Occurrence of Repeat Induced Point Mutation in Long Segmental Duplications of Neurospora

David D. Perkins,* Brian S. Margolin,† Eric U. Selker† and S. D. Haedo‡

**Department of Biological Sciences, Stanford University, Stanford, California 94305-5020, †Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403 and ‡Departamento de Química Biológica, Facultad Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5016-Córdoba, Argentina*

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ABSTRACT

Previous studies of repeat induced point mutation (RIP) have typically involved gene-size duplications resulting from insertion of transforming DNA at ectopic chromosomal positions. To ascertain whether genes in larger duplications are subject to RIP, progeny were examined from crosses heterozygous for long segmental duplications obtained using insertional or quasiterminal translocations. Of 17 distinct mutations from crossing 11 different duplications, 13 mapped within the segment that was duplicated in the parent, one was closely linked, and three were unlinked. Half of the mutations in duplicated segments were at previously unknown loci. The mutations were recessive and were expressed both in haploid and in duplication progeny from *Duplication* × *Normal*, suggesting that both copies of the wild-type gene had undergone RIP. Seven transition mutations characteristic of RIP were found in 395 base pairs (bp) examined in one *ro-11* allele from these crosses and three were found in ~750 bp of another. A single chain-terminating C to T mutation was found in 800 bp of *arg-6*. RIP is thus responsible. These results are consistent with the idea that the impaired fertility that is characteristic of segmental duplications is due to inactivation by RIP of genes needed for progression through the sexual cycle.

ANALYSIS of the fate of transforming DNA in *Neurospora crassa* revealed the existence of a genetic mechanism that silences duplicated sequences in the haploid nuclei of premeiotic cells (SELKER *et al.* 1987; SELKER and GARRETT 1988). This mechanism, named RIP (repeat induced point mutation), results in numerous C to T mutations. In addition, most of the cytosines remaining after RIP are typically found methylated (see SELKER 1990 for a review). Duplicated gene-size segments can sometimes escape RIP in any given sexual cycle, but when discovered by the RIP machine, both copies are affected. If a sequence is repeated in one of the nuclear types of the premeiotic heterokaryon, but not the other, the nucleus with the single copy serves as a safe haven for the sequence, where it is not subject to mutation. This is consistent with indications that RIP operates in a pairwise manner (SELKER and GARRETT 1988; FINCHAM *et al.* 1989) and only before karyogamy (SELKER *et al.* 1987).

The extent and density of the C to T changes is variable. For example, in a survey of eight defective alleles of the *am* gene that resulted from the operation of RIP on an unlinked duplication of a 2.6-kilobase (kb) segment, 0.6–12% of the G:C pairs were mutated in a single passage through the sexual cycle (SINGER *et al.* 1995a). This duplication escaped RIP at frequencies ranging from 72 to 98% (SINGER *et al.* 1995b). Another unlinked duplication of similar size escaped RIP at a lower frequency (21–58%; IRELAN *et al.* 1994). The effects of RIP, while usually drastic, are sometimes mild. For example, *am* mutations generated by RIP can be so mild that the product is still functional (FINCHAM 1990). Mild RIP is apparently rare. In the *al-3* gene, which apparently specifies an essential product, only 70 *al-3* mutants were recovered in 32,000 ascospores subject to RIP (0.42%); as expected, these were all leaky (BARBATO *et al.* 1996). Closely linked repeated sequences rarely escape RIP (SELKER *et al.* 1987; BOWRING and CATCHESIDE 1993; IRELAN *et al.* 1994). Unduplicated sequences that are adjacent to duplicated regions detected by RIP sometimes also become mutated (FOSS *et al.* 1991). Mutations characteristic of RIP have been found at sites almost 1 kb removed from a duplication, and restriction site changes that presumably resulted from RIP have been found at least 4 kb from a duplicated sequence (IRELAN *et al.* 1994).

RIP has been subjected to extensive molecular and genetic examination, and it has been used extensively to determine whether null mutations at a given gene locus are viable and whether they are phenotypically mutant [SELKER *et al.* 1989; for examples see BELL-PEDERSON *et al.* 1992 (*eas*), BOWMAN *et al.* 1992 (*vma-1*), CHARY *et al.* 1994 (*sod-1*), FUENTES *et al.* 1994 (*T*), GLASS and LEE 1992 (*mat A1*), HARKNESS *et al.* 1994 (*mom19*), NELSON and METZENBERG 1992 (*asd-1*), and YARDEN and YANOFSKY 1991 (*chs-1*)]. Until now, the studies have all involved small duplicated segments ap-

Corresponding author: David D. Perkins, Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020.
E-mail: perklab@leland.stanford.edu

