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High Frequency Repeat-Induced Point Mutation (RIP) Is Not Associated With Efficient Recombination in Neurospora

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ABSTRACT

Duplicated DNA sequences in *Neurospora crassa* are efficiently detected and mutated during the sexual cycle by a process named repeat-induced point mutation (RIP). Linked, direct duplications have previously been shown to undergo both RIP and deletion at high frequency during premeiosis, suggesting a relationship between RIP and homologous recombination. We have investigated the relationship between RIP and recombination for an unlinked duplication and for both inverted and direct, linked duplications. RIP occurred at high frequency (42–100%) with all three types of duplications used in this study, yet recombination was infrequent. For both inverted and direct, linked duplications, recombination was observed, but at frequencies one to two orders of magnitude lower than RIP. For the unlinked duplication, no recombinants were seen in 900 progeny, indicating, at most, a recombination frequency nearly three orders of magnitude lower than the frequency of RIP. In a direct duplication, RIP and recombination were correlated, suggesting that these two processes are mechanistically associated or that one process provokes the other. Mutations due to RIP have previously been shown to occur outside the boundary of a linked, direct duplication, indicating that RIP might be able to inactivate genes located in single-copy sequences adjacent to a duplicated sequence. In this study, a single-copy gene located between elements of linked duplications was inactivated at moderate frequencies (12–14%). Sequence analysis demonstrated that RIP mutations had spread into these single-copy sequences at least 930 base pairs from the boundary of the duplication, and Southern analysis indicated that mutations had occurred at least 4 kilobases from the duplication boundary.

REPEAT-INDUCED point mutation (RIP) is a process that detects duplications in the genome of *Neurospora crassa* and makes multiple G:C to A:T mutations in both copies of the duplicated DNA (SELKER 1990; CAMBARERI *et al.* 1989; SELKER and GARRETT 1988). RIP occurs only during the sexual cycle, at the stage between fertilization and meiosis, when both parental nuclear types are proliferating within a common cytoplasm (SELKER *et al.* 1987). A sequence that has been altered by RIP is usually heavily methylated at cytosines, even when present as a single copy in the genome (SELKER and GARRETT 1988).

RIP inactivates repeated sequences in a pairwise fashion (SELKER and GARRETT 1988), even when there are more than two copies (FINCHAM *et al.* 1989), suggesting that RIP, like homologous recombination, involves pairing of like sequences. A direct duplication was observed to undergo, in addition to RIP, high frequency deletion of one copy of the duplicated sequence and the region between the elements of the duplication, apparently as the result of intrachromosomal recombination (SELKER *et al.* 1987). This rearrangement was shown to have occurred just before meiosis, at the same time in the sexual cycle that RIP occurs. The similar timing of RIP and deletion in this direct duplication raised the possibility that RIP and recombination utilize common machinery to pair homologous sequences and that these processes may act sequentially or simultaneously on the same homologously paired structure. This early study established a temporal correlation between RIP and recombination but did not attempt to determine whether deletion had occurred more often in the molecules that were altered by RIP than in those that were not.

RIP can occur in the absence of pairing of homologous chromosomes in meiosis (FOSS and SELKER 1991), indicating that premeiotic DNA-level homologous pairing is separable from meiotic chromosome-level pairing in Neurospora. RIP and premeiotic deletion are distinct in the sense that they seem to be differentially affected by genetic background and by degree of sequence homology. *rec-2*, a gene that affects meiotic recombination, influenced the frequency of loss of a genetic marker located between the elements of a direct duplication at *his-3* but did not influence the frequency of RIP (BOWRING and CATCHESIDE 1993). Furthermore, a study that followed a direct duplication through several crosses revealed that deletion was more sensitive than RIP to sequence divergence (CAMBARERI *et al.* 1991).

We have investigated the relationship between RIP and recombination for both linked and unlinked duplications. We expected to see a positive correlation between RIP and recombination if these two processes share an intermediate and can operate on that intermediate either sequentially or simultaneously.

TABLE 1

Neurospora strains

Name	Genotype	Source
N408	*lys-1 A*	V. Miao
N534	*mtr SR62 col-4 trp-2 a*	D. Stadler
N566	*mtr SR62 col-4 his-5 trp-2 A*	This work
N648	*am::mtrP-Swa*I *trp-2 a*	This work
N649	Same as N648	This work
N650	*am::mtrA-Swa*I *trp-2 a*	This work
N651	Same as N650	This work
N24	am_{132} *inl A*	R. Metzenberg
N204	*his-2 nuc-1* am_{132} *inl a*	R. Metzenberg
N528	am_{132} $(am^{+}\ hph^{+})^{ec51}$ p^{JI1} *his-2 nuc-1 inl a*	This work
N571	am_{132} $(am^{+}\ hph^{+})^{ec53}$ p^{JI1} *his-2 nuc-1 inl a*	This work
N583	am_{132} $(am^{+}\ hph^{+})^{ec28}$ p^{JI2} *his-2 nuc-1 inl a*	This work
N584	am_{132} $(am^{+}\ hph^{+})^{ec42}$ p^{JI2} *his-2 nuc-1 inl a*	This work
N585	am_{132} $(am^{+}\ hph^{+})^{ec43}$ p^{JI2} *his-2 nuc-1 inl a*	This work
N637	am_{132} $(am^{+}\ hph^{+})^{ec28}$ p^{JI2} *inl A*	This work
N638	am_{132} $(am^{+}\ hph^{+})^{ec42}$ p^{JI2} *inl A*	This work
N639	am_{132} $(am^{+}\ hph^{+})^{ec53}$ p^{JI1} *inl A*	This work
N640	am_{132} $(am^{RIP17}\ hph)^{ec53}$ p^{JI1} *his-2 nuc-1 inl a*	This work
N641	am_{132} $(am^{RIP54}\ hph)^{ec53}$ p^{JI1} *his-2 nuc-1 inl a*	This work
N642	am_{132} $(am^{RIP4}\ hph)^{ec42}$ p^{JI1} *his-2 inl A*	This work
N643	am_{132} $(am^{RIP37}\ hph)^{ec42}$ p^{JI2} *nuc-1 inl a*	This work
N645	am_{132} $(am^{RIP56}\ hph)^{ec51}$ p^{JI1} *his-2 inl A*	This work

The nature and status of the transgenic DNA is indicated in parentheses. Within the parentheses, the superscripted information designates allele type (when due to RIP) and number. Outside the parentheses, the superscripted information designates transformant type (ectopic) and number, and the name of the plasmid.

We anticipated a negative correlation if they share an intermediate that can be processed to either a recombinant or a product of RIP, but not both. No correlation was expected if the two processes are independent of each other. In any case, we anticipated a built-in bias toward a negative correlation due to the established negative influence of one event on the other; RIP decreases homology, resulting in lower probabilities of subsequent recombination, while some recombination events (such as deletion) result in loss of the duplication required for RIP. We also tested the hypothesis that direct repeat deletions occur by intrachromosomal crossing over, by assessing the frequency of such events in inverted duplications, where the crossover recombinant would contain an inversion rather than a deletion.

