SAD-2 is required for meiotic silencing by unpaired DNA and perinuclear localization of SAD-1 RNA-directed RNA polymerase

Patrick K. T. Shiu^{*†}, Denise Zickler[‡], Namboori B. Raju[§], Gwenael Ruprich-Robert[‡], and Robert L. Metzenberg^{*†¶}

^{*}Division of Biological Sciences, University of Missouri, Columbia, MO 65211; [‡]Institut de Génétique et Microbiologie, Université Paris-Sud, 91405 Orsay Cedex, France; [§]Department of Biological Sciences, Stanford University, Stanford, CA 94305; and [¶]Department of Biology, California State University, Northridge, CA 91330

Edited by David D. Perkins, Stanford University, Stanford, CA, and approved December 7, 2005 (received for review October 11, 2005)

A gene unpaired during the meiotic homolog pairing stage in *Neurospora* generates a sequence-specific signal that silences the expression of all copies of that gene. This process is called Meiotic Silencing by Unpaired DNA (MSUD). Previously, we have shown that SAD-1, an RNA-directed RNA polymerase (RdRP), is required for MSUD. We isolated a second gene involved in this process, *sad-2*. Mutated *Sad-2^{RIP}* alleles, like those of *Sad-1*, are dominant and suppress MSUD. Crosses homozygous for *Sad-2* are blocked at meiotic prophase. SAD-2 colocalizes with SAD-1 in the perinuclear region, where small interfering RNAs have been shown to reside in mammalian cells. A functional *sad-2⁺* gene is necessary for SAD-1 localization, but the converse is not true. The data suggest that SAD-2 may function to recruit SAD-1 to the perinuclear region, and that the proper localization of SAD-1 is important for its activity.

epigenetics | meiosis | MSUD | *Neurospora* | RNA interference

Coenocytic organisms, in which nuclei coexist in a common cytoplasm, are probably at especially high risk from proliferation of detrimental retrotransposons. In the haploid ascomycete *Neurospora crassa*, several gene-silencing mechanisms exist to maintain its genome integrity. Quelling, which defends the organism during the vegetative phase, is an RNA interference (RNAi) system that suppresses the expression of transgenes occurring in more than one copy (1, 2). Another surveillance system, known as repeat-induced point mutation (RIP), is a premeiotic process that scans the genome for duplicated sequences and targets them for C to T mutations (3). A third silencing mechanism, named meiotic silencing by unpaired DNA, scans the genome and monitors the pairing of DNA segments with their homologs during meiotic prophase (4–6). This mechanism probably prevents the expression and transposition of invasive sequences, and serves the organism in its need to counter exogenous elements and perhaps to regulate endogenous elements. Deletion or extensive mutation in an RdRP-encoding gene, *sad-1* (suppressor of ascus dominance), reduces meiotic silencing to a low level. RdRP plays an important role in some RNAi systems (2). For example, if foreign nucleic acids trigger the production of aberrant RNA (aRNA), the single-stranded aRNA can be replicated into a double-stranded species (dsRNA) via the activity of an RdRP. The dsRNA is then processed into small interfering RNA (siRNA) duplexes by Dicer. The siRNAs subsequently guide the cleavage of mRNA via the RNA-induced silencing complex. The fact that an RdRP is required for meiotic silencing suggests that the synthesis of dsRNA, its amplification, or both, are essential for the process.

We have now identified an additional gene, *sad-2*, which is also required for meiotic silencing. Dominant mutations (*Sad-2*) can suppress the meiotic silencing of unpaired loci with efficiency comparable to that of *Sad-1*. A *Sad-2* mutation does not give any obvious abnormal phenotype during vegetative growth and, correspondingly, *sad-2⁺* mRNA can only be detected in the sexual phase. A cross homozygous for the *sad-2* mutation is

Fig. 1. The strategy for the identification of strains carrying dominant mutations that affect meiotic silencing. The expression of *asm-1⁺* is required for ascospore maturation, whereas the expression of *r⁺* is required for the production of spindle-shaped ascospores. Thus, crosses involving unpaired copies of the *asm-1⁺* and *r⁺* lead to the production of white, round ascospores (Upper). However, a dominant mutant of meiotic silencing (*Sad-x*) can bypass the pairing requirements of *asm-1⁺* and *r⁺*, thus allowing the recovery of black spindle-shaped ascospores (Lower).

arrested at meiotic prophase I. The *sad-2⁺* gene encodes a protein not previously identified as a component of the RNA-based silencing pathways. SAD-2 colocalizes primarily with SAD-1 in the perinuclear region from karyogamy to the end of meiotic pairing, suggesting that the two proteins interact. The perinuclear localization of SAD-1 depends on the presence of SAD-2 but not vice versa, suggesting that SAD-2 may recruit SAD-1 to this region and that the proper localization of SAD-1 (an RdRP) is crucial for its function.