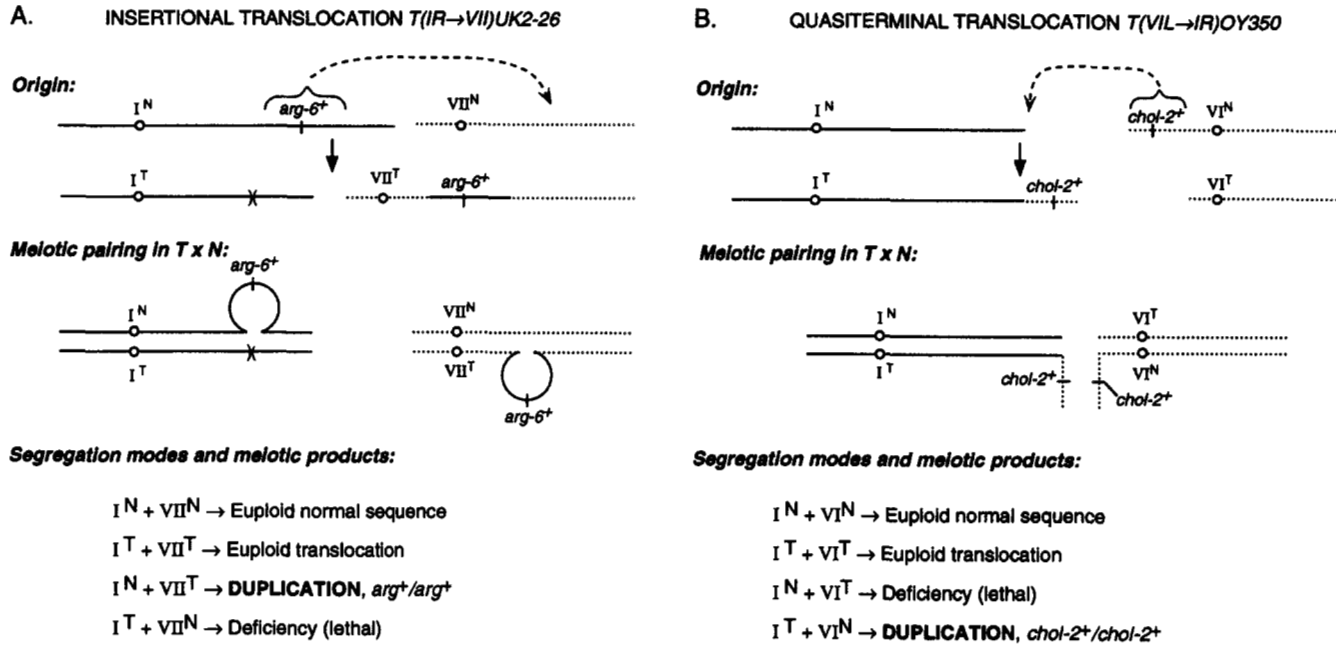

FIGURE 1.—Examples of two types of duplication-generating translocations, showing origin of the rearrangement, meiotic pairing in crosses of $T \times N$, and segregants from $T \times N$. One third of viable meiotic products are partial diploids ("duplications") with two copies of all genes located in the translocated segment (see PERKINS and BARRY 1977). For simplicity, only two noncrossover chromatids are shown. *T*, translocation sequence; *N*, normal sequence. (A) Insertional translocation *T(IR→VII)UK2-26*. Reverse parentheses indicate position of the excision. For simplicity, the duplicate segments are shown unpaired at meiosis. (B) Quasiterminal translocation *T(VIL→IR)OY350*.

proximately the size of individual genes. They have not addressed the question of whether RIP occurs in larger duplications.

In haploid organisms such as Neurospora, partial-diploid strains with duplications of chromosome segments of the order of megabases can be obtained as segregant progeny from crosses that are heterozygous for insertional and quasiterminal translocations (Figure 1). For each rearrangement, the duplication corresponds to the transposed chromosome segment in extent and content. With the rearrangements now available, duplications produced in this way collectively cover four fifths of the *Neurospora crassa* genome (see Figure 2). Curiously, when a duplication strain is crossed by a euploid strain, perithecia are unproductive. Sexual development is arrested to various degrees, and at different times, depending on the aberration. The duplication-induced arrest is seen cytologically to occur during the same period as RIP or shortly thereafter (RAJU and PERKINS 1978). A few exceptional asci may be able to progress far enough so that a few viable ascospores can be obtained from crosses parented by most of the duplications.

RIP seemed for the first time to offer a reasonable explanation for the barrenness that is characteristic of crosses involving segmental duplications (SELKER 1990). If loci within long duplicated segments are subject to RIP, as might be expected by extrapolation from the results with single-gene duplications, genes with nucleus-limited functions that are essential for progression through the sexual cycle might be inactivated. Defective products of genes mutated by RIP could also poison the cell. The result might be to interrupt development and impair fertility. However, an occasional nuclear lineage might escape RIP, or at least heavy RIP, and might retain its ability to proceed through meiosis and to complete development. This line of reasoning suggested that among the surviving progeny one might hope to find mutant alleles at individual loci in the duplicated segment.

The primary aim of the present study was to determine whether gene loci in extensive duplicated chromosome segments are subject to RIP. Progeny were examined from crosses parented by duplications that differed in length and gene content. A demonstration that RIP occurs at gene loci within duplicated chromosome segments would be of interest for its evolutionary implications. It could also lead to significant practical applications. If segmental duplications are indeed subject to RIP, they should be useful as a source of new alleles at known loci, as a means of identifying previously unknown loci within defined chromosome regions and as an alternative to recombination for introducing markers into rearrangement sequence. Conceivably, a saturation map of the duplicated region could be obtained. New light might also be thrown on the mechanism of RIP.

MATERIALS AND METHODS

Genetic analysis: General methods and media were as described by DAVIS and DE SERRES (1970) and by PERKINS (1959,

1986). Crosses were carried out on synthetic crossing medium (SC) containing 1.5% agar and 1% sucrose. The productive rearrangements are listed in Table 1. Figure 2 shows the segments that are duplicated in duplication progeny from each of these rearrangements.

Duplication strains were obtained by crossing each rearrangement by a normal-sequence Oak Ridge strain (usually FGSC 2489, 4200, 4317 or 4347) and testing f_1 progeny for barrenness of perithecia in test crosses with *fl*(OR)*A* or *fl*(OR)*a* testers (FGSC 4317 or 4347; PERKINS and POLLARD 1989). Individual duplication strains were used as male parents to fertilize 6- or 7-day-old normal-sequence cultures. Many of the duplication stains were highly infertile when crossed with an OR wild type, producing perithecia but few or no ascospores. Lowering the crossing temperature from 25 to 15° did not increase fertility. The ascospore yield was often increased significantly by using a normal-sequence *fluffy* strain in place of wild type as protoperithecial parent and by making crosses in petri dishes rather than in culture tubes. These were incubated at 25° with a 12-hour light 12-hour dark cycle. Ten days after fertilization, the original petri dish lids were replaced with lids containing EDTA storage medium (METZENBERG 1988) solidified with 3% agar. The plates were wrapped in plastic film to avoid desiccation, inverted, and incubated in the dark for 20 days or longer.

Mature ascospores from productive crosses were picked up on small pieces of storage medium, transferred to slants of complete medium in 12 × 75 mm tubes, activated by heatshock, and incubated at 25°. After inspection for visible mutant phenotypes, the f_1 cultures were transferred to minimal medium and tested for growth at 34° to detect auxotrophic and heat-sensitive mutants. Putative new mutants were tested for linkage to an appropriate duplication-linked marker. Whether a mutant gene is located within the transposed segment was ascertained by testing for duplication-coverage (PERKINS 1986) or for allelism with a mutant gene that was known to be located in the duplicated segment.