The linked duplication constructs employed here included a marker gene in the interstitial single-copy sequences in order to facilitate detection of deletion events. It has been previously shown that mutations due to RIP can occur outside of the boundary of a duplication (Foss *et al.* 1991), as can the cytosine methylation associated with RIP (Selker *et al.* 1993), so that loss of activity of the interstitial marker can result from the effects of RIP as well as from deletion. Tests for loss of function of this marker, without deletion, allowed a determination of the frequency at which RIP and the associated cytosine methylation affect genes located in single-copy sequences near a duplication.

MATERIALS AND METHODS

Basic procedures, media and materials: Culture and crossing of Neurospora were carried out as described by Davis and De Serres (1970), using the crossing medium of Russo *et al.* (1985). Neurospora strains are described in Table 1. DNA was purified using the protocol of Irelan *et al.* (1993) or Oakley *et al.* (1987). Electrophoresis of DNA in agarose gels was followed by depurination and alkaline vacuum transfer to Zetabind nylon membrane (CUNO). DNA labeling and hybridization were as described by Selker and Stevens (1987).

Construction of unlinked duplication strains: The *mtr* gene, carried on a single-stranded version of the plasmid pCVN2.9 (Stuart *et al.* 1988) was mutated by synthesizing a second strand *in vitro*, using as a primer a 20-base oligonucleotide (5′-CGGCTTGATTTAAATCTTCA-3′) containing a single mutation (underlined). The change is a G to T nonsense mutation at position +220 in the sequence of Koo and Stuart (1991), and creates a unique *Swa*I restriction site (ATTTAAAT) within the *mtr* gene. DNA chain extension was done following the protocol of Sambrook *et al.* (1989), except using only one primer in a 30-min Sequenase (U.S. Biochemical Corp.) reaction. The reaction mix was used to transform *Escherichia coli* SMR104 (594 *mutL211::*Tn*5*; S. M. Rosenberg) and a plasmid carrying the desired *Swa*I site was named pAH30.

The altered *mtr* gene was subcloned from pAH30 into the Neurospora *am* gene in pMS3 (Miao *et al.* 1994), in both orientations. The plasmid in which *am* and *mtr* are oriented in the same direction was made by ligating an *Eco*RV-*Bam*HI fragment carrying *mtr* with a 2.5-kb *Eco*RI-*Bgl*II fragment of pMS3 in which the *Eco*RI site had been filled in using the Klenow fragment of DNA PolI (NEB). Similarly, the other orientation of *mtr* in *am* was constructed by excising *mtr* with *Eco*RI (which cuts next to *Bam*HI in the polylinker) and *Eco*RV, and ligating it with the same fragment of pMS3, in this case with a filled *Bgl*II end. These alleles of *am* were named *am::mtrP-Swa*I (*P* for parallel orientations of *am* and *mtr*, in pAH31) and *am::mtrA-Swa*I (*A* for antiparallel orientations of *am* and *mtr;* in pAH32).

The *mtr*-disrupted *am* genes were liberated from plasmids pAH31 and pAH32 and used to transform Neurospora strain N408 by homologous integration, using a scheme developed

by MIAO *et al.* (1994). Am$^-$ transformants were analyzed by Southern hybridization to verify the presence of a single copy of the *Swa*I-cuttable allele of the *mtr* gene within the *am* gene. Transformants were crossed with N534 to introduce a *trp-2* marker and remove the *lys-1* marker.

Analysis of unlinked duplications: Two Mtr$^+$ isolates carrying *am::mtrP-Swa*I (N648, N649) and two carrying *am::mtrA-Swa*I (N650, N651) were crossed with N566. N566 is deleted for the native *mtr* locus and carries mutations in *col-4* and *his-5*, both of which are linked to *mtr*. Progeny carrying the *mtr* gene from the *mtr* duplication parent were isolated by selecting for *his-5*$^+$, and then further screened by discarding those that carried the *col-4* (*col*onial morphology) marker, which is very tightly linked (>99%) to the *mtr* deletion allele from the non-duplication parent. The progeny were analyzed genetically for RIP and physically for evidence of recombination. To assay RIP, colonies were transferred to medium selecting for *mtr*$^+$ strains (0.01 mg/ml tryptophan, 0.6 mg/ml arginine). Those that failed to grow were counted as products of RIP. In some cases, colonies were also transferred to medium selecting for *mtr* strains (0.05 mg/ml *p*-fluorophenylalanine); in this case colonies that grew were scored as products of RIP. There was complete agreement between the two tests for *mtr* function. All media were supplemented with 0.06 mg/ml alanine, to allow vigorous growth of *am* strains (higher concentrations of alanine interfered with the Mtr$^+$ and Mtr$^-$ selections), and 0.05 mg/ml anthranilic acid (except when selecting for *mtr*$^+$ strains), to allow growth of *trp-2* strains.

Lyophilized mycelial mats from cross progeny were weighed and equal amounts were combined in pools of five (total of 25–50 mg of material). DNA was prepared from the pools, and 1 µg from each was digested with *Swa*I and with *Eco*RV, which is insensitive to cytosine methylation and cuts sites flanking *mtr* and *am*. The digests were subjected to electrophoresis and Southern analysis using the 2.5-kb *Bam*HI-*Eco*RV fragment containing *mtr* as a probe. Gene conversion resulting in transfer of the *Swa*I site to the native copy of *mtr* would result in a conspicuously altered band pattern, as would crossing over. Mixing experiments indicated that 1 recombinant in a pool of 10 is detectable using this assay (data not shown).

Creation of linked duplication strains: Plasmid pES201 was created by inserting a 2.6-kb *Bam*HI fragment containing the *am* gene (derived from pJR2; KINSEY and RAMBOSEK 1984) into the single *Bam*HI site of pES200 (STABEN *et al.* 1989), such that *am* would be transcribed toward *hph* (which encodes hygromycin B phosphotransferase, conferring resistance to hygromycin B). A 2.0-kb *Bam*HI-*Eco*RI segment of the 2.6-kb *Bam*HI fragment, containing the *am* gene, was made blunt-ended by filling-in and was inserted into the single *Sma*I site of pES201. The resulting plasmids contain a duplication of *am* flanking 2.3 kb of DNA containing *hph*, where *am* is in either the inverted (pJI1) or the direct (pJI2) orientation. These plasmids were linearized by *Pvu*II digestion and used to transform strain N203 by the protocol of AKINS and LAMBOWITZ (1985). Transformants were obtained by plating on medium containing 1.5 mg/ml glycine at 25° to select for *am*$^+$ strains. Homokaryons were purified by three cycles of conidiation, selecting for *am*$^+$ in the first round and for *hph*$^+$ (on 200 µg/ml hygromycin B from Calbiochem) in the next two rounds. Single copy transformants were identified by Southern analysis using a variety of restriction enzymes and several overlapping probes.

