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Meiotic Silencing by Unpaired DNA

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Summary

The silencing of gene expression by segments of DNA present in excess of the normal number is called cosuppression in plants and quelling in fungi. We describe a related process, meiotic silencing by unpaired DNA (MSUD). DNA unpaired in meiosis causes silencing of all DNA homologous to it, including genes that are themselves paired. A semidominant *Neurospora* mutant, *Sad-1,* fails to perform MSUD. *Sad-1* suppresses the sexual phenotypes of many ascus-dominant mutants. MSUD may provide insights into the function of genes necessary for meiosis, including genes for which ablation in vegetative life would be lethal. It may also contribute to reproductive isolation of species within the genus *Neurospora*. The wild-type allele, *sad-1*$^+$, encodes a putative RNA-directed RNA polymerase.

Introduction

In *Neurospora*, as in other eukaryotes, each gene or DNA segment is normally represented once in the haplophase and twice in the diplophase. Violation of the normal ploidy in either stage is usually a harbinger of trouble, perhaps most commonly because a virus or transposon is on the move. A number of mechanisms can detect and deal with DNA segments that are present in an inappropriate number of copies. These include mechanisms that alter the coding potential of the repeated DNA itself (Selker, 1990), other mechanisms that prevent mRNA from being transcribed from repeated genes, and still others that degrade mRNA after it has been made (Wolffe and Matzke, 1999). Silencing may occur in somatic cells during vegetative life, either as a direct (Pal-Bhadra et al., 1999) or indirect consequence of the presence of abnormal numbers of DNA copies, and has variously been called cosuppression in plants, RNAi interference in animals, and quelling in filamentous fungi (Fire et al., 1998; Wolffe and Matzke, 1999; Kennerdell and Carthew, 1998; Cogoni and Macino, 2000; Carthew, 2001). There has been impressive progress in elucidating the biochemistry of silencing, most recently in the activation of posttranscriptional degradation of specific mRNA molecules. In at least one well-studied case, a double-stranded RNA species is produced by the action of an RNA-directed RNA polymerase (Schiebel et al., 1998). This dsRNA is cut into fragments 21–23 base pairs in length (small interfering or siRNA), which act as guide RNAs for an ATP-dependent cleavage of homologous mRNA (Zamore et al., 2000).

The filamentous fungus *Neurospora crassa* exhibits several types of gene silencing. It is haploid during vegetative growth, and the sexual phase includes only a transiently diploid cell, the zygote (Figure 1). Few genes are present in more than one copy per nucleus other than the tandemly repeated rDNA units, the repeated but scattered 5S rDNA genes, and transfer RNA-encoding genes (Perkins et al., 2001). If other DNA sequences are present in multiple copies, they are likely to represent a transposable element that has the potential for future mischief. *Neurospora* has a silencing mechanism, called quelling, that specifically recognizes mRNAs from such repeated sequences and targets them for degradation through a mechanism involving an intermediate formation of dsRNA (Cogoni and Macino, 1999).

Another silencing process called RIP (repeat-induced point mutation) occurs before karyogamy and consists of a genome-wide scan for duplicated sequences, followed by extensive introduction of C to T mutations in any such sequence. Unlike quelling, RIP is irreversible. Tandemly repeated rDNA sequences within the nucleolus organizer are spared, while DNA sequences encoding 5S RNA and tRNA are below the size threshold of the RIP machinery. One plausible role of RIP is the inactivation of transposons that have already been duplicated at least once (Selker, 1990, 1997).

Here we describe a different system of targeted gene silencing that operates after karyogamy. Aramayo and Metzenberg (1996) had previously reported that a cross between a dominant deletion mutant (*Asm-1*$^\Delta$ [ascus maturation]) and wild-type (*asm-1*$^+$) resulted in nearly all the ascospores, including those carrying the wild-type allele, being unmelanized and inviable, even though they were formed in normal numbers. The introduction of an ectopic copy of wild-type *asm-1*$^+$ into the deletion strain did not correct the sterility of such a cross; and yet the cross of two deletion mutants, each of them complemented by an identically-placed ectopic copy of *asm-1*$^+$ and thus able to pair throughout, gave abundant viable black spores (Aramayo and Metzenberg, 1996). Because these results obey the same formal rules as transvection in the soma of *Drosophila melanogaster* (Wu and Morris, 1999), we previously referred to the phenomenon as "meiotic transvection" (Aramayo and Metzenberg, 1996). Transvection denotes the phenomenon in which genes are expressed differently when they are paired than when they are unpaired and ordinarily describes a process that occurs in somatic tissues. We now prefer to use the more neutral term "meiotic silencing by unpaired DNA" (MSUD). If quelling and RIP act to silence or destroy non-single-copy DNA sequences in tissues that are properly haploid, meiotic silencing can be thought of as silencing of non-two-copy sequences in

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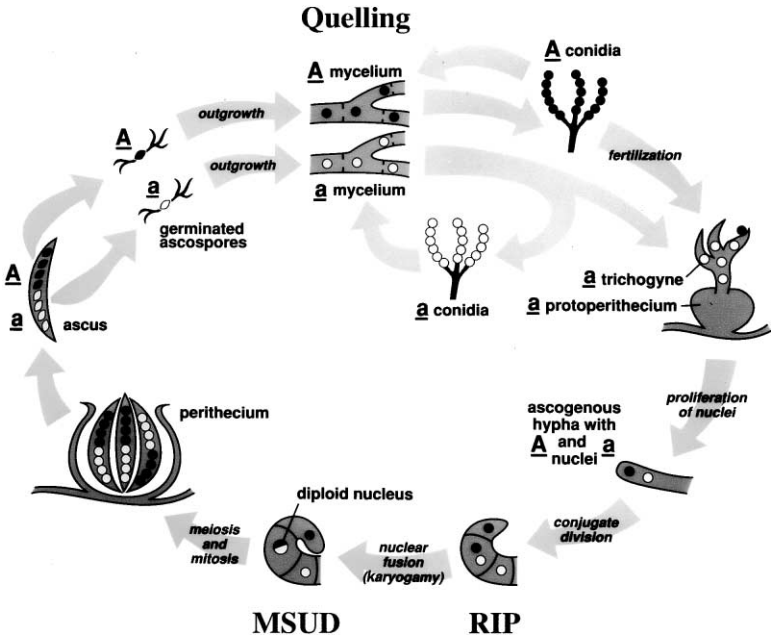


Figure 1. The Life Cycle of *N. crassa*

Indicated are the sites for quelling, RIP (repeat-induced point mutation), and MSUD (meiotic silencing by unpaired DNA). Mating occurs only between strains of *mat A* and *mat a*. After fertilization, the haploid nuclei proliferate in the premeiotic ascogenous tissue, which gives rise to asci within the developing ascocarp (perithecium). In the young ascus, the haploid nuclei derived from the *mat A* and *mat a* parents fuse. The resulting diploid zygote nucleus immediately undergoes the two meiotic divisions and a postmeiotic mitosis in the common cytoplasm of the ascus. The resulting eight haploid nuclei are then sequestered into eight spindle-shaped (American football-shaped) ascospores, which are held in linear order in the narrow ascus. Maturing ascospores become multinucleate and pigmented. About 200 asci are produced in each perithecium (see Raju, 1980, for photos).

the diploid zygote. We report here that unpaired segments are silenced, as are genes present in any odd number of copies or homologous genes that are single-copy in each parent but that occupy nonhomologous positions. We find that the gene *sad-1*$^{+}$ is required for meiotic silencing. *Sad-1*^{UV} and the deletion mutant *Sad-1*$^{\Delta}$ can suppress the near-absolute barren phenotype of crosses between wild-type and a variety of strains in which a segment of DNA is duplicated. In addition, these *Sad-1* mutants suppress several long-known, conventionally arisen "ascus-dominant" mutants and a variety of newly constructed dominant mutants containing an ectopic transgene. This strongly suggests that these, too, owe their ascus dominance to the MSUD mechanism. Finally, interspecific crosses within the genus *Neurospora* that are normally almost completely infertile become much more fertile if the *N. crassa* parent is a *Sad-1* mutant, suggesting that MSUD due to numerous small mispairings may play a role in reproductive isolation of these species.