Results and Discussion

Isolation of *Sad-2*, a Suppressor of Meiotic Silencing. To isolate mutants that fail to silence the expression of genes whose DNAs are unpaired during meiotic prophase, we used random insertional mutagenesis of a pantothenate-requiring mutant with a *pan-2⁺* plasmid. The rationale is that an insertion into a *sad* gene should disrupt its pairing with a wild-type homolog in a suc-

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Freely available online through the PNAS open access option.

Abbreviations: RNAi, RNA interference; RIP, repeat-induced point mutation; siRNA, small interfering RNA; RFP, red fluorescent protein; RdRP, RNA-directed RNA polymerase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY303388).

See Commentary on page 2007.

[†]To whom correspondence may be addressed. E-mail: shiup@missouri.edu or rmetzenberg@yahoo.com.

© 2006 by The National Academy of Sciences of the USA

ceeding cross, thus silencing the silencer. The affected genes will also be tagged with a selectable marker, thus simplifying subsequent cloning. We screened prototrophic candidates that could simultaneously suppress two different mutations that result in meiotic silencing in the target strain (Fig. 1). These were *asm-1⁺*, which was deleted from its canonical location on linkage group V and relocated to the *his-3* locus on LG I, and *Round spore*. An ectopic *asm-1⁺*, when crossed to a canonically located *asm-1⁺*, causes almost all ascospores (sexual spores) to remain white and inviable unless meiotic silencing is inactivated by mutation (5). A heterozygous cross of *Round spore* (a deletion allele) to its wild-type counterpart gives $\geq 99.9\%$ round ascospores instead of the wild-type spindle-shaped ones. Therefore, the screen, besides being selective for insertion of *pan-2⁺* and for ascospores that were black and viable, was also a visual one for spindle-shaped ascospores. In addition to isolating further alleles of *Sad-1*, we identified *Sad-2*, a mutant deficient in meiotic silencing (see *Materials and Methods*).

SAD-2 Is Essential for Sexual Development and Sporulation. The *Sad-2* mutant strain has no obvious defects during vegetative growth. Meiosis and sporulation are also completely normal in *Sad-2* $\times$ wild type crosses (see ref. 7 for wild-type meiosis). In contrast, development in crosses homozygous for the *Sad-2* mutation lags behind by ≈ 1 day, and the perithecia (fruiting bodies) are barren. At 5 days after fertilization, development of perithecia appears normal, although they contain $< 50\%$ of the usual number of asci (spore sacs) (50–75 vs. 100–200). Early ascus development and ascus morphology appear normal but the asci become arrested in meiotic prophase (Fig. 2). Among at least 100 pachytene-stage asci examined, chromosome pairing is only partial, with large segments unpaired in all nuclei (compare Fig. 2B with D). At 6 days after fertilization, 75–80% of the older asci are swollen (paddle-shaped) and contain a single nucleus, which shows diffuse chromatin (Fig. 2A and C), whereas $> 80\%$ of the wild-type asci had already delimited ascospores by this time. Fewer than 5% of the mutant asci ever progress beyond the meiotic prophase arrest, and even these become vacuolated without ever having delimited any ascospores. Occasionally (about one in every 10 plates) a few ascospores are seen, whereas a typical wild-type cross usually yields up to 10^6 ascospores per plate. A similar, but 100% barren, phenotype is seen in crosses homozygous for *Sad-1* (5). The mechanism by which fertility is lost is unknown. It is possible that some degree of meiotic silencing is a required checkpoint for cell cycle progression. Alternatively, SAD-1 and SAD-2 may be needed for other functions in meiotic prophase.

Molecular Cloning of *sad-2⁺*. The *Sad-2* mutation induced by the insertional mutagenesis was mapped to the cosmid X1:B12 (see *Materials and Methods*). This cosmid complements the barrenness of crosses homozygous for *Sad-2*, suggesting the presence of the *sad-2⁺* gene (see Figs. 7–9, which are published as supporting information on the PNAS web site). The *sad-2* gene is interrupted by an intron of 78 bp (Fig. 8). The predicted SAD-2

Fig. 2. Meiotic arrest in asci of *Sad-2* $\times$ *Sad-2*. Acriflavin staining is shown. (A) Ascus development is arrested in meiotic prophase, following an apparently normal karyogamy in the young asci. (B) Chromosome pairing is grossly abnormal in early pachytene stage asci; arrow shows two unpaired segments of a chromosome. (C) An arrested ascus at 5–6 days after fertilization contains a single nucleus with diffuse chromatin. (D) Normally paired pachytene chromosomes in a wild-type ascus at 4 days after fertilization. (Scale bars, 10 μ m.)

protein is a 1,097-aa polypeptide made up of 40% nonpolar and 22% charged residues. A BLAST search (8) revealed similarity between SAD-2 and several proteins of unknown function (19–20% identity; Fig. 9), including XP453995 of *Kluyveromyces lactis* (formerly *Saccharomyces lactis*), AAL68190 of *Drosophila melanogaster*, NP505150 of *Caenorhabditis elegans*, and the N terminus (position 8–437) of a predicted protein (EAA49305) of the ascomycete *Magnaporthe grisea* (26% identity). Because a SAD-2-like protein appears to be encoded in the genome of other organisms, it may represent an unrecognized but widespread component of the RNAi machinery.