Analysis of linked duplications: Ascospores were germinated on medium containing alanine (permissive for *am*$^-$ strains), and the germlings were transferred to permissive slants and allowed to conidiate. The resulting conidia were then tested by spotting onto plates containing either 1.5 mg/ml glycine at 25° (which allows growth of *am*$^+$ but not *am*$^-$ strains) or alanine plus 100 µg/ml hygromycin B. For the analysis of progeny that escaped RIP in direct duplication crosses, ascospore germlings were transferred directly to glycine plates and those that exhibited growth were transferred to permissive slants and tested as described. The assay for recombination involved digestion of genomic DNA with *Bcl*I, which is insensitive to cytosine methylation (NELSON *et al.* 1993), followed by Southern analysis. For some analyses, DNA methylation was prevented using the drug 5-azacytidine, as described previously (SELKER and STEVENS 1985).

Amplification and sequencing of interstitial sequences: A 573-bp segment of single-copy sequences (see Figure 2) ending 384 bp from the edge of the duplicated region was amplified using degenerate primers complementary to bases 873–889 of *hph* (GRITZ and DAVIES 1983) and bases 2400–2418 of *am* (KINNAIRD and FINCHAM 1983), with primer sequences of 5'-GGAATTCGGGGATTCCYATA-3' and 5'-GGAATTCCG-ATRTCGCCCGTTT-3', respectively. Polymerase chain reactions were carried out using buffer provided with the *Taq* polymerase (Promega), 100 ng of Neurospora DNA, 200 pmol of each primer, 200 mM of each dNTP, and 2 units of *Taq* polymerase. Thirty cycles of 1-min incubations at 94°, 40° and 72° were carried out, and the resulting fragments were cloned into the single *Eco*RI site of pBluescript KS+ (Stratagene), and then subcloned as a *Bam*HI-*Pst*II fragment. Each subcloned region was sequenced at least once on each strand on an Applied Biosystems, Inc. model 373A DNA sequencer at the Center for Gene Research and Biotechnology, Oregon State University.

RESULTS

Recombination and RIP of an unlinked duplication: To assess whether RIP and recombination are mechanistically associated, we monitored both processes among progeny of a cross involving a marked, unlinked duplication. The duplicated region contained the *mtr* gene, which encodes a neutral amino acid permease. This gene has the feature that either function or lack of function can be selected (STADLER 1967). Due to the need to assay recombination at *mtr* in the presence of mutations due to RIP, a physical recombination assay was employed.

The experimental strategy is diagrammed in Figure 1. The strains carrying the duplications have a wild-type allele of *mtr* at the native locus, on linkage group IV, and a defective *mtr* allele, containing a single base change that creates a unique restriction site and results in a nonsense mutation, integrated in the *am* gene on linkage group V. The sequence of the restriction site can be neither destroyed nor created by RIP at this location. The *mtr* duplication strains were crossed with a Neurospora strain (N566) deleted for *mtr* and carrying mutations in *his-5* and *col-4*, which are closely linked to *mtr*. His$^+$ Col$^+$ progeny were selected, effectively selecting for the native locus of *mtr* from the duplication strains, and RIP of the duplication was assayed by testing for *mtr* function. Recombination was assayed by Southern analysis, using the duplicated part of the *mtr* region as a probe and looking for the predicted novel restriction fragments (Figure 1) that should result from gene conversion (used here to simply indicate transfer of information at the marker within *mtr* without rearrangement of flanking sites) and crossing over.

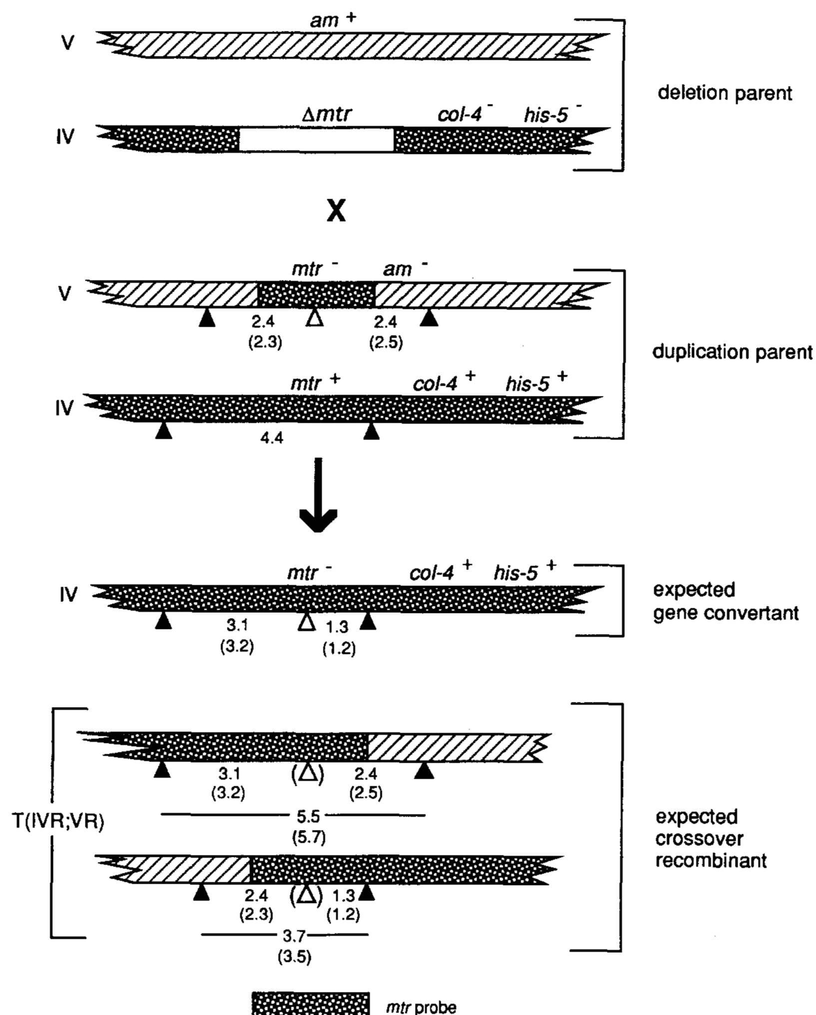

FIGURE 1.—Strategy for detecting recombination between elements of an unlinked duplication. The deletion parent carries no *mtr* sequences, whereas the duplication parent carries two copies of *mtr*, the native one on linkage group IV (spotted) and the ectopic one on linkage group V (striped). Restriction sites are indicated with triangles: the *Swa*I site (open) serves as a marker for conversion, and *Eco*RV sites (filled) serve as flanking markers. Sizes of fragments in kilobases are indicated for the orientation of ectopic *mtr* in N650 and N651, and, in parentheses, the sizes for the opposite orientation, in N648 and N649. The expected gene conversion and crossover products are shown, along with the probe used. Parentheses around the *Swa*I sites in the crossover recombinant indicate that these may or may not be present, which will be determined by the position of the crossover and the presence or absence of conversion. Sizes of fragments for crossover recombinants appear on either side of the *Swa*I site for those that carry this site, and below this site for those lacking it. T(IVR;VR) indicates that the crossover recombinant is a reciprocal translocation exchanging the right arms of linkage groups V and IV.