Molecular analyses: DNA was isolated from Neurospora strains as described by OAKLEY *et al.* (1987). Procedures for restriction digestions, gel electrophoresis, molecular cloning, and Southern hybridizations were essentially as described by SAMBROOK *et al.* (1989). Fragments of defective *ro-11* and *arg-6* genes were amplified from genomic DNA by PCR in 50–100 μl reactions containing: 1× Promega *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 200 μM of each nucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 200 ng genomic DNA, 360 ng of each primer, and 2.5 units *Taq* polymerase (Promega). The sequences of primers used to amplify segments of the *ro-11* allele are as follows: 5′CTTCACGACAGCGACGAC3′ and 5′CGTAGGATAATCCCTC3′ for the region 113–501 segment; 5′GAGCAGGAAAGAGAACG3′ and 5′GACTGGTTA GCCTTGTG3′ for the region 966–1393 segment; and 5′CAT CAGCTACAGCACAG3′ and 5′CCAAAAAGTTCCTGTCC3′ for the region 2237–2626 segment (coordinates correspond to those of wild-type sequence kindly provided by MICHAEL PLAMANN). The following primer pairs were used to amplify segments of *arg-6* (GESSERT *et al.* 1994): 5′CGCTCGCTCGAC CGATC3′ and 5′CCCACTTCTCCTTGTTCAGGTAGTC3′ for the region 1430–1879 segment; 5′GCCATCATCCATCCT GAGGAAC3′ and 5′GCCTTGAACGGGTTCTCCTTG3′ for the 2230–2450 segment; and 5′CCGCTTTGACAACAAGTG GACC3′ and 5′TAACCTCACGCTCGTGGATGTG3′ for the region 3285–3562 segment. Samples were heated initially to 94° for 5 min in a Hybaid Omnigene machine and then subjected to 28 or 30 cycles of the following regime with the machine set for "tube control": 2 sec at 94°; 10 sec at 50°; 20 or 30 sec at 72°. The samples were finally incubated 5 min at 72° and then fractionated by gel electrophoresis. The PCR fragments were gel purified and cloned into a pT7Blue T-Vector (Novagen) following the protocol provided with the T-Vector kit. Sequence information for both strands was obtained by sequencing double-stranded plasmid DNA with an ABI 377 automated sequencer at The University of Oregon Biotech Facility using standard sequencing primers (T7 and M13 forward).

RESULTS

Choice of duplication parents: There existed a wide choice of segmental duplications that might be examined for their susceptibility to RIP (PERKINS 1997). The map locations of duplications from 56 different rearrangements are shown in Figure 2. The duplications are not all suitable for testing occurrence of RIP, however. Some are too unstable, others are insufficiently fertile. With unstable rearrangements, fertility is restored by deletion of the duplicated segment, either before or during the critical period between fertilization and fusion of the haploid nuclei. Sequences in the abundant progeny may not have been subjected to RIP. Instability is more frequently a problem with quasiterminal duplications than with insertional duplications. At the other extreme, the fertility of duplications from stable rearrangements is sometimes reduced so drastically that it is difficult to obtain enough progeny for a meaningful test. Between these extremes are duplications that are both sufficiently stable and sufficiently productive for testing the RIP hypothesis.

The spectrum of duplications: Even though ascospore production was often very low, distinct putative RIP mutations were obtained in progeny from 11 relatively stable duplications. These are listed in Table 1 and highlighted in Figure 2. Seven of the productive duplications were interstitial insertions, and all but two are relatively short. This does not necessarily mean that genes inactivated by RIP are more likely to be recovered using short interstitial duplications rather than longer duplications or those that are terminal. The preponderance of short interstitial duplications among those yielding analyzable mutations may merely reflect our decision to use interstitials preferentially because of their greater stability and to use short duplications because of the possibility that mutations at single loci might be recovered more frequently when the number of included loci is low.

The distinct mutations specify a variety of phenotypes, including altered nutritional requirements, morphology, and pigmentation. Recovery of the heat-sensitive conditional mutant *un-24* is consistent with the observation by FINCHAM (1990) that RIP can produce partial loss-of-function mutations, including some that are temperature sensitive. These are presumably lightly mutated.

The search for mutations was not limited to the 11 duplications that produced distinct mutations among their progeny. Exploratory experiments were also con-

