partner on the opposite chromosome. If this were the case, then the “silencing” that we observe could be due, at least in part, to this epigenetic transfer. Three pieces of evidence strongly argue against this: First, all Rsptep alleles tested can be suppressed entirely by Sad-1tep. If even a fraction of the silencing observed is due to this proposed “epigenetic transfer,” then this fraction would presumably not be suppressed by the

### TABLE 1

<table>
<thead>
<tr>
<th>Gene and allele</th>
<th>No. of mutations</th>
<th>Length of duplicated region</th>
<th>Accession no.</th>
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<td>2644</td>
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<tr>
<td>Puntep1</td>
<td>ND</td>
<td>1874</td>
<td>AF181821</td>
</tr>
</tbody>
</table>

**ND, not determined because the sequence of the wild-type precursor is unknown; NA, not applicable.**

*a* Genes names and alleles designations are: roundspore (rsp-recessive allele or Rsp-dominant allele), suppressor of ascus-dominance-1 (sad-1), and amutation deficient (am).

*b* Number reflects the number of mutations present in the duplicated region. For Rsp alleles, a mutant base was scored only if the identity of that base was unambiguously determined across all Rsp alleles sequenced.

*c* Percentage of identity is relative to the wild-type duplicated region.


% identity to wild type = 100.0 ± 0.05

RIP index I = 0.74 ± 0.01

RIP index II = 1.19 ± 0.03

### TABLE 1

Comparison of the mutational degree of different RIP alleles
SAD-1A allele. We would have detected this difference. Second, the epigenetic transfer would have to be unidirectional and highly efficient to be the primary silencing mechanism. The efficiency of transfer reported by Colot et al. (1996) was low. Third, the maintenance of this "epigenetic transfer" would have to be transient and confined only to ascus development, since the segregation of RIP phenotypes that we observe among the progeny is Mendelian, unless there is a mechanism for erasing the transfer only from the newly silenced allele.

This work was possible due to the observation that while highly identical to its wild-type allele, RspRIP clearly shows a dominant genetic behavior in the presence of DNA methylation vs. a semirecessive genetic behavior in its absence. Why would this be? RIP alleles differ from their wild-type counterparts in essentially four ways: First, they contain point mutations. Second, they tend to be methylated. Third, they can be associated with trimethylated histone H3 lysine-9 (H3-K9) containing chromatin. Fourth, they are generally transcriptionally inactive. In the absence of DNA methylation (e.g., as when RspRIP is propagated in a dim-2 mutant background), some of these differences can be erased. The DNA methylation is always lost (Kouzminova and Selker 2001). The H3-K9-trimethylated mark has also been observed to disappear at some loci (e.g., amRIP), but not at others (e.g., Punt; see Table 1; Tamaru and Selker 2001, 2003; Tamaru et al. 2003). Finally, the transcriptional elongation that is highly perturbed in the presence of DNA methylation can be restored in its absence, at least for some loci (Rountree and Selker 1997). In this work, we clearly show that DNA methylation is one of the parameters responsible for the dominance displayed by some of the RIP alleles studied here. We also show a correlation between DNA identity and dominance. These observations, however, do not discount the possibility that other factors, like chromatin state, might play a role in the sensing step.

But what is involved in homolog transsensing? Or how do chromosomal regions evaluate their degree of equivalence with opposite regions? We think that at least two different, but potentially interrelated mechanisms, are involved: DNA identity and chromatin identity. The first model is simple and attractive and can potentially explain all our observations. It considers that DNA identity is the only parameter involved during chromosome sensing. Under this model, a 5-methylcytosine (5-mC) base is "seen" as a "fifth base." This means that, to a guanine, 5-mC would be as different as an adenine. It follows that the level of DNA methylation of a given chromosomal region will determine its degree of perceived identity when compared to an equivalent wild-type region. For example, if the identity between a demethylated RspRIP and rbp + allele is 94%, in the presence of DNA methylation their identity would be 55%, assuming all cytosines become methylated. This model is supported by the moderate correlation that we detected between sequence identity and dominance in the absence of DNA methylation.