We chose to look for gene conversion only at the native locus, since only 50% of the selected progeny were expected to carry ectopic *mtr* and because conversion there to mtr^+ would look like partial digestion by *Swa*I. Although gene conversion was expected to occur in strains with either orientation of ectopic *mtr*, crossovers were expected from only the strains with ectopic *mtr* oriented in the same direction, relative to the centromere, as the native *mtr* gene. Crossovers between genes in opposite orientations relative to their centromeres would produce pairs of dicentric and acentric chromosomes that would likely perish in premeiosis or meiosis (PERKINS 1972). Because the orientation of native *mtr* is unknown, both orientations of ectopic *mtr* were tested. In the strains carrying both *mtr* genes in the same orientation relative to their centromeres, crossing over would create translocation chromosomes that would only be recovered when both crossover products segregate together, which should occur with 50% probability. Therefore, only half of the total crossovers should be recoverable.

RIP occurred efficiently in the unlinked *mtr* duplications (Table 2). For ectopic *mtr* in one orientation, RIP occurred at frequencies of 66 and 79% in two different strains, whereas two strains carrying the opposite orientation experienced RIP at frequencies of 42 and 52%. The RIP frequencies for these two orientations are significantly different from each other ($P < 0.001$ by contingency chi square). Analysis of a total of 548 mtr^-

TABLE 2

Frequencies of RIP and recombination involving an unlinked duplication of *mtr*

Duplication parent	Mtr^-/total	RIP frequency (%)	Recombinants/total	
			RIP class	Non-RIP class
N648	245/311	79	0/100	0/100
N649	208/314	66	0/100	0/100
N650	130/310	42	0/206	0/59
N651	161/310	52	0/142	0/93
Totals			0/548 (<0.18%)	0/352 (<0.28%)

Progeny carrying native *mtr* from the duplication parent were selected and tested for *mtr* function and for evidence of recombination. N648 and N649 are siblings of identical genotype, as are N650 and N651, and these two pairs of strains differ in the orientation of ectopic *mtr* in *am*. All strains listed were crossed with N566.

TABLE 3

Loss of am and hph function in linked duplication crosses

Cross	Hyg^R Am^+	Hyg^R Am^-	Hyg^S Am^-	Hyg^S Am^+	Percent Hyg^R loss	Percent RIP
A. Heterozygous crosses						
DR1 (N24 × N583)						
Cross 1	0	53	102	0	32	100
Cross 2	4	35	101	0	44	90
DR2 (N24 × N584)						
Cross 1	5	47	76	0	19	90
Cross 2	1	25	65	0	43	96
DR3 (N24 × N585)						
Cross 1	3	40	78	0	29	93
Cross 2	1	5	20	0	54	83
IR1 (N24 × N528)	4	105	137	0	11	96
IR2 (N24 × N571)	7	83	113	0	11	92
B. Homozygous crosses						
DR4 (N637 × N583)	6	118	22	0	15	95
DR5 (N638 × N584)	7	109	29	0	20	94
IR3 (N639 × N571)	11	73	11	0	12	87

DR and IR indicate direct and inverted repeat crosses, respectively. For heterozygous crosses, loss of *hph* function was estimated by comparing the number of Hyg^R progeny to the number expected (50%) according to the formula $(0.5T-H)/0.5T$ where T = total progeny and H = Hyg^R progeny. The RIP frequency in these crosses was determined by scoring loss of the Am^+ marker among Hyg^R progeny and taking the ratio of Hyg^R Am^- to total Hyg^R progeny. For homozygous crosses, loss of *hph* function was determined from the proportion of Hyg^S to total progeny and the RIP frequency was determined from the proportion of Am^- to total progeny. An additional set of progeny from homozygous crosses DR4 and DR5 were tested for Hyg^R to assess recombination frequency among Am^+ progeny (Table 4). Of 2396 additional DR4 progeny tested, 148 were Am^+ giving a RIP frequency of 94%. Of an additional 3316 DR5 progeny tested, 133 were Am^+, giving a RIP frequency of 96%.

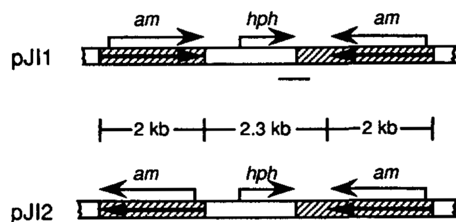

FIGURE 2.—Structure of linked-duplication constructs. Filled boxes indicate *N. crassa* sequences containing the *am* gene. The arrows within the filled boxes indicate the orientation and end points of the duplicated sequences. The location and direction of transcripts are indicated by arrows above the boxes. The horizontal lines below the boxes indicate the region isolated for sequence analysis.

progeny and 352 mtr^+ progeny by restriction enzyme digestion and Southern hybridization revealed no recombinants (Table 2). No progeny were recovered that displayed the expected transfer of the unique restriction site that served as a marker for gene conversion, nor did any display the rearranged flanking restriction sites diagnostic of crossing over. A number of other restriction site alterations were observed among the Mtr^- class, as expected from the high frequency of mutation in this class due to RIP, and no such alterations were observed in the Mtr^+ class.

Recombination and RIP of linked duplications: To investigate RIP and recombination in linked duplications, we constructed duplications of the *am* gene (encoding the NADP-specific glutamate dehydrogenase) in either orientation flanking the *E. coli hph* gene (encoding hygromycin B phosphotransferase) (Figure 2). These constructs allowed us to detect RIP by scoring for loss of *am* function, and to detect recombination resulting in deletion of interstitial sequences by initially scoring for loss of *hph* function (loss of resistance to hygromycin B) and then checking for the absence of *hph* sequences by Southern analysis. Plasmids pJI1 and pJI2 were introduced into a Neurospora strain deleted for the *am* locus (N203), homokaryons were isolated, and single-copy strains were identified by Southern analyses. Three of 12 direct (N583, N584 and N585) and 2 of 35 inverted (N528 and N571) duplication transformants clearly contained a single, intact copy of the transforming DNA and were chosen for further study. Since the transformation host did not share any DNA sequences with the plasmids, and the restriction pattern of the integrated DNA was different in each transformant, it is assumed that the insert DNA is located in different regions of the genome in each of these transformants.