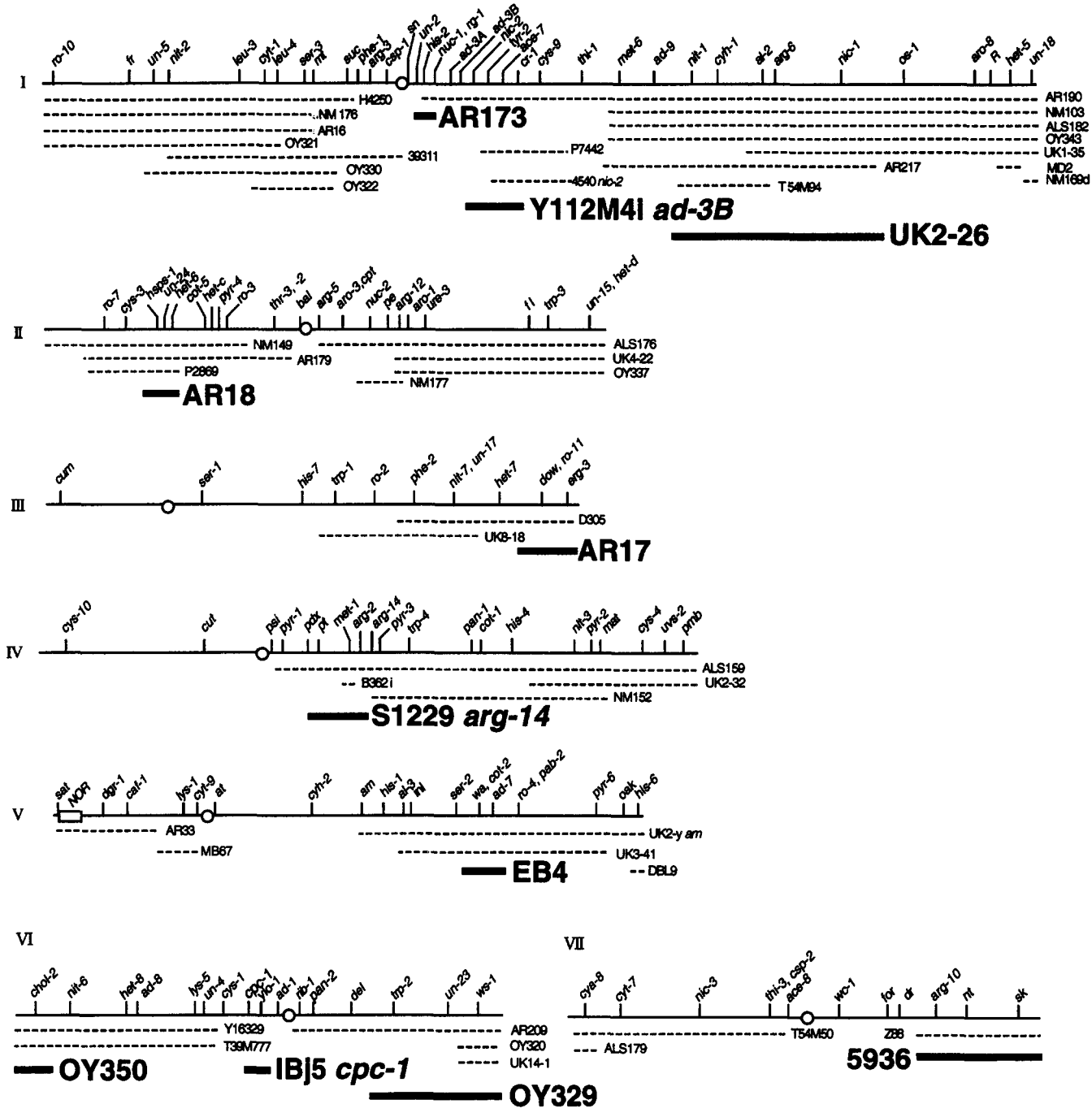

FIGURE 2.—Segments of the *Neurospora crassa* genome that can be obtained as viable nontandem duplications in progeny of crosses heterozygous for insertional or quasiterminal chromosome rearrangements, as shown in Figure 1. Segments duplicated in the crosses used for Table 1 are highlighted. Other duplications, identified by numbers in small print, may be useful in the future for obtaining mutations in other regions. The number adjoining each dashed line identifies the insertional or quasiterminal rearrangement that was crossed to a normal-sequence strain to build the corresponding duplication strain. The extent of each transposed, duplicated segment can be determined precisely from tests of duplication coverage (PERKINS 1986). For identification of symbols and description of genes see PERKINS *et al.* (1982) or PERKINS (1992).

ducted using 13 other duplications. With several of these, fewer than 100 progeny were obtained for testing (DBL9, T54M94, B362i, 4540, NM177, and P7442). Other duplications that produced many ascospores were clearly unstable (MB67) or were suspected of having been deleted, with the result that one or more perithecia became fertile (UK3-41, Z88, NM169d, ALS179, OY320, and AR33).

Only well defined, distinct mutations are listed in Table 1. Phenotypically abnormal strains of other types were at least equally numerous among progeny of the same crosses. Some of these strains stopped growing

TABLE 1

Mutations obtained in progeny of *Duplication* × *Normal*

Duplication	Mutation[a]	Fraction of mutant progeny from *Dp* × *Normal*[b]	Is mutation included in duplicated segment?	Evidence[c]
Dp(IR → VII)UK2-26	*arg-6*	2/225	Yes	Covered. Crosses × *arg-6* (29997) are barren or produce only white ascospores. 1/57 × *al-2.*
	mo(VP103)[d]	2/225	Yes	Covered. Right of *arg-6* (5/39).
Dp(VR → VII)EB4	*ad-7*[e]	2/63	Yes	Covered. Mostly white ascospores × *ad-7* (Y175M256); no Ad$^+$ among 14 viable ascospores.
Dp(VIL → IR)IBj5	*vvd*[d,f]	14/31	Yes	Covered. 2/29 × *ylo-1.*
Dp(IIIR → [IR;IIR])AR17	*dow*	13/274	Yes	Covered. 0/109 × *dow* (P616).
	ro-11[d]	3/274	Yes	Covered. 0/46 × *dow* (P616).
Dp(IIL → IIIR)AR18	*un-24*[d,g]	1/173	Yes	Covered. Included in same cosmid with *het-6*[h].
	cys[g]	1/173	No	Unlinked to *un-24*[i].
Dp(IR → IIIR)Y112M4i	*mo(P4247)*	1/83	No	Unlinked to *Dp Y112M4i* or to *alcoy; csp-2. ropy*-like morphology.
Dp(IR → VIL)AR173	*his-2*	1/66	Yes	Covered. 0/83 × *his-2 (Y152M14).*
	nic[e]	1/66	No	Unlinked to *Dp AR173* or to *nic-2* (43002), which it resembles phenotypically.
Dp(VIR → III)OY329	*col-18*[d]	1/207	Yes	Covered. 3/28 × *un-23* (64D).
Dp(VIL → IR)OY350	*chol-2*[e]	1/74	Yes	Covered. 0/200 × *chol-2* (47904).
	lgd[d]	5/74	Yes	Covered. 7/32 × *chol-2* (47904).
Dp(IVR → VII)S1229	*pt*	2/435	Yes	Covered. 0/32 × *pt* (NS1).
	ad-6	4/435	No	Linked but not recovered. 1/23 × *T S1229;* 10 of 21 Ad$^-$ progeny are barren. Mostly white ascospores × *ad-6* (Y175M130), which is not covered.
Dp(VIIR → IL)5936	*tns*[d]	1/54	Yes	Covered. 2/52 left of *arg-10* (B370).

[a] Gene names and phenotypes are as follows: *ad,* adenine; *arg,* arginine; *chol,* choline; *col,* colonial morphology, *cys,* cysteine; *dow,* downy morphology; *his,* histidine; *lgd,* laggard (slow growth and conidiation); *mo,* morphological abnormality; *nic,* nicotinic acid; *pt,* phenylalanine plus tyrosine; *ro,* ropy morphology; *tns,* tenuous (very slow growth and conidiation); *un,* unknown heat-sensitive lesion; *vvd,* vivid (bright carotenoid pigmentation). (The word "requirement" is omitted from names of auxotrophs). For fuller information on map locations and phenotypes see PERKINS (1992) and PERKINS *et al.* (1982).