The other, nonmutually exclusive, chromatin identity model states that the meiotic transsensing machinery determines the pairing potential of two opposite regions at the level of chromatin. Two euchromatic or two heterochromatic regions would be considered homologous. In contrast, if a euchromatic region on one chromosome is compared to a heterochromatic region on the other, the regions would be considered heterologous and meiotic silencing would be triggered. Under this model the observed variance in the dependence on DNA methylation for allele dominance can be explained by the different abilities of the alleles to maintain heterochromatin in the absence of DNA methylation. Similarly, other loci also differ in their maintenance of trimethylated H3-K9 in a dim-2 background (Tamaru et al. 2003). Furthermore, the correlation detected between sequence identity and dominance could be superficial, reflecting more a difference in AT richness as opposed to simply reflecting changes in nucleotide identity. Given that the AT richness of a region correlates with de novo DNA methylation (Tamaru and Selker 2003) and that DNA
methylation requires trimethylated H3-K9 (Tamaru and Selker 2001), it is possible that the Rsp^{RIP64} alleles that we isolated differ in their ability to recruit the silencing complexes that recognize this AT-rich signature, as described in the model proposed by Selker et al. (2002). Given that RIP mutates CpA dinucleotides preferentially, the mutation level of a DNA region can be easily predicted by using two RIP indexes [TpA/ApT and (CpA + TpG)/(ApC + GpT), Table 1; Selker et al. 2003]. If the silencing complexes that recognize and methylate demethylated RIP regions de novo also recognize qualitatively this preference, alleles with strong RIP indexes would also be predicted to maintain silencing better in the absence of DNA methylation.

This latter model could also explain the anomalous behavior of the Rsp^{RIP102} and Rsp^{RIP103} alleles. On the basis of DNA identity alone, Rsp^{RIP103} should be more dominant than Rsp^{RIP102} (i.e., 92.8 vs. 94.0%, Table 1). Biologically, however, both alleles have statistically identical levels of dominance (Figure 2A). It is therefore possible that both of these alleles maintain similar levels of heterochromatin. Perhaps the quality of the AT-rich regions in Rsp^{RIP102} is more successful at recruiting heterochromatin than those in Rsp^{RIP103}. The RIP indexes calculated for Rsp^{RIP102} and Rsp^{RIP103} are consistent with this idea (Table 1). Similar arguments can also explain the drastic differences in dominance observed for Rsp^{RIP103} and Rsp^{RIP104} alleles (Figure 2A). The difference in percentage of identity to wild type of these last two alleles is similar to the difference in percentage of identity to wild type of Rsp^{RIP102} and Rsp^{RIP103} [i.e., 1.1 (92.8–91.7) vs. 1.2 (94.0–92.8), Table 1].

DNA methylation is known to inhibit meiotic recombination in Ascobulus (Maloisel and Rossignol 1998) and can interfere with V(D)J recombination in mammals (Engler and Storb 1999). Chromatin structure is also known to affect the positioning of the double-strand breaks associated with recombination (Wu and Lichten 1994). Sequence homeologies are established barriers for DNA recombination in a mismatch-repair-dependent manner (Rayssiguier et al. 1989; Radman and Wagner 1993; Hunter et al. 1996). In Saccharomyces cerevisiae, recombination inhibition may occur by both an inhibition of double-strand-break formation at sequence heterology (Xu and Kleckner 1995; Rocco and Nicolas 1996) and a rejection of heteroduplex DNA in homeologous regions (Alani et al. 1994; Chambers et al. 1996; Hunter et al. 1996). It is therefore possible that the inhibition of recombination associated with the DNA methylation and/or sequence homeology of RIP alleles triggers meiotic silencing. If this is the case, then recombination may promote or facilitate meiotic trans-sensing, which in turn reduces meiotic silencing. This model predicts that the “sensing” of recombination-deficient chromosomal regions is impaired and that meiotic silencing might not occur in these neighborhoods. This model also predicts that regions that are recombination proficient might be more sensitive.
to being unpaired. Finally, the model predicts that at least some components of the meiotic recombination apparatus might be part of the meiotic trans-sensing machinery.