Transformants bearing linked duplications were first crossed to a Neurospora strain deleted for the *am* locus (N24) and progeny were analyzed for *am* and *hph* function (Table 3A). In each of these crosses, the frequency of RIP was estimated from the ratio of Am^- progeny to Hyg^R (hygromycin resistant) progeny, and the deletion frequency was estimated from deviations in the proportion of Hyg^R progeny from 50% of the total progeny, which was the Mendelian expectation. In all cases *am* was inactivated by RIP at high frequency (83–100%), as expected for closely linked duplications. The expectation that *hph* function would frequently be lost among progeny from the direct duplication crosses was also fulfilled. The proportion of Hyg^R progeny in all six experiments involving the three direct duplications (crosses DR1, DR2 and DR3; each cross was performed twice) was significantly reduced, suggesting loss of *hph* function at

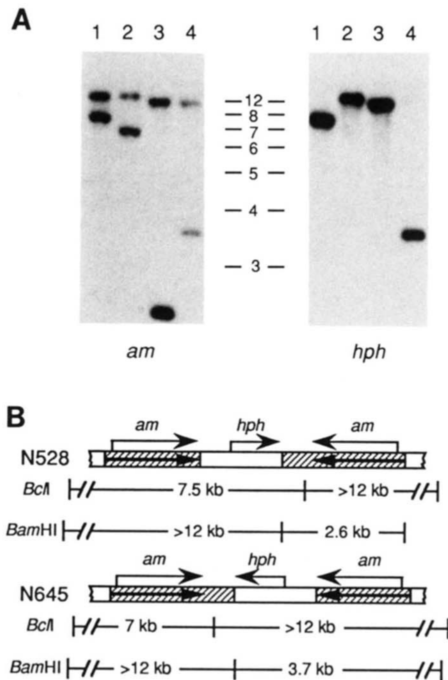

FIGURE 3.—Recombination in an inverted duplication. (**A**) Southern analysis of genomic DNA from the transgenic parent (N528) and the single recombinant strain (N645) from cross IR1 treated with 5-azacytidine to eliminate cytosine methylation, using *am* (left panel) or *hph* (right panel) probes. *Bcl*I digests of N528 (lane 1) and N645 (lane 2) and *Bam*HI digests of N528 (lane 3) and N645 (lane 4) are shown. The positions of size standards (in kilobases) are indicated. (**B**) Restriction maps of the transforming DNA in N528 and in a reciprocal recombinant. See Figure 2 for symbols. *am* and *hph* probes are coincident with the long shaded and central open boxes, respectively.

frequencies of 19–54%. The hypothesis that deletions in direct duplication strains occur by intrachromosomal crossing over predicts that strains with the inverted duplication would not lose *hph* function, since recombinants of the latter would retain the *hph* sequences. In the two crosses involving inverted duplications (IR1 and IR2), the proportion of Hyg^R progeny was less than 50%, yet not significantly so ($0.1 > P > 0.05$ by chi square).

Crosses homozygous for these linked duplications were performed in order to measure loss of *hph* function directly, rather than relying on estimates based on Mendelian expectations. Hyg^R Am^+ progeny from two of the direct (DR1 and DR2) and one of the inverted (IR2) duplication crosses were backcrossed to the duplication parent. As expected, *hph* function was lost in the direct duplications and the frequency of RIP was high (Table 3B). Contrary to expectation, *hph* function was also lost in 12% of the inverted duplication progeny, indicating that either some event other than an intrachromosomal crossover resulted in loss of *hph* function, or *hph* was not expressed in the expected recombinant.

To assess the recombination frequency in inverted duplications, Hyg^R progeny from the heterozygous crosses

TABLE 4

Recombination in linked duplications

Cross	Recombinants/total RIP class	Recombinants/total non-RIP class
IR1 (N24 × N528)	1/73 (1.4%)	0/4
IR2 (N24 × N571)	0/83	0/7
DR4 (N637 × N583)	1/140 (0.71%)	0/154
DR5 (N638 × N584)	10/138 (7.2%)	0/140

Recombination frequency in inverted duplications was determined by Southern analysis of Hyg^R progeny from heterozygous crosses IR1 and IR2. Recombination frequency among the RIP class of progeny from direct duplications was determined by Southern analysis of Hyg^S Am^- progeny from homozygous crosses DR4 and DR5. Recombination frequency among the non-RIP class was determined by Southern analysis of Hyg^S Am^+ progeny, most of which were obtained from an additional set of DR4 and DR5 progeny that were first tested for the Am^+ phenotype, then for the Hyg^S phenotype.

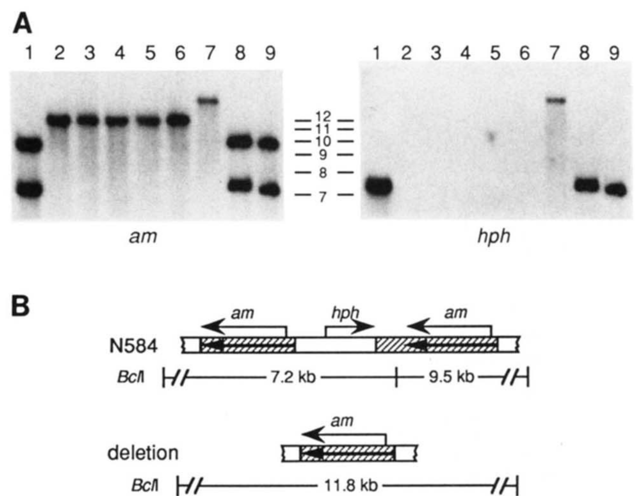

FIGURE 4.—Deletion and restriction site loss in a direct duplication. (**A**) Southern analysis of genomic DNA from cross DR5 parents and progeny, using *am* (left panel) or *hph* (right panel) probes. *Bcl*I digests of the parents, N584 (lane 1) and N638 (lane 9), and progeny representative of deletion (lanes 2–6), loss of the central *Bcl*I site (lane 7), and no alteration (lane 8) are shown. The positions of size standards (in kilobases) are indicated. (**B**) Restriction maps of the transforming DNA in N584 and a deletion product. See Figure 2 for symbols. *am* and *hph* probes are coincident with the shaded and central open boxes, respectively.

were analyzed by Southern hybridization following digestion with *Bcl*I (Figure 3), which is insensitive to cytosine methylation (NELSON *et al.* 1993). One of 77 IR1 and none of 90 IR2 Hyg^R progeny had undergone inversion of the interstitial sequences (Table 4), indicating that although these events are rare, they can occur, and that both orientations of *hph* confer Hyg^R. Recombination frequencies in direct duplications were assessed by Southern analyses of all Hyg^S Am^- progeny from the two homozygous, direct-duplication crosses (Figure 4). Cross DR4 produced 22 Hyg^S progeny, but only one was a deletion, giving a deletion frequency of less than 1%. Cross DR5 produced 29 Hyg^S progeny, of which 10 were deletions, giving a deletion frequency of 7.2% (Table 4).