Results

An Unpaired Copy of *Asm-1* Affects Expression of All Paired and Unpaired Copies

Aramayo and Metzenberg (1996) left unanswered the question of whether normal ascospore maturation requires that there be paired *asm-1*$^{+}$ alleles or that there not be unpaired ones. We therefore made crosses involving three copies of *asm-1*$^{+}$ (Table 1). A strain carrying two copies, one at its endogenous locus and one at *his-3*, was crossed to two strains, each with a single copy of *asm-1*$^{+}$, one carrying it at the endogenous locus and one at *his-3*. Thus, in each cross, two copies were paired with a homolog at the same chromosomal position and one was unpaired. Both crosses gave ascospores which were more than 99.5% white and inviable, suggesting that the presence of paired copies is insufficient for ascospore maturation and that the real requirement is the absence of unpaired copies. This was verified in crosses involving parents with all combinations of zero, one, or two copies. Crossing two strains, each carrying two wild-type copies, should result in the production of mature ascospores. This expectation was qualitatively fulfilled. The shortfall from a fully wild-type level of fertility can be explained by the fact that both parents were subject to RIP of the *asm-1*$^{+}$ gene.

Isolation of a Suppressor of Ascus Dominance (*Sad-1*^{UV})

To explore the mechanism of silencing, we devised a selection for mutants that were able to pass through a cross in which *asm-1*$^{+}$ is not paired (unpublished data). One such mutant, *Sad-1*^{UV}, was studied in detail. It has no obvious phenotype in vegetative life nor in crosses to wild-type. However, homozygous *Sad-1*^{UV} crosses were completely barren. Cytological analysis of perithecia from such crosses showed that karyogamy occurred normally and that normal numbers of asci were formed, but 100% of the asci were arrested in meiotic prophase at pachytene or diplotene (Figure 2A; compare with wild-type asci in Figure 2B).

The wild-type gene was cloned from a cosmid library by its ability to restore ascospore production in a *Sad-1*^{UV} homozygous cross. Two complementing cosmids of overlapping sequence were further subcloned as a 6847 bp *Xba*I-*Cla*I fragment (GenBank accession number AY029284). BLAST-P searches (Altschul et al., 1990) revealed that SAD-1 has significant homology with many presumptive RNA-directed RNA polymerases (RdRP), including SDE1 from *Arabidopsis thaliana* (Dalmay et al., 2000), EGO-1 from *Caenorhabditis elegans* (Smardon et al., 2000), and QDE-1 from *N. crassa* (Cogoni and Macino, 1999). RdRP has been implicated in posttranscriptional gene silencing (PTGS), in which mRNAs from gene repeats are targeted for degradation (Carthew, 2001).

Table 1. Meiotic Silencing by Unpaired DNA (MSUD)

Parent 1 (*mat A*)		Parent 2 (*mat a*)		Percent Black Ascospores	Nature of Cross[a]	MSUD?
his-3:: Locus	*asm-1* Locus	*his-3::* Locus	*asm-1* Locus			
	Asm-1$^+$		*Asm-1*$^+$			
I —	V —	I —	V —	98	C × C	No
Asm-1$^+$	( )	*Asm-1*$^+$	( )			
I —	V —	I —	V —	94.6	E × E	No
Asm-1$^+$	*Asm-1*$^+$	*Asm-1*$^+$	*Asm-1*$^+$			
I —	V —	I —	V —	13.6[b]	CE × CE	No
I —	V —	I —	V —	No spores	Δ × Δ	N.A.
			Asm-1$^+$			
I —	V —	I —	V —	1.1	Δ × C	Yes
	Asm-1$^+$	*Asm-1*$^+$				
I —	V —	I —	V —	0.028	Δ × E	Yes
	Asm-1$^+$	*Asm-1*$^+$	*Asm-1*$^+$			
I —	V —	I —	V —	0.024	Δ × CE	Yes
	Asm-1$^+$	*Asm-1*$^+$				
I —	V —	I —	V —	0.01	C × E	Yes
	Asm-1$^+$	*Asm-1*$^+$	*Asm-1*$^+$			
I —	V —	I —	V —	0.057	C × CE	Yes
Asm-1$^+$	*Asm-1*$^+$	*Asm-1*$^+$				
I —	V —	I —	V —	0.19	CE × E	Yes

Ascospore maturation in crosses with paired and unpaired *asm-1*$^+$ genes.

[a] Abbreviations are as follows: C, canonical (i.e., endogenous) position of the *asm-1*$^+$ gene; E, ectopic location of *asm-1*$^+$; CE, duplication, i.e., *asm-1*$^+$ gene in both canonical and ectopic sites; and Δ, deletion of the *asm-1*$^+$ gene.

[b] The reduced percent black ascospores in the CE × CE cross compared to the C × C and E × E crosses probably reflects the vulnerability of both parents to RIP of the *asm-1*$^+$ genes.

Suppression of the Ascus-Dominant Mutations *Round spore (R), Peak (Pk^D)*, and *Banana (Ban)*

Aramayo and Metzenberg (1996) suggested that these long-known ascus-dominant mutations might be explained by a failure of the mutant allele and its wild-type homolog to pair, as postulated for *Asm-1*$^\Delta$, rather than their being hypermorphs or by making a toxic analog as had been previously supposed. Crosses of wild-type × *Round spore* (*R*) produce 100% round-spored asci (Mitchell, 1966), whereas crosses of *Sad-1*^{UV} × *Round spore* give abundant asci containing all spindle-shaped ascospores (Figure 2C versus 2D; compare with 2E). *Sad-1*^{UV} also suppresses *Pk*^{D} and *Ban. Peak* is a mutation that causes asci to be swollen sacs of disordered octads of ascospores rather than being linearly arrayed. Most *peak* alleles are recessive *(pk)*, consistent with their being point mutations. One dominant allele, *Pk*^{D}, is inseparable from one breakpoint of a reciprocal translocation. When *Pk*^{D} is crossed to wild-type, it gave about 10% ordered asci and the other 90% were unordered sacs (Figure 2F), whereas a cross of *Sad-1*^{UV} to *Pk*^{D} gave virtually 100% ordered asci (data not shown but indistinguishable from wild-type, Figure 2E). Crosses heterozygous for *Ban* give asci in which individual ascospores fail to be delimited; the nuclei from all four meiotic products and their mitotic progeny are confined to one giant banana-shaped ascospore (Figure 2G; see also Raju and Newmeyer, 1977). A cross of *Sad-1*^{UV} to *Ban* gave roughly 50% normal asci with eight ascospores instead of the 1%–2% seen in crosses to wild-type (data not shown). Each perithecium produced both normal and *Ban* asci. We propose that the mutant loci contain rearrangements that prevent them from pairing with *r*$^+$, *pk*$^+$, and *ban*$^+$, thus silencing these wild-type alleles.