Nature of *Sad-2* Mutations. Sequence analysis shows that the *sad-2* gene in the original *Sad-2* mutant was rearranged (see *Materials and Methods*) and, in addition, inactivated by RIP mutations (including the translation terminator mutation Q80 $\rightarrow$ stop and the translation initiator mutation M1 $\rightarrow$ I, for isolates 42 and 92, respectively). The RIP mutations in this *Sad-2* mutant cannot have originated from the “spreading” of a RIP mutation of the inserted *pan-2⁺* gene into the neighboring regions of *Sad-2*, because the intactness of this gene was part of the basis of selection. Because the *sad-2⁺* allele in a heterozygous *sad-2* cross can be expected to have trouble pairing with a RIPed sequence, the silencer will be autogenously silencing itself to some low, nonzero level. The same mechanism has been invoked in the case of *Sad-1* (5).

Isolation of Dominant, Semidominant, and Recessive *sad-2* Mutants by RIP. Because the original *Sad-2* isolates contained at least one rearrangement that might affect the expression of nearby genes,

Table 1. Phenotypes and sites of *sad-2* mutations

Strain	Percent dominant, %	Mutation map	GC $\rightarrow$ AT changes	Null mutation (*)
<i>sad-2^{RIP23}</i>	0		171	M1 $\rightarrow$ I
<i>Sad-2^{RIP107}</i>	27.0		195	Q140 $\rightarrow$ stop
<i>Sad-2^{RIP32}</i>	90.5		325	Q17 $\rightarrow$ stop

The percent dominance of a *sad-2* allele is correlated with the number of mismatches with wild type. The dominance of an allele is defined as the percent spindle-shaped ascospores produced when it is crossed to a *Round spore* tester. The sequence from position 813 to 4860 is shown (see Fig. 8).

Table 2. Meiotic silencing by ectopic transgenes and its suppression by *Sad-2*

Function encoded	Parent 1 (<i>mat A</i>), ectopic gene inserted at <i>his-3</i> ⁺	Parent 2 (<i>mat a</i>), allele at <i>sad-2</i>	Yield of ascospores, × 10 ³	Predominant ascus phenotype
Actin	<i>act</i> ⁺	<i>sad-2</i> ⁺	6.2	Lollipop asci, banana spores*
	<i>act</i> ⁺	<i>Sad-2</i>	412	Normal
β -tubulin	<i>Bml</i> ^R	<i>sad-2</i> ⁺	9.2	Arrest before metaphase I
	<i>Bml</i> ^R	<i>Sad-2</i>	526	Normal
Histones H3, H4-1	<i>hH3hH4-1</i> ⁺	<i>sad-2</i> ⁺	37.4	Ascospores do not mature
	<i>hH3hH4-1</i> ⁺	<i>Sad-2</i>	434	Normal

The unsuppressed strains show a much-reduced fertility when crossed with wild type as a result of meiotic silencing of the essential genes coding for actin (*act*⁺), β -tubulin (*Bml*^R), and histone H3H4 (*hH3hH4-1*⁺), respectively, by the copy inserted at the *his-3* locus. This reduction in fertility is not seen in crosses in which one parent carries *Sad-2*.

*The drastic reduction in actin during meiosis also causes a partially penetrant defect in ascospore delimitation, which leads to the production of giant banana-shaped ascospores.

it was important to perform a defined disruption of the *sad-2* gene candidate. We identified several putative *sad-2* RIP mutants, based on the observation that these candidates were barren when crossed to the *sad-2 fl* tester strains (see *Materials and Methods*). These RIP isolates were crossed to *Round spore* for testing the suppression of meiotic silencing. Candidates that gave spindle-shaped ascospores were presumably mutated in the *sad-2* locus. The percentage of spindle-shaped ascospores ranged from 0% to >90%. Three of the RIP candidates (alleles 23, 32, and 107) were subjected to sequence analysis (Table 1). All three contain putative null mutations in *sad-2* that terminate translation early or fail to initiate it. Crosses homozygous for any of these three *sad-2*^{RIP} mutations are arrested at the same stage as those for the original *Sad-2* allele (data not shown). The percent dominance of a strain seems to correlate with the number of GC → AT changes, suggesting that a greater mismatch with wild type leads to a greater degree of self-silencing. This is similar to the behavior of the *Sad-1*^{RIP} alleles (5).