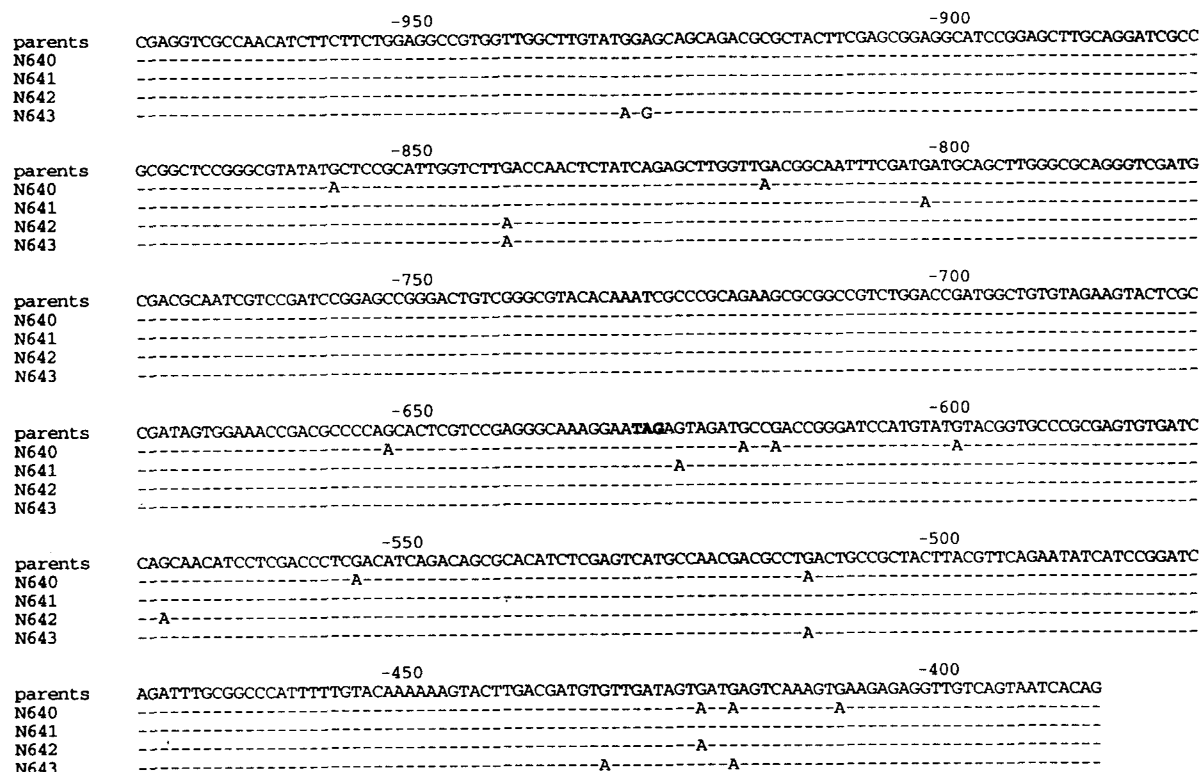

FIGURE 5.—Extension of RIP into unique sequences flanked by inverted and direct duplications. A 573-bp region covering portions of *hph* and *am* was examined from four Hyg^S progeny derived from an inverted (N571) and a direct (N584) duplication. The top line indicates the sequence of the parental strains, which was not altered. Differences from the parental sequence for the two inverted (N640 and N641) and the two direct (N642 and N643) duplication progeny are indicated below the parental sequence. Numbering is relative to the edge of the nearest element of the duplication, the *Eco*RI site of *am* (KINNAIRD and FINCHAM 1983). Bold type indicates the *hph* stop codon.

Thus, loss of *hph* sequences by deletion was infrequent and was not the only cause of loss of *hph* function.

An additional set of progeny was isolated from each of the direct duplication crosses to assess the frequency of deletion among progeny that had escaped RIP. All of the 154 Am$^+$ progeny of cross DR4 and all of the 140 Am$^+$ progeny of cross DR5 were Hyg^R, indicating that none had undergone deletion (Table 4). For cross DR5, the deletion frequency among Am$^+$ progeny was significantly less than the deletion frequency observed in the Am$^-$ class ($P < 0.01$ by contingency chi square), indicating a correlation between RIP and deletion.

Extension of RIP into single copy sequences: In each homozygous, linked-duplication cross, a significant proportion (12–14%; calculated from Tables 3 and 4) of the total progeny had lost *hph* function yet retained the sequences containing *hph*. One possible explanation for this result is that RIP extended into the *hph* sequences. This would require that RIP mutations occur farther outside of a duplication than those previously reported (FOSS *et al.* 1991), since the *hph* coding sequences are located more than 600 bp from the edges of the duplication. Consistent with this hypothesis, loss of restriction enzyme sites was observed in sequences located far beyond the duplication boundary. Southern analysis of the eleven Hyg^S progeny from the homozygous inverted duplication cross (IR3) revealed patterns indicative of loss of *Bcl*I sites in the interstitial sequences and/or flanking the duplicated sequences in seven progeny (data not shown). The *Bcl*I site in the interstitial sequences is 321 bp from the edge of the duplication, while the flanking sites are approximately 3 and 4 kb from the edge of the duplication. Several of the progeny from each homozygous cross involving direct duplications also lost *Bcl*I sites in single-copy sequences (see Figure 4).

To determine directly whether RIP was creating mutations in *hph*, we cloned and sequenced a 573-bp region starting 384 bp from the nearest edge of the duplication from each of two inverted (N640 and N641) and two direct (N642 and N643) duplication Hyg^S progeny as well as from their parents (N638 and N639). These progeny were obtained from crosses IR3 and DR5, respectively. The region sequenced includes 347 bp of the 3′ end of the *hph* coding sequences (see Figure 2). One of the two Hyg^S strains of each duplication type (N641 and N642) had lost the *Bcl*I site located in the interstitial sequences. All four Hyg^S progeny contained mutations of the type created by RIP, while the sequence from the parents remained unaltered (Figure 5). The location of the most distant RIP-type mutation detected in each allele ranged from 802 to 930 bp from the edge of the nearest element of the duplication. All of the RIP mu-

tations were of the same polarity, resulting in G to A changes on the *hph* coding strand. One direct-duplication strain, N643, also showed a single mutation of a type not created by RIP. It is possible that this mutation occurred during the amplification of these sequences for cloning; this type of mutation (A to G) is the most frequent type made by *Taq* DNA polymerase (Keohavong and Thilly 1989).