Not all ascus-dominant mutations are suppressed by *Sad-1*^{UV}. The compendium of Perkins et al. (2001) lists other loci at which mutations are formally dominant. These include *Four spore (Fsp)-1* and *-2*, which yield four rather than eight spores, and *Spore killer (Sk-2* and *-3),* meiotic drive elements which kill all spores not carrying the killer allele. None of these is suppressed. Presumably the basis for ascus dominance for these mutants is something other than MSUD.

The Basis for Semidominance in *Sad-1*^{UV}

To identify the changes in the *Sad-1*^{UV} mutant, we sequenced the mutant allele. Approximately the 3′ half of the gene was deleted, and Southern blots showed that it is not present elsewhere in the genome (Table 2). The remaining 5′ end also shows eight RIP changes. The deletion of the 3′ portion of the *sad-1* gene would leave the 3′ portion of the wild-type partner unpaired in meiosis and presumably subject to silencing. Thus, in a heterozygous cross, *sad-1*$^+$ could be silenced by a negative feedback, autogenous mechanism, limiting the expression of *sad-1*$^+$ to some low level and giving rise to semidominance of the mutant allele.

To test this hypothesis, we constructed a knockout mutant strain (*Sad-1*$^\Delta$) with a gene replacement of the entire *sad-1* ORF (see Experimental Procedures). It was barren in homozygous *Sad-1*$^\Delta$ crosses and in crosses with *Sad-1*^{UV}, as expected. The phenotype in crosses was qualitatively identical with that of the UV-induced mutant, but the dominance of *Sad-1*$^\Delta$ was more nearly complete (Table 2). This fact eliminates any possibility that *Sad-1*^{UV} is dominant because of negative complementation by the N-terminal peptide of the mutant or because a new neighbor is juxtaposed to the remaining *Sad-1*^{UV} sequence.

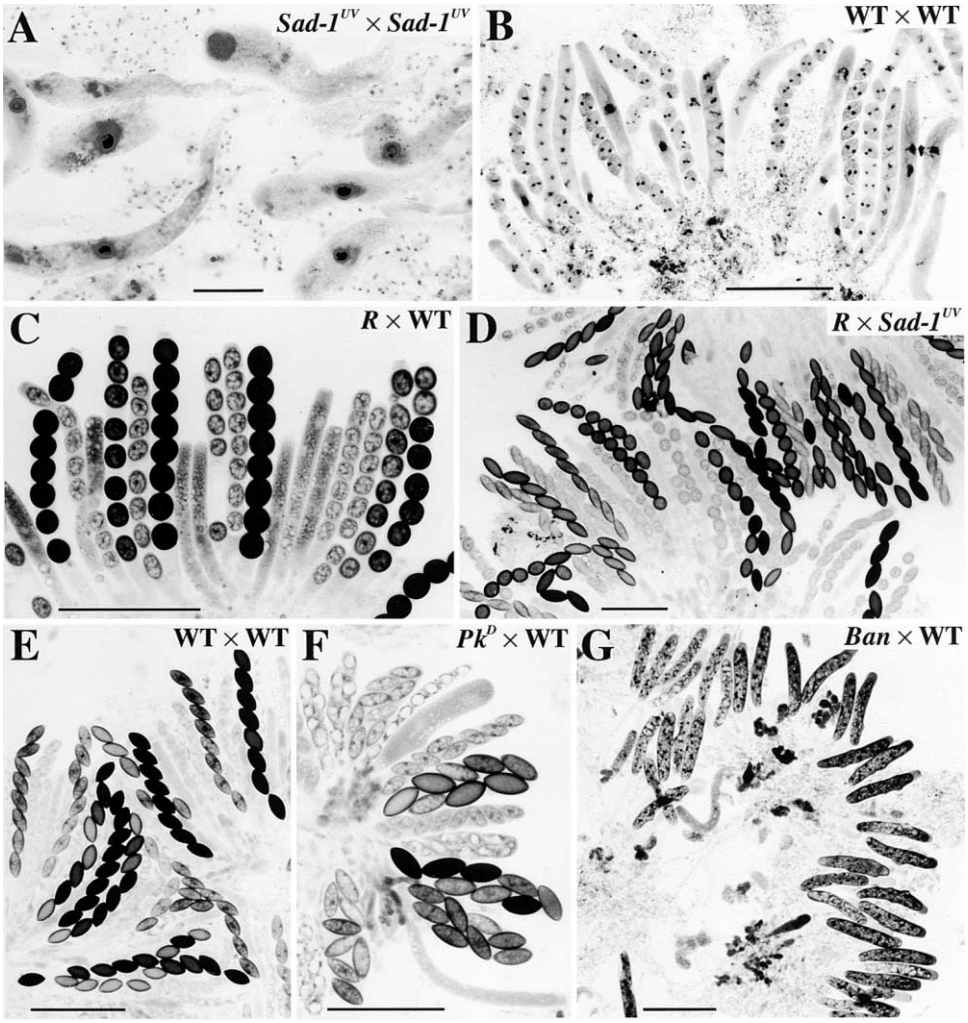


Figure 2. Meiotic Arrest in a Homozygous Cross of *Sad-1*^{UV} × *Sad-1*^{UV} and Suppression of the Dominance of the *Round spore (R)*, *Peak* (*Pk*^{D}), and *Banana* (*Ban*) Phenotypes by *Sad-1*^{UV}

(A) *Sad-1*^{UV} × *Sad-1*^{UV} cross, 7 days postfertilization. Ascus development is arrested in meiotic prophase; the asci abort and degenerate. The bar equals 25 μm.

(B) Wild-type × wild-type. Normally developing asci from perithecium 5 days postfertilization; many asci have already formed individual ascospores.

(C) *Round spore* × wild-type. Maturing asci from a perithecium 8 days postfertilization. Every ascus produces eight round spores, although four of the spores are genotypically wild-type.

(D) *Round spore* × *Sad-1*^{UV}. The round spore phenotype is suppressed in many asci, which now contain eight normal, spindle-shaped spores.

(E) Wild-type × wild-type. Normally maturing cylindrical asci in which the eight ascospores are linearly ordered.

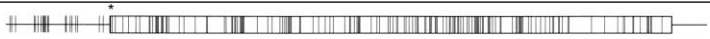
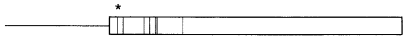
(F) *Pk*^{D} × wild-type. Swollen asci in which the ascospores are nonlinearly arranged. Asci of *Pk*^{D} × *Sad-1*^{UV} are not shown, but they are virtually indistinguishable from the wild-type cross shown in (E).