Specific Expression of *sad-2*⁺ in the Sexual Phase. The expression pattern of *sad-2*⁺ was detected by RT-PCR. *sad-2*⁺ mRNA samples prepared from vegetative cells and from perithecia were used as templates. Primers specific for *sad-2*⁺ were used to amplify sequences from these mRNAs (see *Materials and Methods*). The cDNA product of *sad-2*⁺ mRNA (the identity of which was confirmed by DNA sequencing) could be detected in the two perithecial preparations (3 and 6 days after fertilization), but not in the vegetative mRNA. It seems likely that the expression of *sad-2*⁺ is limited to the sexual phase, as is that of the *sad-1*⁺ gene (6).

Suppression by *Sad-2* of Meiotic Silencing Induced by DNA Mismatches at Various Loci. Insertion at an ectopic locus of any of a variety of wild-type genes encoding essential metabolic functions required for sexual development, and crossing such strains to wild type, results in silencing all copies of those genes. The result is a drastic reduction of fertility (5). For example, strains containing an ectopic copy of actin, β -tubulin, or histone H3H4 produce abundant perithecia in crosses to wild type, but produce very few ascospores. *Sad-2* suppresses this effect: crosses of *Sad-2* with these insertion strains produce roughly normal numbers of mature ascospores (Table 2). *Sad-2* also suppresses the barren phenotype of crosses heterozygous for large duplications (see *Materials and Methods*). The self-silencing nature of *Sad-2* suggests that transcripts of the meiotically active genes we tested are also required after the homologous pairing stage and that whatever gene products may be made before that stage are insufficient to execute the necessary function.

To examine the suppressing phenotype of *Sad-2* visually instead of functionally, we crossed a *Sad-2*-null mutant to a strain

containing an extra copy of histone H1 (*hH1*)-GFP or, alternatively, β -tubulin (*Bml*)-GFP. In the three diagnostic crosses, *Sad-2 A* × *hH1-GFP a*, *Sad-2 a* × *hH1-GFP A*, and *Sad-2 A* × *Bml-GFP a*, the GFP-tagged genes were expressed throughout meiosis and ascospore delimitation. Unlike in wild type × *hH1-GFP*, in which *hH1-GFP* was silenced (Fig. 3A), the nuclei in *Sad-2* × *hH1-GFP* fluoresced throughout meiosis and post-meiotic mitosis (Fig. 3B). Similarly, in a cross of *Bml-GFP* × *Sad-2*, β -tubulin-GFP was expressed, and the asci and ascospores developed normally (data not shown). The suppression of meiotic silencing of the two GFP-tagged genes by *Sad-1* was similar to that by *Sad-2* (data not shown).

Perinuclear Localization of SAD-1 and SAD-2. To localize SAD-2, we linked the *sad-2* gene to the sequence encoding GFP, as well as to the sequence encoding red fluorescent protein (RFP). Either a *sad-2-GFP* or a *sad-2-RFP* fusion construct inserted into a *sad-2*-null strain complements the barren phenotype of a *Sad-2* × *Sad-2* cross, indicating that the chimeric proteins are functional. SAD-2-GFP is not seen in vegetative mycelia, conidia (asexual spores) or perithecial cells. It is first seen as irregular aggregates converging toward the nuclear periphery after fertilization when the two nuclei of opposite mating type become isolated in the croziers (hook-shaped sexual cells). At karyogamy the protein forms two spotted rings around each of the fusing nuclei (Fig. 4A–C). Spots coalesce after nuclear fusion (Fig. 4D–F). At leptotene, SAD-2-GFP is always seen as two bright crescents on opposite sites of the nucleus (Fig. 4G–I). During zygotene and pachytene, SAD-2-GFP spreads over the nuclear surface in an irregular network of patches (Fig. 4J–N). These patches vary from one ascus to another in the same perithecium in brightness, thickness, and number (3 to >20), but the spreading from crescent to complete envelopment of the nucleus is

Fig. 3. Suppression of meiotic silencing of GFP-tagged histone H1 (*hH1*) by *Sad-2*. (A) The expression of unpaired *hH1-GFP* is completely silenced during meiosis and postmeiotic mitosis by meiotic silencing. Consequently, the nuclei do not fluoresce. (B) Meiotic silencing of *hH1-GFP* is suppressed by *Sad-2*, and the nuclei fluoresce throughout meiosis and until after ascospore delimitation. (Scale bars, 50 μ m.)

Fig. 4. Perinuclear localization of SAD-2. (A–C) Before karyogamy, SAD-2 is seen as from 1 to >20 foci (arrows) around the two fusing nuclei (arrowheads). (D–F) After karyogamy, foci (arrows) fuse around the nucleus (arrowhead). (G) At leptotene, SAD-2 is seen as crescents (arrows) and cytoplasmic foci (arrowheads). (H) Corresponding DAPI showing the nucleolus (arrow; nu) and mitochondria (arrowhead; m). (I) Nucleolus faces the upper crescent (arrow); the cytoplasmic spots of SAD-2 (arrowheads) do not coincide with mitochondria. (J–N) Four Z sections through a pachytene nucleus and the corresponding DAPI. Arrows point to SAD-2-GFP around the nucleus in J and above nucleus in M. (O and P) SAD-2 forms 20–100 small, irregular foci (O) around the diffuse stage nucleus (P). (Q and R) Two ascospores with SAD-2 foci (arrows) located in the vicinity of their nuclei (arrowheads). (S–U) RFP is covalently attached to SAD-2 (S). The overlay (U) between DAPI (T) and red signals shows that the ring formed by SAD-2-RFP (S) is located outside of the nucleus. (Scale bar, 3 μ m in A–Q and S–U, and 10 μ m in R.)