DISCUSSION

Previous studies on the fate of repeated sequences during the sexual cycle of *N. crassa* suggested a relationship between the processes of RIP and genetic recombination. A linked, direct duplication was observed to undergo both RIP and deletion of one element of the duplication and the interstitial sequences at high frequency in the stage between fertilization and premeiotic DNA synthesis (Selker *et al.* 1987). We have further investigated this relationship by assessing RIP and recombination frequencies in three types of duplications: unlinked, direct, and inverted. Unlinked duplications of *mtr* were mutated by RIP in 42–79% of sexual progeny. No recombination was detected between the two copies of this duplication among a total of 900 progeny in the Mtr^+ and Mtr^- classes (Table 2). Based upon these results, it seems likely that RIP and recombination between unlinked duplications are not mechanistically associated. The linked duplications of *am* were mutated by RIP in 83–100% of sexual progeny (Table 3), yet deletion frequencies in the direct duplications were 7.2% or less, and recombination frequencies in the inverted duplications were 0.71% or less (Table 4). Together, these results demonstrate that it is possible to obtain high frequency RIP without high levels of recombination.

The low frequency of recombination observed in this study was surprising based on the high frequency of deletion (67%) seen with the duplication of "flank" by Selker *et al.* (1987). A possible explanation for this difference in frequency is the difference in sizes of the repeats used; "flank" is 6 kb whereas the duplications employed here were 2–2.5 kb. Although Bowring and Catcheside (1993) observed up to 42% loss of a marker located between elements of a 1.5-kb direct duplication of *his-3*, they did not verify that these were deletions. The frequency of these putative deletion events at *his-3* was influenced by *rec-2*, a regulator of meiotic recombination that is thought to act on *his-3* via a *cis*-acting element, *cog* (Catcheside 1977). Thus, an alternative explanation for the low frequency of recombination in the current study could invoke differences in *cis-* and/or *trans*-acting factors that influence recombination frequency. The fact that deletion frequencies in direct duplication crosses DR4 and DR5 were significantly different from each other ($P < 0.05$ by contingency chi square) suggests that *cis*-acting elements influence recombination, since these crosses differed only in the location of the transforming DNA in the transgenic parent. However, it is also possible that this difference is due to normal variation in recombination frequency from cross to cross.

The influence of the meiotic recombination regulator *rec-2* on direct duplication deletions hypothesized by Bowring and Catcheside could be due to the occurrence of some of these events during meiosis, rather than premeiosis. Recombination frequencies in the current study were too low to allow a determination of the timing of the events; however, it should be noted that both meiotic and premeiotic events would be expected to contribute to the recombinant classes. The absence of recombinants in nearly 1,000 progeny of the unlinked duplication is perhaps not surprising when it is considered that meiotic, intragenic recombination at *mtr* occurs at frequencies of 1 in 7,000 to 1 in 30,000, depending on the markers used (Stadler and Kariya 1969).

Deletion between copies of a direct repeat was correlated with RIP. In cross DR5, 7.2% of the progeny that had inactivated the duplicated *am* gene showed deletions, whereas none (<0.75%) of the progeny that escaped RIP showed deletions. Because deletions can occur by either a nonreciprocal or a reciprocal mechanism (Figure 6), this event might be permitted when reciprocal events are prohibited or are lost from the analysis. Of all the duplications studied here, only the linked, direct duplication can produce a viable recombinant from a single, nonreciprocal event, and only this duplication recombined at a frequency high enough to allow us to detect a correlation with RIP. Nonreciprocal recombination is thought to be the source of deletion events in linked, direct duplications in a variety of organisms (Maryon and Carroll 1991; Ozenberger and Roeder 1991; Takahashi *et al.* 1992; Fishman-Lobell *et al.* 1992). A direct comparison between inverted and direct duplications in yeast suggested the existence of a nonreciprocal recombination pathway that produces viable recombinants from only the direct duplication (Rudin *et al.* 1989). Recombination between sister chromatids can also account for the observed deletions and inversions in linked duplications; however, one of the expected products of unequal sister chromatid exchange between direct duplications is a triplication, which has not been observed in either this or previous studies on direct duplications in Neurospora (Selker *et al.* 1987; Cambareri *et al.* 1991).

One motivation for seeking a correlation between RIP and recombination was to investigate the possibility that there is a common intermediate for these two processes; however, in light of our findings that only deletions are demonstrably correlated with RIP, it now seems more realistic to propose that RIP and deletion are associated for some other reason. Deletion could be stimulated by RIP if RIP caused damage that resulted in breaks in the DNA, possibly due to overlapping excision repair tracts