(G) *Ban* × wild-type. Each ascus consists of a single giant ascospore, which encloses all four meiotic products and their mitotic derivatives. The giant-spore phenotype of *Ban* is suppressed by *Sad-1*^{UV} in up to 50% of asci, which produce eight normal-sized ascospores (photo not shown). In (B)–(G), the bar equals 100 μm.

sad-1^{RIP} and *Sad-1*^{RIP} Mutants

We have isolated 26 *sad-1*-RIP candidates (unpublished data). All of these were fertile in crosses to wild-type but barren in crosses to *Sad-1*^{UV}, suggesting that they are probably *sad-1*^{RIP} null mutants. These candidate strains were also tested for suppression of *Round spore* by examining the spore prints for spindle-shaped spores. They ranged from 0% spindle-shaped (four strains) to near 100% (six strains), with the rest giving intermediate levels. Three of these, RIP-candidate alleles 73, 154, and 141, which gave 0%, 50%, and almost 100% suppression of *Round spore*, were studied further. All three showed GC → AT changes typical of RIP and no other changes. All three RIP alleles had, among other changes, an ATG → ATA mutation in the first position of the open reading frame, indicating that not even a short N-terminal *sad-1* peptide could be synthesized. The RIP mutations summarized in Table 2 allow two conclusions. First, the full recessiveness of allele 73 disproves any model in which the *Sad-1*$^{\Delta}$ mutant is dominant because one normal copy of the gene is insufficient for its expression. Second, the partially dominant and

Table 2. Phenotypes and Sites of *sad-1* Mutations

Strain	Percent Dominant	Mutation Map	GC→AT Changes	*Null Mutation
sad-1^{RIP73}	0%		194	M1→I
Sad-1^{UV}	35%		8	Q26→STOP
Sad-1^{RIP154}	50%		522	M1→I
Sad-1^{RIP141}	~100%		828	M1→I
Sad-1$^{\Delta}$	~100%		None	Δ

The dominance of a particular strain is defined as the percent of spindle-shaped ascospores when that strain is crossed to a *Round spore sad-1*$^{+}$ tester (92-36 or 92-37). The sequence from position 479 to 6682 is shown. The GenBank accession numbers for the first four sequences are (from top to bottom) AF411016, AY032878, AF411018, and AF411017.

fully dominant RIP alleles, 154 and 141 (522 and 828 changes, respectively), have a markedly higher number of RIP changes than does the recessive allele 73 (194 changes). It is possible that, in alleles 154 and 141, there are enough nucleotides mismatched with wild-type that the parental copies are interpreted in the zygote as "unpaired."

Suppression of Barrenness of Crosses of Duplications to Wild-Type, and Evidence that RIP Still Occurs in *Sad-1*^{UV} Crosses

Stable duplications of an internal segment or terminal segment of a chromosome arm constitute another class of dominant "mutations," though they are not usually so designated (Perkins, 1997, and references therein). Heterozygous crosses of such duplications to wild-type are barren; the number of perithecia is normal, but they produce no asci or very few (Perkins, 1997; Raju and Perkins, 1978). It was demonstrated that such duplications are targets for RIP (Selker 1990; Perkins et al., 1997). However, RIP does not easily explain the almost complete barrenness of the heterozygous crosses, because the wild-type euploid parent should not be a target of RIP and about half the normal number of ascospores might be expected to come through the cross intact. On the basis of other evidence, Noubissi et al. (2000) have also suggested that RIP may not be the primary cause of the barren phenotype. The MSUD model suggests an alternate interpretation: the many genes within the duplication are present in three copies in the cross, and one copy of each such gene is necessarily unpaired. This would lead to silencing of all three copies of each gene, with the almost inevitable result that one or more genes necessary for meiosis and/or ascospore development would not be expressed.

To test this hypothesis, we crossed *Sad-1*^{UV} and *Sad-1*$^{\Delta}$ to eight different duplication strains chosen for their low rate of reversion to euploidy and examined the fertility of these crosses in comparison to crosses of wild-type to the duplications. The results were striking. All of the duplications were much more fertile in crosses to *Sad-1* mutants than in crosses to wild-type, ranging from 50-fold to 8,000-fold (see Supplemental Table S1 http://www.cell.com/cgi/content/full/107/7/905/DC1). The cross of a duplication with *Sad-1*^{UV} or *Sad-1*$^{\Delta}$ was not macroscopically distinguishable from a homozygous wild-type cross. However, examination of the contents of individual perithecia from several crosses revealed that, as expected, four spores in each ascus were somewhat smaller than normal, poorly pigmented or unpigmented, and obviously inviable. Presumably these were victims of RIP that occurred because of the presence of the duplicated segment in one parent. In the above tests, *Sad-1*^{UV} entered the crosses from the nonduplication parent, but we expected that *Sad-1*^{UV} would also suppress barrenness if it entered with the duplication parent. This expectation was confirmed. We found that if a given duplication is present in both parents in a cross and therefore both are subject to RIP, *Sad-1*^{UV} does not confer fertility (data not shown).

Duplication strains (Dp) of *Neurospora* vary tremendously in their residual fertility in crosses to wild-type, from virtually none to almost completely fertile. This variation results mostly if not entirely from differences in the rate of loss of the duplicated segment, which regenerates the euploid condition and restores fertility. The degree of instability depends on overall genetic background and on alleles at particular loci (Schroeder, 1986, and references therein). It was conceivable that *Sad-1*^{UV} rendered otherwise barren strains fertile by speeding up this loss. Accordingly, we tested whether a representative duplication (Dp) generated from the translocation *T(IIL → VR)NM149*, which is normally stable, could pass through an entire vegetative and sexual cycle even in a *Sad-1*^{UV} background. The results indicated that the duplication is not destabilized by *Sad-1*^{UV} (data not shown).

Can a Wide Variety of Genes Be Meiotically Silenced If They Are Unpaired?

Our model predicts that if any gene coding for any function required during meiosis is inserted ectopically into a wild-type or innocuously-marked strain and the resulting strain is crossed to wild-type, this heterozygous cross should be barren. The model makes two additional predictions. If an otherwise identical insertion is made into a strain of the opposite mating type and these two strains are crossed, their ectopic transgenes will pair in meiosis and the cross will be fertile. Finally, a heterozygous cross of a transgenic strain with *Sad-1*^{UV} or *Sad-1*$^{\Delta}$ should be fertile.

These three predictions have been tested by inserting genes of obviously important function via a double crossover at the *his-3* locus. The genes tested were ones coding for the globally important proteins β-tubulin (*Bml*), actin (Tinsley et al., 1998), histones H3 and H4-1

Table 3. Silencing by Heterozygosity of Ectopic Genes and Suppression by Homozygosity of the Ectopic Genes and by *Sad-1*

	Parent 1 (*mat A*)	Parent 2 (*mat a*)			
Function Encoded	Ectopic Gene Inserted at *his-3::*	Ectopic Gene Inserted at *his-3::*	*Sad-1* Allele	Ascospores	Predominant Ascus Phenotype
APSES-domain transcription factor	*asm-1*$^+$	-	*sad-1*$^+$	abundant, almost all white	white, aborted ascospores
	asm-1$^+$	-	*Sad-1*^{UV}	abundant, mostly black	normal
	asm-1$^+$	*asm-1*$^+$	*sad-1*$^+$	abundant, many black	normal
β-tubulin	*Bml*^{R}	-	*sad-1*$^+$	very few	arrest before metaphase I
	Bml^R	-	*Sad-1*^{UV}	abundant	normal
	Bml^R	*Bml*^{R}	*Sad-1*$^+$	abundant	normal
Actin	*act*$^+$	-	*sad-1*$^+$	very few	lollipop asci
	act$^+$	-	*Sad-1*^{UV}	abundant	normal
	act$^+$	*act*$^+$	*Sad-1*$^+$	abundant	normal
Histones H3, H4-1	*hH3hH4-1*$^+$	-	*sad-1*$^+$	very few	ascospores do not mature
	hH3hH4-1$^+$	-	*Sad-1*^{UV}	abundant	normal
	hH3hH4-1$^+$	*hH3hH4-1*$^+$	*sad-1*$^+$	abundant	normal
Plasma membrane ATPase	*pma-1*$^+$	-	*sad-1*$^+$	very few	bubble asci do not mature
	pma-1$^+$	-	*Sad-1*^{UV}	abundant	normal
	pma-1$^+$	*pma-1*$^+$	*sad-1*$^+$	abundant	normal
RecA/RAD51 homolog	*mei-3*$^+$	-	*sad-1*$^+$	very few	blocked in meiosis I
	mei-3$^+$	-	*Sad-1*^{UV}	moderately abundant	normal
	mei-3$^+$	*mei-3*$^+$	*sad-1*$^+$	moderately abundant	normal

Scoring was by inspection, but the designations "very few" and "abundant" differed by at least two orders of magnitude.