[b] All the listed mutations were detected in single-mutant progeny. More than one independent RIP event is known to be represented among the *dow* and *ro-11* isolates.

[c] "Covered" signifies that duplication progeny are phenotypically nonmutant (and therefore heterozygous) when a normal-sequence mutant strain is crossed by the parental translocation. Fractions are the numbers of recombinant/total progeny in normal-sequence crosses of the new mutation by the indicated marker. Allele numbers of the markers are in parentheses.

[d] Gene locus previously unknown.

[e] Originally recovered in a phenotypically mutant f_1 duplication. Obtained in euploid sequence as shown in Figure 3B.

[f] The high frequency of *vvd* progeny might be explained if ascospores originated from one perithecium in which RIP had occurred soon after fertilization.

[g] Obtained and linkage tested by DAVID J. JACOBSON. Cloned by SMITH and GLASS (1996).

[h] SMITH and GLASS (1996).

[i] D. J. JACOBSON, personal communication.

soon after ascospore germination, while others were characterized by slow growth, poor conidiation, or subtle differences in vegetative morphology and pigmentation. Some of these abnormalities may have resulted from RIP at more than one locus. The decision was made not to attempt analysis of these potentially complicated variants but to give priority instead to the mutants that appeared to be clear and simple.

Genetic evidence that the mutations were produced by RIP: Distinct mutations at 17 loci were recovered among the rare progeny from crosses heterozygous for the 11 duplications (Table 1; Figure 2). Both the high mutation frequency and the chromosomal location are what would be expected if most of these mutations originated by RIP. Frequencies ranged from 2 of 435 to 14 of 31. Thirteen of the 17 mutated loci map inside the segment that was duplicated in the parent, one is outside the duplication but closely linked, and three are

unlinked (Table 1). Seven of the mutations are at previously unknown loci within the duplicated segments.

When a locus undergoes RIP in a cross of *Duplication* by *Normal*, both copies of the gene should be mutated (SELKER *et al.* 1987). Therefore, new mutations should be found not only in fertile euploid progeny but also in barren duplication progeny. Progeny from *Duplication* × *Normal* should be equally likely to be duplication sequence or normal sequence, provided that the two progeny types are equally viable, that anaphase I segregation is unbiased, and that the duplication is stable and remains intact. (see Figure 3B) To test this expectation, duplication strains obtained by crossing a sample of five different translocations were crossed by normal-sequence testers and the progeny from these test crosses were scored for fertility as an indicator of the presence or absence of the duplication. The duplications chosen to be tested were all known to be relatively barren, producing few ascospores when crossed. Thirty-eight of 81 progeny from these test crosses were fertile nonduplications and 43 were barren duplications. Apparently all five of the duplications are readily transmitted. Twenty-three of the mutant strains attributed to RIP in Table 1 were test crossed. Of these, 11 were barren.

Transfer of putative RIP mutations from duplication progeny into rearrangement sequence and into normal sequence: RIP can be used to insert mutant markers into transposed segments that are too short to permit introduction by crossing over. This is accomplished most directly by crossing a duplication strain by its parental translocation and recovering new mutations in the euploid translocation-sequence progeny (Figure 3A). In practice, most of our crosses have been *Duplication* × *Normal* rather than *Duplication* × *Translocation.* From such a cross, a new mutation may be recovered either in a normal-sequence f_1 or in a duplication f_1 (Figure 3B, class 2 or class 4 segregant), but none of the f_1s would be expected to have the euploid rearrangement sequence. However, when a new mutation is recovered in one of the f_1 duplication progeny (Figure 3B, class 4), the mutant allele can be obtained in a euploid translocation-sequence strain by crossing mutant *Duplication* × *Translocation* (Figure 3A, class 2).

With the crosses for Table 1, when a new mutant type was represented more than once and both barren and fertile mutant strains were available among the F_1 progeny, usually only one of the fertile representatives was analyzed and the barren siblings were put aside. But when the only representative of a new mutant type was barren, as occurred with *ad-7, chol-2,* and *nic,* the barren strains were crossed by normal sequence to obtain the mutant allele in a euploid normal-sequence background (Figure 3B, class 2).

The expectations outlined in Figure 3 were tested by crossing the original *chol-2* mutant strain, which included the *OY350* duplication, both by translocation *OY350* and by normal-sequence. After several months