always observed at mid prophase in >300 asci examined from three different crosses. Optical sections show that the protein is located outside of the nucleus, and overlays with DAPI sections confirm that no SAD-2-GFP is visible inside the nucleus or along the chromosomes (Fig. 4 F and I). The patches become more irregular and numerous (30 to >100) during the diffuse stage (Fig. 4 O and P) and the perinuclear localization is no longer seen after diplotene. The localization described through meiotic prophase I is not materially different in crosses in which *sad-2* is tagged with GFP or with RFP, nor in crosses that contain zero, one, or two unmodified *sad-2*⁺ alleles in their canonical locations distributed between the two parents. In addition to the prophase perinuclear location, SAD-2-GFP is seen from karyogamy to metaphase I in the ascus cytoplasm as 1 to >30 irregular spots covering a range of brightness (Fig. 4G, arrowheads) and is always present as 1–3 spots in each ascospore (Fig. 4 Q and R). Comparison with DAPI staining shows that these cytoplasmic spots overlap vacuoles, not mitochondria (Fig. 4I). It is unclear what the cytoplasmic foci represent. Although they could be

Fig. 5. Colocalization of SAD-1 and SAD-2. (A–C) Colocalization of SAD-1-GFP and SAD-2-RFP in both the nuclear periphery (arrows) and the two cytoplasmic spots (arrowheads). (E–G) In some asci, the two proteins do not completely colocalize (arrows). (D and H) Corresponding DAPI of A and E, respectively. (Scale bars, 3 μ m.)

storage vesicles or molecules in the process of degradation by lysosomes, other possibilities abound.

In all analyzed asci (>300), the SAD-2-GFP or SAD-2-RFP “ring” (Fig. 4S) is seen outside of the nucleus (Fig. 4 T and U). We cannot, of course, exclude the possibility that some SAD-2-RFP or -GFP might be inside the nucleus in quantities not detectable by confocal microscopy. Unmodified GFP and RFP (data not shown) do not accumulate in the perinuclear area, eliminating the possibility that the GFP or RFP motif alone is sufficient to direct any protein containing them to the perinuclear region.

To test whether SAD-1 and SAD-2 are physically associated in the meiotic silencing pathway, we studied the localization of SAD-1. SAD-1-GFP, which complements the barren phenotype of *Sad-1* $\times$ *Sad-1* crosses, shows a highly similar localization to that of SAD-2-GFP (see Fig. 5). However, in both fixed and living material, both the perinuclear and the cytoplasmic SAD-1-GFP spots are 2–3 times less numerous than the SAD-2-GFP spots. Because the SAD-1-GFP spots are, in general, less bright than SAD-2-GFP spots, the numerical difference may merely reflect the existence of an appreciable threshold for detection of less intense fluorescence.

Colocalization of SAD-1 and SAD-2. The localization of both SAD-1 and SAD-2 to the perinuclear region is consistent with the possibility that they work together. We therefore examined crosses of strains encoding SAD-1-GFP and SAD-2-RFP and inquired whether they are colocalized during sexual development. When both tags are expressed in a given ascus, the perinuclear localization of SAD-1-GFP and SAD-2-RFP is coincident for most (85%) of their patches from karyogamy to the diplotene stage (Fig. 5 A–D). However, some spots (up to 50% in some asci) are different in shape, localization, or brightness (Fig. 5 E–H). These differences are not stage-specific. The cytoplasmic aggregates are either coincident (arrowheads in Fig. 5 A–C) or not; in several cases, no RFP spots are detectable where GFP spots are seen, and vice versa.

SAD-2 Is Required for the Perinuclear Localization of SAD-1. The colocalization of SAD-1 and SAD-2 suggests the possibility that the two proteins may form a complex and that they could be codependent for their proper localization. Therefore, we asked whether SAD-1-GFP is localized in the perinuclear region in the

Fig. 6. The effect of *sad-2* mutation on the localization of SAD-1 and the converse. (A–G) SAD-1-GFP is not localized to the perinuclear region in a *Sad-2* × *Sad-2* cross. (A and B) SAD-1-GFP (arrows) appears at the correct time in croziers, when the two prekaryogamy nuclei (arrowheads) are not yet fused. (C and D) In 5% of the asci, SAD-1-GFP (arrow) is seen near the nucleus. (E–G) In 95% of the asci, SAD-1-GFP (arrow) is seen in 1–20 spots distributed throughout the cytoplasm. (H–J) SAD-2-GFP is correctly localized in a *Sad-1* × *Sad-1* cross. During early pachytene, brighter spots are formed (arrows in H). These face the telomeres, which are grouped in a restricted area at the bouquet stage (delimited by arrowheads in I and J). (Scale bars, 3 μ m.)