(*hH3hH4-1*$^+$) (Woudt et al., 1983), and plasma membrane ATPase (*pma-1*) (Hager et al., 1986). We also tested one gene known to be essential specifically in meiosis because homozygous mutant crosses are barren: the *Neurospora RecA/RAD51* ortholog *meiotic-3 (mei-3)* (Schroeder, 1975). All of these genes are normally single-copy with the exception of *hH4-1* (Perkins et al., 2001). The predictions of the working model were fulfilled in all cases (Table 3). It should be noted that, despite the nearly complete loss of fertility in crosses to wild-type, all of these strains appear normal during vegetative growth.

The phenotypes given by heterozygous crosses of actin and β-tubulin insertion mutants to wild-type are shown in Figures 3 and 4. There are three findings of note. First, as expected, β-tubulin (compare Figures 4A and 4C) and actin (compare Figures 4E and 4F) can no longer be seen after meiotic silencing of their genes has occurred. Second, the silencing effect of MSUD occurs after karyogamy (Figures 4B–4D). Third, absence of actin leads to lollipop-shaped asci with a disorganized microtubular structure (Figure 4G).

It was necessary to test whether insertion at *his-3* of the vector itself or of sequences irrelevant to meiosis could somehow impair fertility. Insertion of the *his-3* vector alone was completely benign, and we expected that heterozygous crosses between structurally wild strains and strains with an insertion at *his-3* of various genes not required in meiosis would also be fully fertile. This prediction was tested with constructs carrying insertions of *pho-4*$^+$ (a prototroph), and of the simple auxotrophs *pan-2*$^+$, *inl*$^+$, and *am*$^+$ (Perkins et al., 2001). The basis for thinking that they would not be directly required in meiosis was that null mutants of these genes gave an abundant production of ascospores even in homozygous crosses (supplemented, of course, with their required nutrients). Normal fertility of crosses heterozygous for the insertions was seen in all cases.

Are Any Genes Immune to MSUD?

The genome contains about 180 tandemly arrayed rDNA repeat units of 9.4 kbp, and the number of copies differs from one strain to the next (Butler and Metzenberg, 1993). rDNA might be mechanically protected from silencing by envelopment in the nucleolus. In addition, the mating type genes, *mat A* and *mat a*, are highly dissimilar in sequence, yet the expression of *mat A* and probably *mat a* continue to be required during meiosis (Shiu and Glass, 2000). These, too, are probably immune to silencing, at least in their normal location. Crosses of wild-type *mat a* to a strain carrying its *mat A* gene in an ectopic location give very few ascospores, suggesting that at least one of the *mat* genes is silenced. In harmony with this interpretation, a cross of *Sad-1*^{UV} *mat a* to the ectopic *mat A* strain was at least 100 times more fertile than the cross of its *sad-1*$^+$ counterpart. This is most easily explained if the immunity to silencing in the endogenous location is conferred by the normal flanking sequences rather than by the *mat* sequences themselves.

Suppression of the Sterility of Some Interspecific Crosses

The genus *Neurospora* includes four heterothallic, eight-spored species in which the ascospores give rise to self-sterile cultures because they carry only *mat A* or *mat a*. These are *N. crassa*, *N. sitophila*, *N. intermedia*, and *N. discreta*. There is also one highly diverse pseudohomothallic, four-spored species, *N. tetrasperma*, in which each ascospore is normally self-fertile because it is heterokaryotic with respect to *mat A* and *mat a* (Perkins and Turner, 1988). *N. crassa* yields some ascospores in crosses to at least some isolates of each of these species except *N. discreta*, ranging from very rare viable ascospores with *N. sitophila* and *N. tetrasperma* to an appreciable minority of black, viable spores with many isolates of *N. intermedia*.

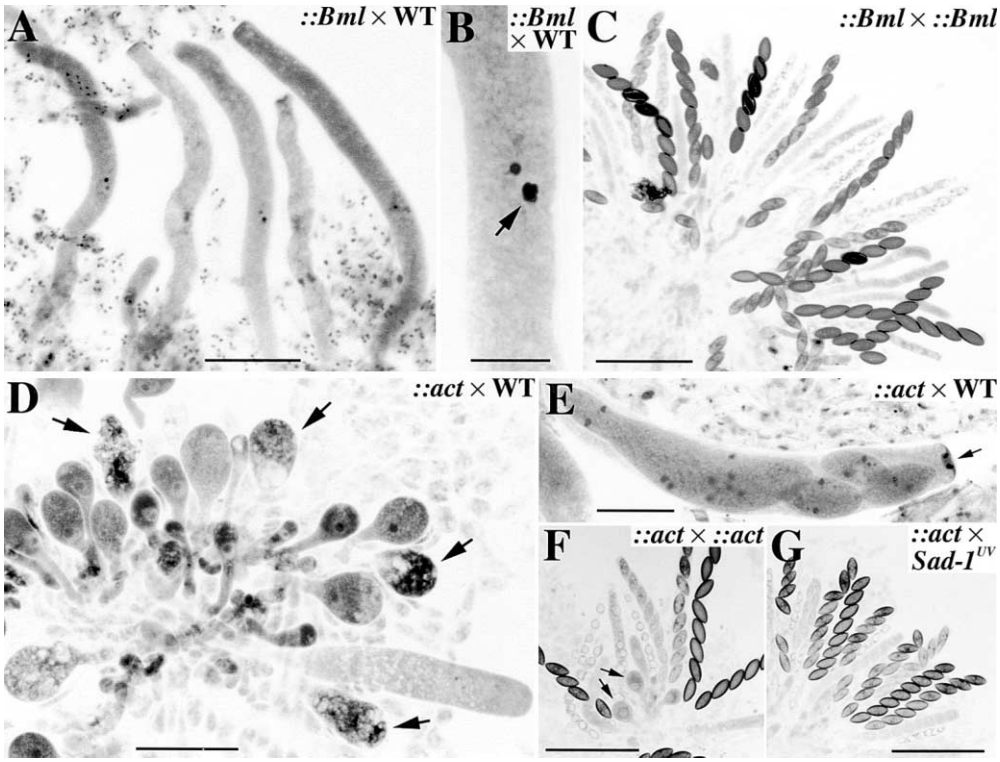


Figure 3. Abnormal Ascus Development and Meiotic Arrest in Ectopic *Bml* (β-Tubulin Gene) and *act*$^+$ (Actin Gene) Insertion Strains (*::*) and Restoration of Their Fertility by *Sad-1*^{UV}

(A and B) *Bml* insertion × wild-type. Ascus development is arrested prior to metaphase I. The asci are long and irregularly shaped. The chromatin is highly condensed (arrow) into a small dense-staining body (arrow) with a small nucleolus adjacent to it. No metaphase/anaphase spindle or spindle pole bodies are visible (also see Figure 4D). The bar equals 50 μm.

(C) *Bml* insertion × *Bml* insertion. Ascus development and nuclear divisions are almost completely normal, and many of the asci produce eight viable ascospores. Ascus development and fertility are also fully restored in *Bml* insertion × *Sad-1*^{UV} (not shown). The bar equals 100 μm.