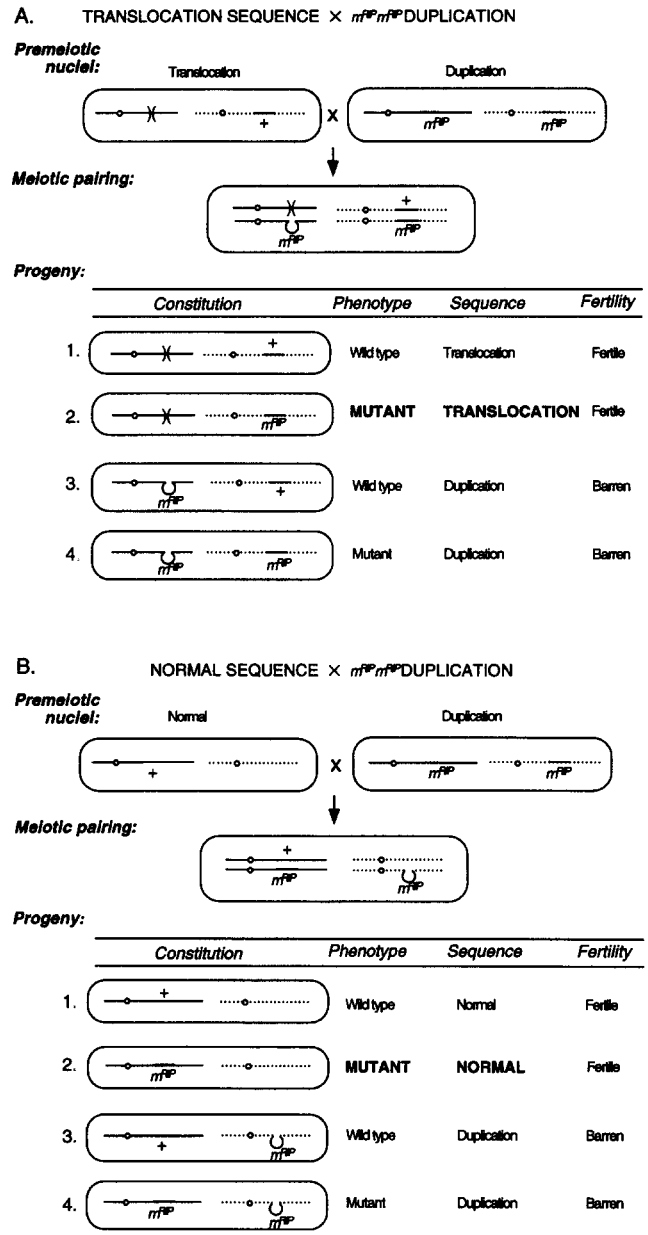

FIGURE 3.—Recovery of a euploid mutant strain among progeny of crosses heterozygous for an insertional duplication in which both copies of a gene (*m*) have undergone RIP. The segment involved in the duplication is drawn as a thick line. (A) *Translocation* × *Duplication.* The mutation is present in translocation sequence in one of the four classes of progeny. (B) *Normal* × *Duplication.* The mutation is present in normal sequence in one of the four classes of progeny. Expectations would be similar with a quasiterminal duplication.

incubation, enough ascospores were obtained to provide the results in Table 2, which confirm predictions for both of the test crosses, as diagrammed in Figure 3.

Molecular evidence that the mutations were produced by RIP: We wished to test more directly the idea that the mutations found in progeny of the segmental duplication strains were due to RIP. We therefore took advantage of the fact that the wild-type alleles of *arg-6*

TABLE 2

Extraction of a newly arisen mutant *chol-2* allele from the duplication strain in which it was recovered

Progeny phenotype	Sequence	Fertility	No. obtained
A. Translocation *OY350* × *Duplication OY350, chol-2/chol-2*			
Chol$^+$	*T*	Fertile	8
Chol$^-$	*T*	Fertile	2
Chol$^+$	*Dp*	Barren	16
Chol$^-$	*Dp*	Barren	15
B. Normal sequence × *Duplication OY350, chol-2/chol-2*			
Chol$^+$	*N*	Fertile	10
Chol$^-$	*N*	Fertile	2
Chol$^+$	*Dp*	Barren	20
Chol$^-$	*Dp*	Barren	7

See Figure 3 for schematic diagram showing genotypes.

and *ro-11* had been cloned and sequenced (GESSERT *et al.* 1994; M. PLAMANN, personal communication). RIP is usually first detected at the molecular level by testing various restriction sites for mutation and/or methylation by Southern hybridization (SELKER *et al.* 1987). Southern analysis of genomic DNA from one *arg-6* allele failed to show any evidence of mutation or methylation using a variety of restriction endonucleases (*Sma*I, *Sac*I, *Sac*II, *Kpn*I, *Bst*XI, *Cla*I, *Xhx*I, *Xho*I, *Hpa*II, *Hha*I, *Mbo*I, and *Sau*3A1; data not shown). This result would not be unexpected if the mutant phenotype resulted from one, or very few, mutations by RIP. We therefore examined several segments of the *arg-6* and *ro-11* alleles from Table 1 at the DNA sequence level.

Three well-separated segments of *arg-6* allele VP102, totaling >800 base pairs (bp), were isolated and sequenced on both strands. We found one mutation, a C to T change at position 1510 (Figure 4C). This change, which should cause chain termination, occurred at a 5 CpA dinucleotide, the favored target of RIP (SELKER 1990). Nevertheless, because of the possibility that a mutation could have been introduced *in vitro* during the PCR reaction, we isolated and sequenced the same region from a sibling strain that came from crossing the original mutant isolate VP102. The same mutation was found, demonstrating that it was not an artifact.

Two ~400-bp segments of *ro-11* allele 3053-1 were chosen for analysis, one near the middle of the coding region and one spanning its end. These regions were isolated from genomic DNA by PCR, cloned, and sequenced on both strands. Seven mutations, all polarized transition mutations characteristic of RIP, were found in the 395-bp segment of the central region (positions 1001, 1043, 1049, 1080, 1235, 1289, and 1365; Figure 4A). Based on the predicted amino acid sequence, four of these mutations should be silent and three should be missense (Figure 4A). All seven are G to A changes on the coding strand. Three occurred at 5 TpG/5 CpA sites. The other four occurred at the second most common target of RIP, 5 ApG/5 CpT sites. Thus this allele shows convincing evidence of RIP. No mutations were detected in the 354-bp downstream segment of this allele. The same region of *ro-11* allele 3053-4 showed one mutation, however (position 2383; Figure 4B). Like the mutations of allele 3053-1, this mutation was a transition mutation, but in this case it was a C to T change on the coding strand. Two additional mutations, also both C to T changes, were found in an upstream 353-bp segment of allele 3053-4 (positions 474 and 477; Figure 4B). All three of the mutations in the sequenced segments of this allele occurred at CpA dinucleotides. Two are nonsense mutations; the other is a missense mutation (Figure 4B).

The sequence information from the *arg-6* and *ro-11* alleles gives a picture of the mutation frequency within the sequenced regions. The *ro-11* alleles 3053-1 and 3053-4 and *arg-6* allele VP102 show mutation frequencies per sequenced nucleotide of 9.0×10^{-3}, 4.1×10^{-3}, and 1.2×10^{-3}, respectively.

It was unlikely that the multiple mutations characteristic of RIP that were found in the *ro-11* alleles were artifacts, *e.g.*, of the PCR reactions. Nevertheless, since some of the mutations were predicted to change restriction sites, we checked genomic DNA for the mutations by Southern hybridization. The wild-type *ro-11* sequence contains one *Bgl*II site at nucleotide 1042 and one *Pfl*MI site at nucleotide 2388. When probed with the three sequenced regions of *ro-11*, wild type shows two bands when digested with either *Bgl*II (Figure 5, lane 3) or *Pfl*MI (Figure 5, lane 4). The sequence data predict that the *Bgl*II site is destroyed in allele 3053-1 and the *Pfl*MI site is destroyed in allele 3053-4 (Figure 4, A and B). Accordingly, only one hybridizing fragment was detected for allele 3053-1 when digested with *Bgl*II (Figure 5, lane 8) and only one hybridizing fragment was detected for allele 3053-4 when digested with *Pfl*MI (Figure 5, lane 14), confirming the sequence data.