absence of SAD-2, i.e., in a *Sad-2* × *Sad-2* cross. As shown in Fig. 6 A–G, SAD-1-GFP is expressed and can be seen in the cytoplasm of 90% of the 200 asci examined; however, it is no longer present in the perinuclear region. SAD-1-GFP is first seen in the croziers (Fig. 6 A and B) and in the young asci at prekaryogamy but instead of converging upon the nucleus, the SAD-1-GFP spots remain merely in the general vicinity of the nucleus (5%; Fig. 6 C and D) or are dispersed over the entire ascus (95%; Fig. 6 E–G). Clearly, the protein is expressed at the normal time, but is not correctly located when compared to the crosses that contain at least one wild-type allele of *sad-2*. These data suggest that the reaction catalyzed by SAD-1 (presumably the production and/or amplification of dsRNA) must take place in the perinuclear region.

One possible explanation for the previous result is that only a SAD-1-SAD-2 complex can be localized to the perinuclear region, and that individual proteins alone (SAD-1 or SAD-2 taken separately) have no affinity for that locality. Therefore we asked whether a SAD-2-GFP protein could be properly localized in a *Sad-1* Δ background. In contrast to the *sad-2*⁺ requirement for proper localization of SAD-1-GFP, SAD-2-GFP resides in the perinuclear region even in a *Sad-1* Δ × *Sad-1* Δ cross (Fig. 6 H). The protein is seen from crozier to the diffuse stage around the nucleus. It shows the same progression from the crescent to the more dispersed pattern as in a *sad-1*⁺ × *sad-1*⁺ cross. As in the latter, there is a noticeable tendency for SAD-2-GFP to be concentrated in an area facing the telomeres, which are grouped in a restricted region of the nuclear envelope during the bouquet stage (arrowheads in Fig. 6 I and J); this is a typical meiotic configuration seen from late leptotene to early pachytene. Finally, the spots show the same brightness as in a *sad-1*⁺ × *sad-1*⁺ cross. These observations suggest the following normal order of events: SAD-2 migrates from the cytosol to its perinuclear region and then recruits SAD-1 to this location.

Concluding Remarks. In contrast to the viral RdRPs which are located on the cytosolic surface of intracellular membranes (including that of vesicles derived from the endoplasmic reticulum and Golgi apparatus; refs. 9 and 10), or to the fission yeast RdRP, which is associated with silent centromeric heterochromatin (11), SAD-1 is mainly localized in the perinuclear region. It is possible that, in *Neurospora*, single-stranded aberrant RNAs are exported to the cytoplasm from the nucleus. An aberrant RNA molecule can be expected to be converted into dsRNA as it is running the gantlet of SAD-1 molecules in the perinuclear region. At the very least, this should prevent the aberrant RNA from reaching the translation machinery in the bulk cytosol. An immediate conversion of the aberrant RNA to dsRNA in the perinuclear region may be crucial for the effectiveness of the silencing process. siRNA has been shown to accumulate in the perinuclear region of neuronal (mouse-rat) hybridoma cells (12). Similarly, Chiu *et al.* showed that siRNAs are also targeted to the perinuclear region in HeLa cells and that their proper localization is correlated with the efficiency of RNAi (13). It was suggested that siRNAs are sequestered in the perinuclear region for interactions with RNA-induced silencing complex and that this region might represent a center for RNAi factories. Our results are in agreement with this hypothesis. It should be noted that the siRNA in the experiments of Chiu *et al.* were exogenous in origin rather than arising from dsRNA made by RdRP. It seems likely, therefore, that both RdRP and siRNAs reside in the perinuclear region not because they are formed there, but because of their affinity for a perinuclear element. This element may be the SAD-2 protein or its functional equivalent in mammalian cells.

Materials and Methods

Construction and Manipulation of *Neurospora* Strains. The *Neurospora* strains used in the study are listed in Table 3, which is published as supporting information on the PNAS web site. Culturing and crossing media were prepared as described (6). *Round spore* (R), an ascus-dominant mutant containing a deletion between 20 and 30 kb in length (14), gives $\geq 99.9\%$ round ascospores when crossed to wild type. Descriptions of other genetic loci used in this study can be found in Perkins *et al.* (15). Transformation by gene-size plasmids (<15 kb) was done by electroporation of washed conidia (16). Spheroplasts were prepared from germinated conidia and were used as hosts for transformation by cosmids (17).