(D and E) *act*$^+$ gene insertion × wild-type. Over 70% of the asci are short and lollipop-shaped; the rest are elongated. The lollipop asci are usually arrested in meiotic prophase and aborted (arrows), but a few aborting asci contain two or more abnormal nuclei. The bar in (D) equals 50 μm.

(E) An elongated ascus containing three normal-sized spores and one giant spore. Note the normal apical pore (arrow) through which mature ascospores are forcibly ejected. The bar equals 20 μm.

(F) *act*$^+$ gene insertion × *act*$^+$ gene insertion. Over 70% of asci in the homozygous cross are cylindrical and produce eight normal ascospores. The remaining asci are lollipop-shaped (arrows) and aborted. Apparently, the homozygous condition for *act*$^+$ insertion does not restore full fertility. The bar equals 100 μm.

(G) *act*$^+$ insertion × *Sad-1*^{UV}. Almost all asci are cylindrical and develop normally. The bar equals 100 μm.

It seemed possible that one barrier to facile gene flow between these species could be the existence of numerous small rearrangements of gene sequence. These might disrupt intimate pairing of a number of genes in meiosis, with the result that some genes important in meiosis and ascus development would be silenced. We tested this hypothesis by making interspecific crosses between *Sad-1*^{UV} and various isolates of *N. sitophila*, *N. tetrasperma*, and *N. intermedia*, and compared them with crosses to wild-type *N. crassa*. In no case was fertility comparable to that of the intraspecific crosses restored. Nevertheless, with most combinations, a highly significant increase in fertility resulted from the presence of the *Sad-1*^{UV} allele (Table 4). This was least evident in crosses between *N. crassa* and *N. intermedia*, in which the background fertility is already rather high. One strain of *N. tetrasperma* gave no ascospores at all with any strain of *N. crassa*, including *Sad-1*$^{\Delta}$. This strain, T220, has long been recognized as being distinct from other *N. tetrasperma* cytologically (Topper, 1972; N.B.R., unpublished data) and molecularly (Randall and Metzenberg, 1995). Hence, *Sad-1* mutants partially breach the interspecies barrier between *N. crassa* and *N. sitophila*, "typical" *N. tetrasperma*, and (to a limited degree) *N. intermedia*.

Discussion

Meiotic silencing by unpaired DNA is a novel phenomenon. Other workers have described systems in which transcribed DNA present in a greater than normal copy number in vegetative or somatic cells can result in silencing. However, unlike MSUD, these somatic-phase silencers appear to be transcript-counting or transcript-examining mechanisms; they are probably incapable of detecting newly transposed sequences or retroelements that are present only as a single copy. Furthermore, they are not known to operate during meiosis, the

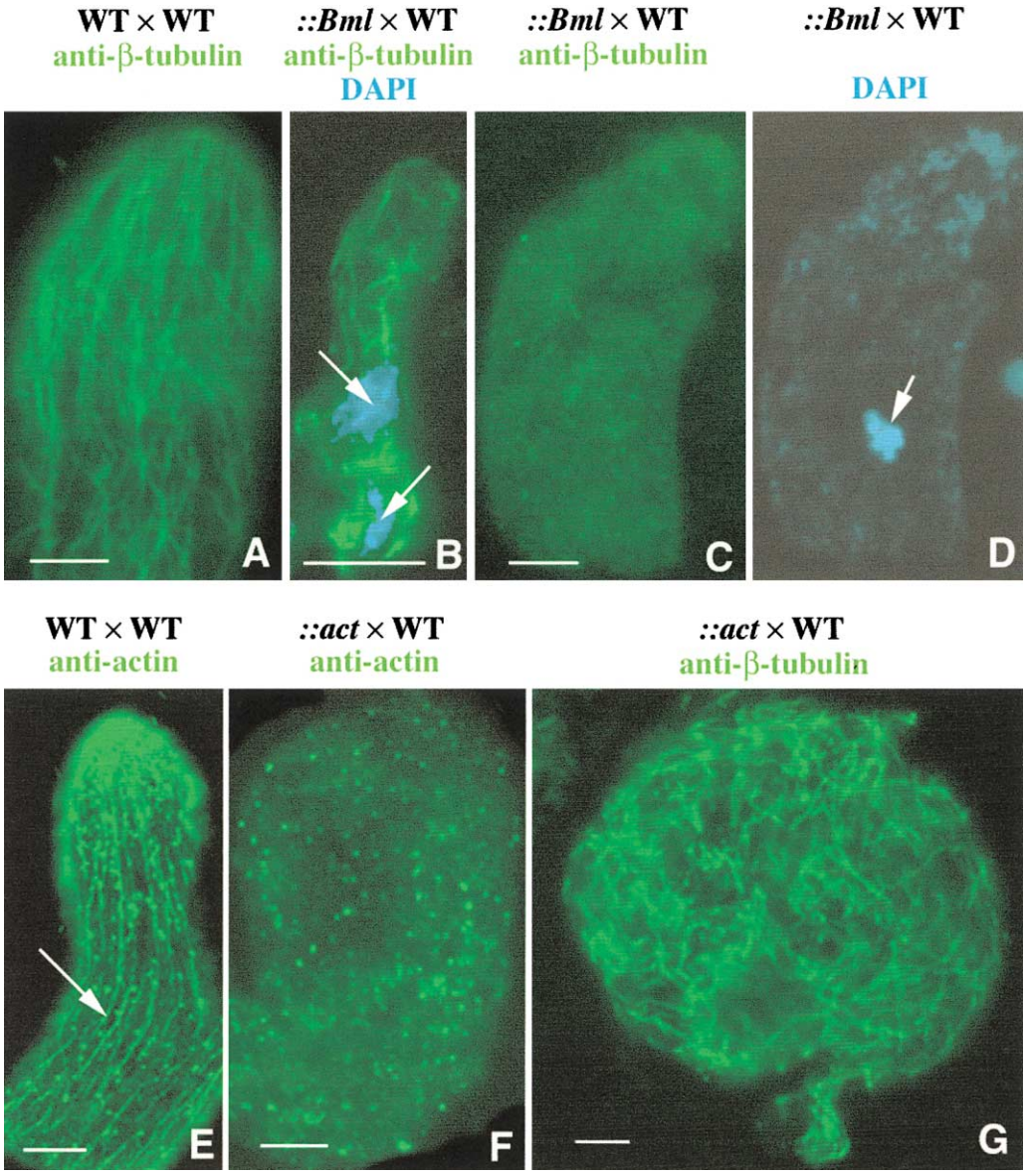


Figure 4. Silencing of β-Tubulin and Actin by Ectopic Transgenes

(A) Wild-type prophase ascus with dense bundles of microtubules (MTs) converging at the tip.
(B) Young ascus from a cross heterozygous for an ectopic copy of the β-tubulin gene (*Bml*) before karyogamy; the two nuclei (arrows, DAPI staining in blue) are still separate, and short MTs are present.
(C) Same cross as (B) after karyogamy. Late prophase (silenced) ascus devoid of MTs.
(D) Corresponding DAPI-staining; note the dense compacted chromosomes (arrow).
(E) Wild-type prophase ascus with orderly longitudinal cortical microfilaments converging to the tip and associated plaques (arrow).
(F) Prophase ascus from crosses heterozygous for insertion of an additional act^+ gene; actin is present only in plaques.
(G) Asci from those crosses are lollipop-shaped and when stained with anti-β-tubulin, they show only disorganized MTs; compare with (A).
The bars equal 5 μm.

time at which the most intimate pairing of chromosomes must occur, and hence, a window of vulnerability to the migration of transposons. Our findings support the idea that DNA unpaired in early stages of meiosis causes both self-silencing and transsilencing of all DNA homologous to it, whether paired or not. As such, even a single-copy DNA sequence that is present in one parent but not in the other will be silenced. Our results suggest that silencing is likely to be posttranscriptional. First, it is hard to construct a credible model in which an unpaired copy of a gene can silence the transcription of two paired copies. Second, RdRP is required for MSUD, and it is known to be also required for PTGS.