To investigate further the level of mutation outside of the sequenced region, we analyzed *Mse*I digests by Southern hybridization. *Mse*I was chosen because of its insensitivity to methylation and because its recognition sequence TTAA should be relatively frequently created by RIP. The wild-type *ro-11* sequence contains no *Mse*I sites and therefore showed only one band when probed with the three sequenced *ro-11* regions (Figure 5, lane 5). Allele 3053-1 showed three *Mse*I bands (Figure 5, lane 10), indicating that at least two *Mse*I sites were created; allele 3053-4 (Figure 5, lane 15) showed two *Mse*I bands, indicating that at least one new *Mse*I site was created.

Commonly, but not invariably, sequences that have been mutated by RIP are methylated in vegetative tissues. We therefore assessed the methylation status of the *ro-11* alleles by Southern hybridization using the

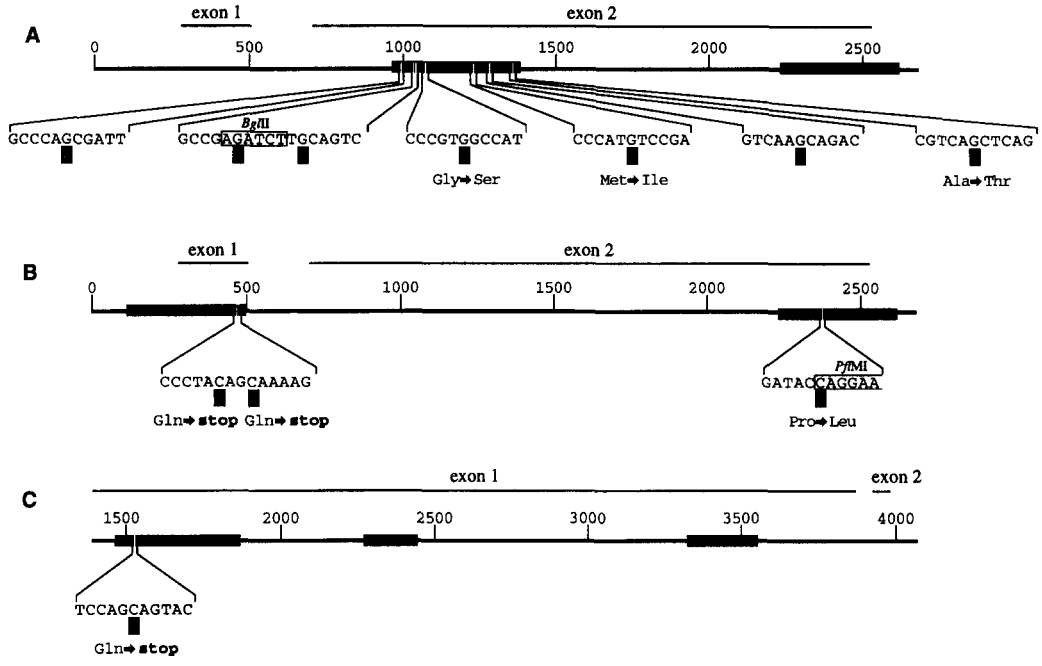

FIGURE 4.—Hallmarks of RIP in *ro-11* (A and B) and *arg-6* (C) alleles isolated from progeny of segmental duplications. The heavy horizontal bars indicate segments of the genes that were amplified by PCR, cloned, and sequenced. Interruptions in the bars mark the positions of mutations, all of which were polarized transition mutations, characteristic of RIP. The changes observed are shown in the black boxes beneath short segments of the wild-type sequences in their vicinity. Predicted coding changes are indicated beneath the mutations. The positions of exons and a nucleotide scale are indicated above each diagram. Coordinates are based upon the published *arg-6* sequence (GESSERT *et al.* 1994) and on *ro-11* sequence information kindly provided by M. PLAMANN (personal communication). The mutant *ro-11* sequences shown are from strains 3053-1 (A) and 3053-4 (B), which were independent progeny of *Dp(IIIR→[IR;IIR])AR17a* × OR23-1VA. The mutant *arg-6* sequence shown (C) was found in both strains 1826*A* and 1827*a*, f_1 siblings from a cross of a mutant produced by crossing *Dp(IR→VII)UK2-26a* × OR23-1VA.

isoschizomers *Dpn*II or *Sau*3AI. Both enzymes recognize the sequence GATC but only *Sau*3AI is sensitive to cytosine methylation (NELSON *et al.* 1993). The *Sau*3AI and *Dpn*II digestion products were identical in the wild-type and *ro-11* strains, indicating that the sites assayed were not methylated (Figure 5). The *Dpn*II and *Sau*3AI digests of *ro-11* allele 3053-1 lacked the 147-bp fragment found in digests of DNA from wild type and instead showed a novel 628-bp fragment (Figure 5, lanes 6 and 7). This is consistent with destruction of the *Dpn*II/*Sau*3AI site at position 1042, as expected from the sequence data (Figure 4A).

We also looked for evidence of new *Mse*I sites or DNA methylation in the two mutant *arg-6* alleles. Neither new sites nor methylation were detected (Figure 6), consistent with the low mutation frequency found by sequencing.

DISCUSSION

Answers have been obtained to the main questions addressed by these experiments: mutations among the progeny of crosses parented by segmental duplications are frequent relative to spontaneous mutations when no duplication is present. The locations, characteristics, and mode of origin of these mutations are generally as expected of RIP. The fact that both duplication and nonduplication progeny show new mutant phenotypes implies that both copies of the gene were inactivated, just as is seen when RIP occurs in the much smaller duplications examined heretofore. Most of the mutations are located in the duplicated segment or nearby. The mutations commonly affect known genes, but a substantial portion of the mutations are at previously unknown loci, as might be expected. Molecular hallmarks of RIP were found in all of the three mutants that were examined at the molecular level.