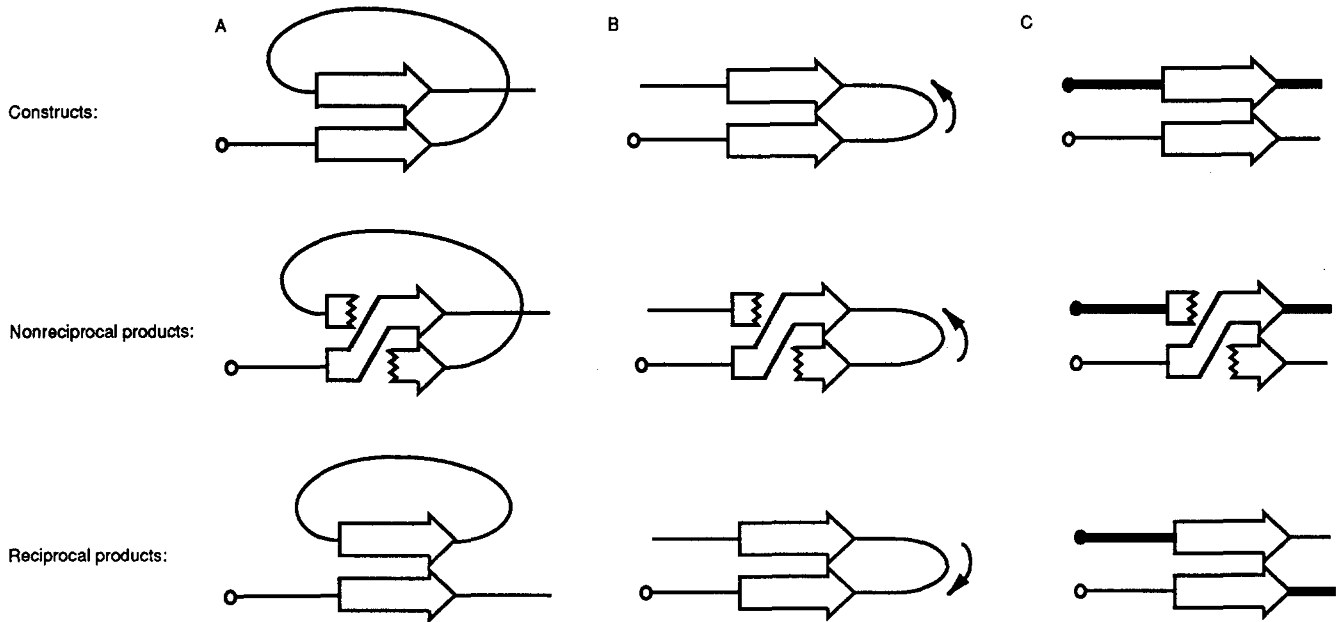

FIGURE 6.—The consequences of reciprocal and nonreciprocal recombination. Chromosomes are shown with duplicated regions aligned (open arrows); lines represent single chromatids and circles represent centromeres. (**A**) Linked, direct duplications may pair intrachromosomally (top panel) and undergo nonreciprocal or reciprocal exchange, the products of which consist of a deletion of one copy of the duplication and the interstitial sequences (middle and bottom panels). (**B**) Linked, inverted duplications may also pair intrachromosomally and undergo nonreciprocal or reciprocal exchange. The former event results in loss of the distal portion of the chromosome (middle panel) while the latter results in inversion of the interstitial sequences relative to the chromosome (bottom panel; the small arrow indicates the orientation of these sequences). (**C**) Unlinked duplications may pair and undergo nonreciprocal or reciprocal exchange. The former results in loss of the distal portion of one chromosome (middle panel), while the latter results in a reciprocal translocation of the chromosome arms (bottom).

provoked by multiple mismatches, a mechanism that has been suggested for creation of breaks under other circumstances (BORTS *et al.* 1990; GLICKMAN and RADMAN 1980). Repair of these breaks may proceed by a single-strand annealing mechanism (LIN *et al.* 1984). The deletion events could also be caused by replication slippage (STREISINGER *et al.* 1966), which has been proposed to account for excision of transposons (EGNER and BERG 1981; FOSTER *et al.* 1981) and could be facilitated by RIP if the RIP machinery held the homologous sequences of the direct duplication in alignment during replication. Both of the above mechanisms would limit recombination associated with RIP to nonreciprocal exchanges.

Recombination between inverted repeats was examined to address the question of whether recombination in linked duplications occurs by a reciprocal or nonreciprocal mechanism. Operation of a nonreciprocal mechanism at significant frequency would result in a higher frequency of recombination for direct duplications than for inverted duplications, since inverted duplications cannot form viable recombinants by a single, nonreciprocal exchange (Figure 6). Pairwise comparison of the recombination frequencies obtained in the crosses listed in Table 4 reveals that only direct duplication cross DR5 is significantly different from any of the other crosses ($P < 0.05$ by contingency chi square). Cross DR5 produced recombinants at a higher frequency than inverted duplication cross IR2, but the recombination frequency was not significantly different from inverted duplication cross IR1. The recombination frequency in cross DR4 was not significantly higher than in either of the inverted duplication crosses. Since there is not a clear difference in recombination frequency between direct and inverted duplications, these data do not support the hypothesis that RIP stimulates deletion by provoking nonreciprocal exchange. However, this hypothesis cannot be ruled out due to the small number of recombinants recovered from most of these crosses.

The frequency of RIP obtained with strains carrying one of the two orientations of ectopic *mtr* (66–79%; Table 2) is higher than had been previously reported for an unlinked duplication. Other unlinked duplications have experienced RIP at frequencies of 5–50% (SELKER *et al.* 1987; FINCHAM *et al.* 1989; FOSS and SELKER 1991; SELKER and GARRETT 1988). This study provides the first information on efficiency of RIP as a function of orientation. Significantly different frequencies of RIP were observed for the two orientations of ectopic *mtr* (66–79% *vs.* 42–52%). Because insertion of *mtr* in the two orientations creates different junction fragments, it would be of interest to determine if orientation, rather than junction sequence, is the relevant variable. Because linked duplications are altered by RIP at a higher frequency than unlinked duplications (this study and SELKER *et al.* 1987), and because sequence divergence reduces the efficiency of RIP (CAMBARERI *et al.* 1991), the

homology search is considered to be the limiting step in RIP. An orientation-dependent difference in RIP frequency would indicate a directional component to the homology search, within the chromosomal context.

While mutations due to RIP are thought to be largely confined to the duplicated sequences, mutations outside of the "flank" duplication have been observed (FOSS *et al.* 1991) as has inactivation, without deletion, of a genetic marker (*am*) located between the elements of the "flank" duplication (see Figure 1 of SELKER *et al.* 1987). We show here by sequence analysis that RIP can extend at least 930 bp from the edge of the duplicated sequence (Figure 5), and Southern analysis indicates that RIP may alter restriction sites as far as 4 kilobases from the duplication. The homozygous crosses involving linked duplications allowed a determination of the frequency with which RIP can inactivate a marker located in single-copy sequences near a duplication. Loss of *hph* function without deletion of *hph* sequences occurred at frequencies of 12–14% (calculated from Tables 3 and 4), indicating that genes neighboring a duplicated sequence can be affected by RIP at a significant frequency. While all four progeny sequenced showed signs of RIP in the *hph* coding sequence, it should be noted that inactivation of *hph* may also occur as a result of the cytosine methylation that has been shown to extend beyond the edge of a region that has been mutated by RIP (SELKER *et al.* 1993). Reversion analyses of the Hyg^S strains obtained from the homozygous, linked-duplication crosses, including tests on the influence of the methylation inhibitor 5-azacytidine, suggest that many of them are Hyg^S due to cytosine methylation (J. IRELAN, unpublished).

RIP involves an efficient, genome-wide search for homologous sequences prior to meiosis. A similar process, methylation induced premeiotically (MIP), occurs in *Ascobolus immersus* and in *Coprinus cinereus* (GOYON and FAUGERON 1989; FREEDMAN and PUKKILA 1993; ROSSIGNOL and FAUGERON 1994). MIP results in methylation, without mutation, of duplicated sequences (RHOUNIM *et al.* 1992). RIP and MIP may play important roles in maintaining proper genome structure, by suppressing the proliferation of transposable elements (SELKER 1990), or by preventing ectopic recombination events that result in chromosome rearrangements (SELKER 1990; KRICKER *et al.* 1992; ROSSIGNOL and FAUGERON 1994). We have shown that Neurospora can efficiently inactivate an unlinked duplication without allowing recombination to occur, as would be desired to prevent genome rearrangements. We have also shown that RIP, apparently unlike MIP, can have important consequences for single-copy genes. The mutations and cytosine methylation that result from RIP often occur far outside the duplication boundary so that a neighboring gene can be inactivated, whereas methylation due to MIP seems to be confined to the duplication (BARRY *et al.* 1993; GOYON *et al.* 1994).

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