MSUD occurs after karyogamy. If self-silencing and transsilencing work by a single mechanism, they might begin after the paired homologs have also become unpaired, i.e., after the end of pachytene. It must have ended not later than the time of ascospore germination, because the genes are reexpressed in vegetative life, as judged by the vegetative normalcy of phenotypically

Table 4. Suppression by *Sad-1* of Sterility of Some Interspecies Crosses

Non-*N. crassa* Species	Strain	*N. crassa* Parent	Ascospores Produced
N. tetrasperma, typical	85 *A*	*sad-1*$^{+}$ *a*	0
		Sad-1^{UV} *a*	2,520
	85 *a*	*sad-1*$^{+}$ *A*	1
		Sad-1^{UV} *A*	4,030
		Sad-1$^{\Delta}$ *A*	42,700
	Ahipara NZ P4371 *A*	*sad-1*$^{+}$ *a*	26
		Sad-1^{UV} *a*	1,160
	Ahipara NZ P4372 *a*	*sad-1*$^{+}$ *A*	80
		Sad-1^{UV} *A*	510
		Sad-1$^{\Delta}$ *A*	4,392
	Lihue-1 v2 *a*	*sad-1*$^{+}$ *A*	0
		Sad-1^{UV} *A*	740
		Sad-1$^{\Delta}$ *A*	10,152
N. tetrasperma, variant	Borneo T220 *A*	*sad-1*$^{+}$ *a*	0
		Sad-1^{UV} *a*	0
		Sad-1$^{\Delta}$ *a*	0
	Borneo T220 *a*	*sad-1*$^{+}$ *A*	0
		Sad-1^{UV} *A*	0
N. sitophila, typical	Arlington *A*	*sad-1*$^{+}$ *a*	0
		Sad-1^{UV} *a*	thousands, some black
	P8085 *A*	*sad-1*$^{+}$ *a*	0
		Sad-1^{UV} *a*	thousands, ~20% black
	P8086 *a*	*sad-1*$^{+}$ *A*	0
		Sad-1^{UV} *A*	thousands, ~50% black
	Europe-2 *a*	*sad-1*$^{+}$ *A*	sparse, almost all white
		Sad-1^{UV} *A*	thousands, ~20% black
N. sitophila, var. *celata*	Adiopodoume-6 *a*	*sad-1*$^{+}$ *A*	very few, almost all white
		Sad-1^{UV} *A*	thousands, ~10% black

There also appeared to be a modest enhancement by *Sad-1*^{UV} of the production of black spores in crosses to various strains of *N. intermedia* (data not shown).

round but genetically wild-type ascospores from crosses heterozygous for *Round spore*. Reexpression might occur even earlier, when the ascospores become individual cells and are no longer dependent on the ascus cytoplasm for their nutrients.

It is not yet clear why RdRP is necessary for completion of meiotic prophase in otherwise wild-type crosses. One possibility is that SAD-1 has an additional function in meiosis. Indeed, in *C. elegans*, mutants homozygous for *ego-1*, a *sad-1* homolog that is expressed almost exclusively in the germ line, have an assortment of defects in both oogenesis and spermatogenesis, though meiosis is not categorically blocked (Smardon et al., 2000). Alternatively, meiosis may fail in the absence of SAD-1 because certain genes must be downregulated. In *C. elegans*, it has been shown that common machinery generates both siRNAs (short interfering RNAs that target mRNA for degradation) and stRNAs (small temporal RNAs that direct repression of mRNA translation; Grishok et al., 2001; Hutvágner et al., 2001). For example, mating-type genes, which are unpaired during meiosis, apparently require tight regulation during sexual development (Coppin and Debuchy, 2000). Small RNAs produced by the silencing system may provide such regulation.

Silencing by an ectopic transgene is potentially a quick and easy tool for investigating the role of genes in meiosis and ascospore development, including genes for which a knockout construct would be lethal in vegetative life. At the same time, the ability to suppress silencing with *Sad-1*^{UV} or *Sad-1*$^{\Delta}$ allows genetic manipulation of the resulting strains. Clearly, a wide variety of genes can be silenced in *Neurospora* to the detriment of meiosis, postmeiotic mitoses, or ascospore maturation. The stage at which development is arrested is characteristic of each individual gene. This is consistent with there being a deficit of the particular product encoded by that gene and excludes the possibility that the arrest of sexual development results from nonspecific inhibition triggered identically by every sort of mispairing. Thus, analysis of the phenotypes of each gene silenced can be informative about the time at which synthesis of any particular macromolecule involved in meiosis becomes important.

Interspecific crosses, especially between *N. crassa* and either *N. sitophila* or *N. tetrasperma*, are normally completely infertile or nearly so, but production of viable ascospores is enhanced by orders of magnitude if a *Sad-1* allele is in the cross. This suggests that microheterogeneity of DNA sequence may play a role in reproductive isolation of species, at least in the genus *Neurospora*, and that occasional occurrences of the *Sad-1* mutation in natural populations could maintain a slow gene flow between species.

How might the silencing of unpaired DNA in meiosis serve a host organism that is haploid most of the time? It would make teleological sense for transposons to have evolved a way of moving from locations present in one parent to the "clean" chromosomes of the other parent in a cross. It would also make sense for the host organism to have evolved defenses against this. Even though transposon mutagenesis may be an essential component of evolution, the great majority of these events are clearly deleterious (Kidwell and Lisch, 2001).

Just as quelling and RIP probably exert surveillance over proliferated invasive elements during stages when all well-behaved genes should be haploid, MSUD could be effective against sequences that are not established in homologous positions of both parents, i.e., that are not diploid when they should be. This silencing mechanism might be important in holding down the genetic load attributable to transposable elements that move during meiosis.

We may ask whether such a system might exist in diploid organisms, including *Homo sapiens*. Silencing by unpaired DNA must not occur regularly in somatic cells, because sizeable heterozygous deficiencies are often viable, but it seems quite plausible that it could occur in meiotic cells. Since the time of Haldane and Muller, population geneticists have been puzzled about how animals with large genomes avoid being extinguished by an excess of "genetic deaths, " i.e., the genetic load imposed by their mutation rates. Recent measures of this mutation rate have emphasized the paradox rather than eliminating it. One way in which animals may have held down the genetic load is by truncation selection—a formulation in which one or a few potentially disadvantageous alleles are harmless or nearly so, but above some threshold number, result in a severe loss of fitness. Thus, each genetic death could remove several deleterious alleles from the gene pool (Crow, 2000). Small chromosomal changes consistent with viability of a conceptus may occur at a specific time, perhaps during meiosis (Crow, 1997). Indeed, a number of newly arisen hemophilia mutations are attributed to *L1Hs (Line-1*) insertions during or very close to the time of meiosis (Dombroski et al., 1991), and the germline of animals is a tissue in which expression of the *Line-1* element is favored (Trelogan and Martin, 1995). In animals, as contrasted with plants, the gametes arise without the haploid genome ever being active, and it seems clear that gametes offer no opportunity for selection against deleterious changes. It is generally assumed that gonial cells and meiotic intermediates such as oocytes and spermatocytes, being diploid, also offer no prospect for lightening the genetic load because almost all mutations are recessive. However, selection during meiosis may have been dismissed too quickly, at least in the case of transposons; a system that detects unpaired DNA during meiotic prophase might allow selection against meiotic cells containing novel DNA sequences, or established sequences in novel positions.