Insertional Mutagenesis and Selection of a Suppressor of Meiotic Silencing. Conidia of strain 96-34 carrying *pan-2* (allele B3, a presumptive point mutation) are transformed to prototrophy with a plasmid carrying the *pan-2*⁺ gene (pOKE102). Strain 96-34, in turn, is a heterokaryon (a cell in which different nuclear types share a common cytoplasm) between the target strain, 96-33, *mep his-3⁺::asm-1⁺*; *Asm-1 Δ* *Round spore*; *pan-2 a* and strain 96-18 (“Helper 5”), which is *mat Δ his-3; hyg^R tk; Bml^R pan-2*. Because strain 96-33 does not grow vigorously even on pantothenic acid, heterokaryosis with a sterile (*mat Δ*) helper strain was necessary to achieve abundant conidiation.

Aliquots of the transformation mixture containing about 16,000 stable *pan-2*⁺ transformants were allowed to conidiate en masse. Harvested conidia were used to fertilize lawns of strain 96-26 (*fl^P; pan-2*). Ascospores shot to the lids of plates were harvested in water, heat-shocked and plated to minimal sorbose-glucose-fructose medium, and the small minority of spindle-shaped *pan-2*⁺ sporelings were isolated to give individual cultures. Conidia from these cultures were put through one more test for the *Sad* phenotype: the ability to suppress the Barren phenotype of a duplication-bearing strain (5). Suspensions were spotted to lawns of strains 18-13 and 18-40 [*fl^P; Dp(VIIR* → *IL*)5936, *mat A*, and *mat a*, respectively]. Thirteen isolates passed

all of these tests. Subsequent genetic and molecular study showed that three of these carried *Sad-1* alleles derived from at least two independent mutational events. The remaining 10 isolates all originated from the same aliquot of transformation mix and, as judged by their molecular properties, originated from a single, although complex, insertional event. Crosses of these strains to *Sad-1* were fully fertile, unlike *Sad-1* × *Sad-1* crosses, which are completely barren. The new mutants were accordingly named *Sad-2*. Conventional mapping placed *Sad-2* between *inv* and *asn* on the right arm of linkage group V.

Cloning of *Sad-2*⁺. Because the *Sad-2* mutation is tagged with the pOKE102 plasmid, the nucleotide sequences immediately adjacent to it could be recovered. Total DNA from two *Sad-2* isolates (42 and 92) were endonuclease-digested and recircularized. Nested PCR was then performed by using primers specific to the insertional plasmid. Sequence analysis showed that the insertional event had induced a chromosomal rearrangement (and probably deletion) around a 14.5-kb region (data not shown). This region corresponds to contig 3.222, bases 72686–87542, according to the Broad Institute's *Neurospora* Genome Database (www.broad.mit.edu). Cosmid X1:B12 of the Orbach and Sachs library (18) covers this region. The million-fold reduction of fertility of crosses homozygous for *Sad-2* allowed an easy identification of the desired cosmid containing *sad-2*⁺ activity. Cosmid X1:B12 potentially containing the sequences disrupted by insertion of pOKE102 into the *Sad-2* isolates restored ascospore production by complementation of *sad-2*. The *sad-2*⁺ activity was further subcloned into a 4,858-bp *Cla*I-*Cla*I fragment (Figs. 7 and 8).

RT-PCR. PCR of the *sad-2*⁺ cDNA was performed by using primers spanning part of the first exon, the intron, and part of the second exon. Vegetative mRNA (from 40–27) and perithecial mRNA (from 40–27 × 81–02) were used as templates. The PCR product of the cDNA (without intron) is 133 bp in length, as compared to 216 bp when amplified from genomic DNA (positions 1205–1420). Actin cDNA was detected as a control in all mRNA preparations.

RIP Mutation. Endogenous genes can routinely be extensively mutated with GC to AT transitions if a second copy of the gene is introduced to the genome and the duplication strain is subsequently crossed. These mutagenesis events, called RIP mutations, occur in the perithecium before the premeiotic S-phase and karyogamy (3). We inserted a 4.1-kb *sad-2*⁺ sequence (positions 788–4883) into the pBM61 vector and used the resulting plasmid to transform a *his-3 A* strain (30–20) to prototrophy. pBM61 contains a truncated fragment of the *his-3*⁺ gene. Transformation at the *his-3* locus can be obtained through

a homologous double crossover event, but not by an ectopic integration (16). After testing the transformant for correct insertion of the *sad-2* ORF, we crossed it to *his-3 a* (41–05). With the now-unwanted ectopic *sad-2* gene being contiguous with the *his-3*⁺ allele, only *his-3* progeny were investigated further. *Sad-2*^{RIP} candidates were those that were barren when crossed to the *Sad-2*; *fl* tester strains (30–05 and 30–06).

***sad-1* and *sad-2* GFP and RFP Fusion Constructs.** PCR-amplified *Neurospora sad-1*⁺ ORF was inserted into *Xba*I-*Pac*I-digested pMF272 plasmid, which encodes the enhanced GFP (19). The *sad-2*⁺ ORF was cloned into the *Bgl*II-*Pac*I site of pMF272 (GFP) and into the *Bgl*II-*Not*I site of pMF334 (RFP) (20). The GFP sequence was linked to the C terminus of the *sad-1*⁺ and *sad-2*⁺ gene, whereas the RFP sequence was linked to the N terminus of the *sad-2*⁺ gene. Both GFP and RFP fusion constructs were targeted to the *his-3* locus of the recipient strain.