Consistent with this, meiotic silencing is partially responsible for the meiotic sterility of Neurospora interspecific crosses (Shiu et al. 2001), an effect similar to the one seen in S. cerevisiae, where the antirecombination effects of mismatch repair lead to meiotic sterility in interspecific crosses (Chambers et al. 1996; Hunter et al. 1996). Recently it has been reported that integration of a reporter gene in a recombinationally impaired region of Neurospora tetrasperma does not result in meiotic silencing (Raju and Jacobson 2004). In maize, a role for Rad51/RecA in chromosome homology recognition during meiosis has been postulated (Pawlowski et al. 2003), and male mice defective for the components of the DNA mismatch repair recognition system exhibit abnormal chromosome synopsis in meiosis (Baker et al. 1995).

In S. cerevisiae, accurate pairing of homologous chromosomes requires Hop2, a protein implicated in homology searching and/or recognition. Mutants in this meiosis-specific recombinase show promiscuous pairing between nonhomologous chromosomes (Leu et al. 1998), a defect that can be partially suppressed by overexpressing the recA homolog Rad51 (Tsubouchi and Roeder 2003). Dmc1, a Rad51 paralogue, works with Rad51 and Hop2 to ensure the legitimacy of the pairing (Tsubouchi and Roeder 2003). Both Hop2 and Dmc1 could not be detected by BLAST searches of the current assembled Neurospora genome (Borkovich et al. 2004). Therefore, if trans-sensing is mechanistically related to meiotic recombination, as we propose here, a different mechanism must exist for homology searching and/or recognition in Neurospora. Alternatively, this function could be fulfilled by the sole Neurospora Rad51 homolog MEI-3. It is noteworthy that, like Neurospora, Caenorhabditis elegans lacks dmc1 and silences unpaired DNA in meiosis (Bean et al. 2004).

Trans-sensing phenomena are relevant to human disease. Pairing abnormalities result in homolog nondisjunction events (i.e., aneuploidy), a major cause of spontaneous abortion and developmental defects in humans, such as Down syndrome (Hassold and Hunt 2001). Normal development and adult phenotype require normal imprinting (i.e., the tissue- and timing-specific functional haplology) of specific human genes (Bennett et al. 1997; Hall 1997; Constancia et al. 1998), and all imprinted regions studied to date consistently show pairing (Lalande 1996; LaSalle and Lalande 1996; Rieselmann and Haaf 1999). Pairing-dependent genetic phenomena have long been known to occur in Drosophila, where homolog pairing influences gene expression (Henikoff et al. 1995; Henikoff and Comai 1998; Pirrotta 1999; Sass and Henikoff 1999; Wu and Morris 1999). They have also been postulated to play important gene regulatory roles in the somatic and meiotic cells of plants (Chandler et al. 2000; Matzke et al. 2001; Stam et al. 2002; Chandler and Stam 2004). In addition, some genes expressed during meiosis and development in the mouse have also been postulated to trans-interact with each other (Duvillie et al. 1998; Herman et al. 2003). Paramutation in maize (Chandler et al. 2000; Stam et al. 2002), transvection of brown dominant (bwD) in Drosophila (Sass and Henikoff 1999), and the trans-rective-like effects observed between lox recombination sites in the mouse (Rassoulzadegan et al. 2002) are all similar to the dominance of RIP alleles in three ways: First, they could represent sensing-dependent phenomena, in which differential DNA methylation or heterochromatin between the loci might be associated with the silencing of the normal allele. Second, these phenomena, with the possible exclusion of the observation in mice, seem to depend on successful sensing to silence the homologous allele whereas meiotic silencing seems to depend on the failure of sensing to silence the homologous allele. Third, these phenomena seem to involve changes in the epigenetic state of the normal allele similar to what is seen in Ascorbulus, rather than posttranscriptional silencing. Importantly, while the output of this trans-sensing may be different, the mechanism of trans-sensing may be universally shared.

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LITERATURE CITED


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