Human oogenesis is, at first glance, a bizarre process. During the first 20 weeks of organogenesis, about seven million diploid oogonia arise in the germ line (Crisp, 1992). At birth, these have all gone through the pairing stages of meiotic prophase and become arrested in diplotene, where they will remain until menarche. During this time, the original seven million are relentlessly culled by atresia so that only about 400–500 of them become Graafian follicles and emerge as an ovum. It is widely assumed that the atretic degeneration of all but about 10^{-4} of the original oocytes is a completely random process. In our view, however, it strains belief that nature would pass up such an opportunity for reducing the genetic load, and the argument that there is no mechanism by which it could happen in a diploid cell is unconvincing. A transposon present in one parent of a human female but not in the other would be unpaired in meiosis, and an oocyte displaying such unpaired DNA, or displaying a gross amount of unpaired DNA above some threshold, might be targeted not only for silencing, but for atresia. Svoboda et al. (2000) have shown that dsRNA can mediate degradation of specific mRNAs in mouse oocytes, indicating that most, if not all, of the machinery necessary for PTGS is present (see also Elbashir et al., 2001; Caplen et al., 2001). The human genome does not appear to contain any homolog or paralog of RdRP, though the current annotation in dbEST does contain 11 cDNAs derived from various human tissues that encode putative RdRPs. MSUD, if it occurs, might make use of RdRP encoded by viruses to prevent accumulation of transcripts encoded by transposons.

Experimental Procedures

Nucleic Acid Methods

Cloning, Southern blotting, etc., were done by standard methods (Sambrook et al., 1989). All DNA sequencing was done by Biotech Core (Mountain View, CA).

Construction and Manipulation of *Neurospora* Strains

The full genotypes of the strains used in this study are reported in Supplemental Table S2. Growth, preparation of conidia, auxanography, and routine manipulations were as described by Davis and DeSerres (1970). Crosses were usually made by simultaneous inoculation of the two mating types into petri dishes of crossing medium. Scoring for mating type, *sad-1*, and *Round spore* was done by spotting conidial suspensions onto *fluffy* tester lawns of the appropriate genotype. Where the spore prints of progeny were to be scored for round or spindle-shaped spores, the spotted lawns were covered with Plexiglas held above the lawns by 1 mm shims. Transformation by plasmids was done according to Margolin et al. (1997) but at 1500 V in a 1 mm gap cell. Transformation by cosmids was done as described by Vollmer and Yanofsky (1986). DNA fragments containing functional copies of *Bml*, *hH3hH4-1*$^+$, *act*, *pma-1*, *mei-3*, *pho-4*, *pan-2*, *inl*, and *am* (Perkins et al., 2001) were cloned into the *his-3* replacement vector pBM61 and targeted to the *his-3* locus (Margolin et al., 1997) of strains *mep his-3; pan-2 a*. Homokaryotic transformants were isolated according to Ebbole and Sachs (1990).

Crosses with Paired and Unpaired *asm-1*$^+$ Genes

Strains 80-22 and 80-24 were heterokaryons with a helper strain because the unassisted deletion mutants do not make protoperithecia (Aramayo and Metzenberg, 1996). The *asm-1*$^\Delta$ strain contains the *mtr*$^+$ gene (instead of the *asm-1*$^+$ gene) at the endogenous *asm* locus (Aramayo et al., 1996). The percent of black ascospores was determined from suspensions of spores ejected to the lids. Ascospores remaining in the perithecia were examined and were similar to those ejected.

Construction of the *Sad-1*$^\Delta$ Strain

A 10,135 bp *Sac*II-*Sac*II fragment containing the *sad-1*$^+$ sequence was cloned from cosmid M11D10 into a modified pBluescript II KS (Stratagene, La Jolla, CA). A 5805 bp *Kpn*1-*Kpn*1 fragment containing the entire *sad-1*$^+$ gene was replaced by a 1.4 kb *Hpa*1-*Hpa*1 fragment carrying a hygromycin resistance cassette from plasmid pCB1004 (Carroll et al., 1994) to give the knockout construct pPKTS001. This carried 1403 bp and 2927 bp of the left and right flanks, respectively, of the *sad-1*$^+$ gene. A *Neurospora* strain, *mep his-3; pan-2 A* (77-29) was transformed with pPKTS001. Transformation mix was plated onto histidine + pantothenic acid medium containing hygromycin, and 100 resistant colonies were picked. These were tested for suppression of *Round spore* without purification by spotting onto lawns of 92-36 and 92-37. Several transformants were found that gave abundant spindle-shaped spores on such lawns. One strain, 95-11, gave over 90% spindle-shaped spores. Southern blotting showed it to be a homokaryon bearing a deletion of the *sad-1* gene (i.e., *Sad-1*$^\Delta$).

Assessment of Fertility of Crosses

Except where noted otherwise, fertility was scored by examining the completed crop of ascospores ejected to the lid of the petri dish. The effects of genetic constitution on the fertility was usually at least two or more orders of magnitude, so that a qualitative designation of fertile or barren could immediately be made by inspection, though "barrenness" was relative except where otherwise stated. In tables where numbers of ascospores are reported, they were counted in their entirety in cases of a high degree of barrenness (zero to a few hundred ascospores); in crosses ranging from a lesser degree of barrenness to full fertility, the entire spore crop in a lid was suspended and an aliquot was counted.

Staining of Asci and Nuclei

Strips of agar medium bearing developing perithecia were fixed at 12 hr intervals (between 5 and 8 days), and the perithecial contents were stained using a propionic-iron-hematoxylin procedure. For rosettes of maturing asci, unfixed 8- to 10-day-old perithecia were opened and photographed after the asci were lightly stained using a 10-fold dilution (in 50% propionic acid) of ferric acetate mordant and hematoxylin (Raju and Newmeyer, 1977). For immunofluorescence studies, asci were fixed in 7.4% paraformaldehyde in 90 mM Pipes (pH 6.9), 10 mM EGTA, 10 mM $MgSO_4$, 0.3% Triton X-100 for 30 min at room temperature and then crushed between a siliconized slide and a polylysine-coated coverslip. After PBS rinsing and extraction with 50 μg/ml myristoyl lysolecithin in the same buffer at 37°C for 1 hr, asci were incubated in primary antibody for 12 hr at room temperature (Thompson-Coffe and Zickler, 1994). Primary antibodies used were anti-β- and anti-α-tubulin at 1:1200 and anti-actin at 1:3000. After two PBS-0.05% Triton rinses, asci on coverslips were incubated in secondary antibody (Caltag FITC, 1:100) at 37°C for 45 min. Chromatin was visualized with DAPI (0.5 μg/ml). Coverslips were mounted in 90% glycerol, 10% 100 mM phosphate (pH 8.7), with 10% w/v 1,4-diazobicyclo (2,2,2)octane (Sigma). Controls were made with primary and secondary antibodies alone. Asci were examined on a Zeiss Axioplan microscope and images captured by a CDD Princeton camera.

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