Staining of Asci and Other Cytological Methods. Unfixed perithecia of crosses homozygous for *Sad-2* that were 4–10 days after fertilization were hydrolyzed and stained with the DNA-specific fluorochrome acriflavin (21). Eight to 10 perithecia were dissected on a microscope slide in a drop of 10% glycerol and the asci were squashed under a cover glass. The acriflavin-stained asci were examined by using blue light excitation (488 nm) and a long-pass emission filter. For studies of suppression of meiotic silencing by *Sad-2*, ascus development in heterozygous crosses of *Sad-2* × *hH1-GFP* or *Sad-2* × *Bml-GFP* was examined from 4–10 days after fertilization (19). Silencing of *hH1* by *hH1-GFP* constructs does not confer any developmental or morphological defect in *Neurospora*. GFP and RFP imaging were performed on a Zeiss Axioplan microscope and images were captured by a charge-coupled device Princeton camera. Chromatin was visualized with DAPI (0.5 μg/ml) after 20-min fixation in 4% formaldehyde. Distribution of both fusion proteins was analyzed by using a Zeiss LSM 510 Confocal Laser-Scanning Microscope. Projections of serial optical sections using Z series were obtained by using LSM software.

We thank Eric Selker and Michael Freitag (University of Oregon, Eugene) for the pMF272 and pMF334 plasmids, as well as for strains carrying the *rid* mutation and an ectopic copy of the gene for histone *hH1-GFP*. P.K.T.S. and R.L.M. are grateful to David Perkins of Stanford University (Stanford, CA), and to Richard Weiss and Gloria Turner of University of California, Los Angeles, for their hospitality and patience during the conduct of this work. This work was supported initially by U.S. Public Health Service Grant GM08995, and more recently by National Science Foundation Grant MCB0234421 (to R.L.M.); by a Centre National de la Recherche Scientifique (Unité Mixte de Recherche 8621) grant (to D.Z. and G.R.-R.); by a Natural Sciences and Engineering Research Council fellowship and University of Missouri Mission Enhancement funds (to P.K.T.S.), and by National Science Foundation Grant MCB 0417282 (in support of N.B.R.).

- Cogoni, C. & Macino, G. (2000) *Curr. Opin. Genet. Dev.* **10**, 638–643.
- Mello, C. C. & Conte, D., Jr. (2004) *Nature* **431**, 338–342.
- Galagan, J. E. & Selker, E. U. (2004) *Trends Genet.* **20**, 417–423.
- Aramayo, R. & Metzberg, R. L. (1996) *Cell* **86**, 103–113.
- Shiu, P. K. T., Raju, N. B., Zickler, D. & Metzberg, R. L. (2001) *Cell* **107**, 905–916.
- Shiu, P. K. T. & Metzberg, R. L. (2002) *Genetics* **161**, 1483–1495.
- Raju, N. B. (1980) *Eur. J. Cell Biol.* **23**, 208–223.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410.
- Schlegel, A., Giddings, J., Ladinsky, T. M. & Kirkegaard, K. (1996) *J. Virol.* **70**, 6576–6588.
- Froshauer, S., Kartenbeck, J. & Helenius, A. (1988) *J. Cell Biol.* **107**, 2075–2086.
- Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I. & Martienssen, R. A. (2002) *Science* **297**, 1833–1837.
- Grünweller, A., Gillen, C., Erdmann, V. A. & Kurreck, J. (2003) *Oligonucleotides* **13**, 345–352.
- Chiu, Y. L., Ali, A., Chu, C. Y., Cao, H. & Rana, T. M. (2004) *Chem. Biol.* **11**, 1165–1175.
- Pratt, R. J., Lee, D. W. & Aramayo, R. (2004) *Genetics* **168**, 1925–1935.
- Perkins, D. D., Radford, A. & Sachs, M. S. (2001) *The Neurospora Compendium* (Academic, San Diego).
- Margolin, B. S., Freitag, M. & Selker, E. U. (1997) *Fungal Genet. Newsl.* **44**, 34–36.
- Vollmer, S. J. & Yanofsky, C. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4869–4873.
- Orbach, M. J. & Sachs, M. S. (1991) *Fungal Genet. Newsl.* **38**, 97.
- Freitag, M., Hickey, P. C., Raju, N. B., Selker, E. U. & Read, N. D. (2004) *Fungal Genet. Biol.* **41**, 897–910.
- Freitag, M. & Selker, E. U. (2005) *Fungal Genet. Newsl.* **52**, 14–17.
- Raju, N. B. (1986) *Mycologia* **78**, 901–906.