The experiments were designed to detect a variety of mutant phenotypes: visibles, auxotrophs, and temperature-sensitive conditionals. Of the distinct mutants, eight were visibly altered in morphology or pigmentation, eight were auxotrophs, and one showed a heat-sensitive conditional growth defect. In addition, numerous subtle variants were encountered that were detectably abnormal in growth rate, vigor, or morphology.

Our studies on survivors from crosses of segmental aneuploids revealed that duplications that are considerably larger than those constructed by transformation are susceptible to RIP. This finding indicates that the attack of sizable duplications by RIP does not necessarily prevent transmission of the affected sequences. Nevertheless, the low frequency of new mutants among survi-

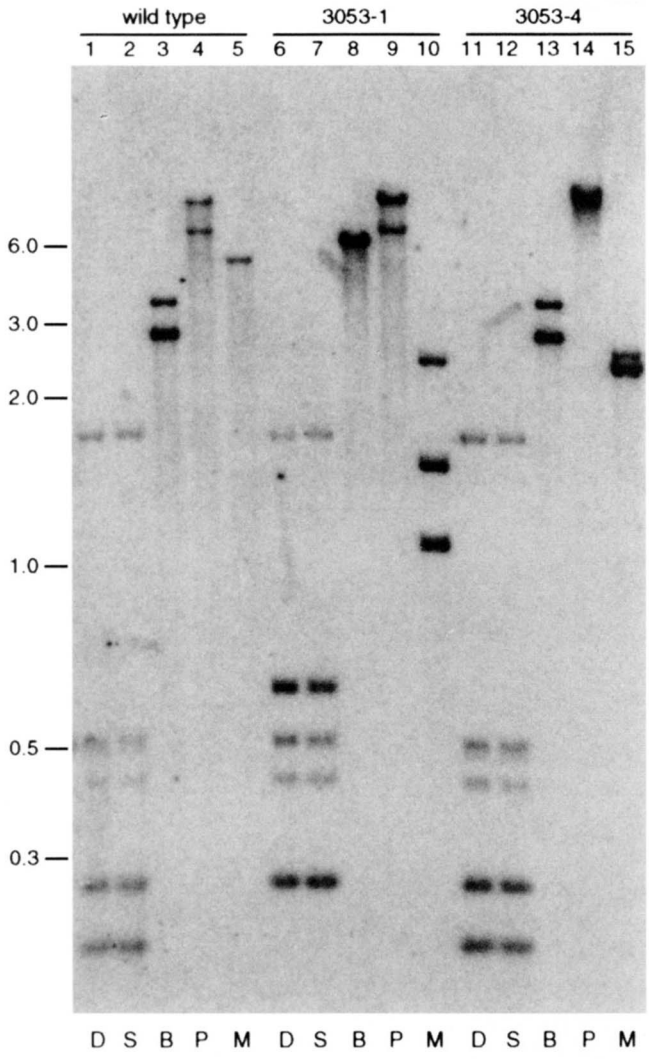

FIGURE 5.—Assay for methylation and polymorphisms at the *ro-11* locus. Genomic DNA from wild type (lanes 1–5), strain 3053-1 (lanes 6–10), and strain 3053-4 (lanes 11–15) were digested with *Dpn*II (D), *Sau3*AI (S), *Bgl*II (B), *Pfl*MI (P), or *Mse*I (M); fractionated on a 1% agarose gel; and simultaneously probed with the sequenced *ro-11* regions (Figure 4, A and B). The positions of molecular weight markers are indicated in kilobase pairs at the left.

vors, relative to what is typically seen when RIP operates on gene-sized duplications, and the low density of mutant sites detected in the genes examined at the sequence level may reflect inefficient operation of RIP on large duplications. It is also possible that the viable ascospores generated do not contain a representative sample of the chromosomes encountered by the RIP machinery. The simplest interpretation would seem to be that the duplications covered essential genes. Consequently, of the duplications detected and affected by RIP, only those that suffered mild mutagenesis, which spared essential genes, were represented in the viable ascospores. If the barren phenotype of segmental duplications is indeed due to RIP acting on genes that produce nucleus-limited or dosage-sensitive products, this could also provide a selection resulting in survivors with relatively low levels of RIP, as was observed.

It may be informative to investigate viable duplication progeny from crosses of segmental duplication strains to learn whether the nontranslocation chromosome of the duplication is generally (or invariably) from the nonduplication parent. Numerous other questions have also been raised by our exploratory study:

- Can the occurrence of closely linked mutations outside the duplication be confirmed as a regular feature? How far from the duplication does the effect extend? (Instances of RIP extending into unique sequences adjoining a duplication were cited in the introduction.) Does the linked but noncovered mutation at *ad-6* have the molecular hallmarks of RIP? If a spreading effect exists outside segmental duplications, is it perhaps more far reaching than that triggered by gene-size duplications?
- Does the presence of a duplication in the premeiotic dikaryotic phase lead to increased mutation at unlinked loci, as might be suggested by recovery of the three unlinked mutations in Table 1?
- Do genes at different loci within the same duplication differ systematically in their susceptibility to RIP? If so, is this an intrinsic property of the locus, as if due to gene size and/or cytosine content? Or is it perhaps due to position in the duplication, as RIP progresses along the chromosome? Could mutations at a given locus fail to appear because the locus is downstream from a gene that is lethal when inactivated? Or could absence of mutation at a locus be due to rank of that gene in a hierarchy such that one or more genes essential for progressing through the sexual cycle are much more likely to be inactivated than is the gene in question?
- With duplications from some rearrangements, one of the duplicated segments undergoes frequent deletion during the period following fertilization (see PERKINS and BARRY 1977). Is there any relation between RIP and this premeiotic deletion of a duplicated segment or between RIP and deletion of tandem repeats observed with transforming DNA (SELKER *et al.* 1987; IRELAN *et al.* 1994) and rDNA (BUTLER and METZENBERG 1989)?
- Can RIP mutations be recovered even from unstable duplications that break down readily in the period between karyogamy and meiosis? Might the time of breakdown indicate whether RIP is equally likely to occur throughout this period, as has been assumed (SINGER *et al.* 1995b)?
- What is the nature of the less well defined slow-growing and poorly conidiating variants commonly seen among the progeny of duplications? Do they result from RIP at multiple loci within the same segment?
- Is the barren phenotype of duplications caused by RIP? The results reported here are consistent with

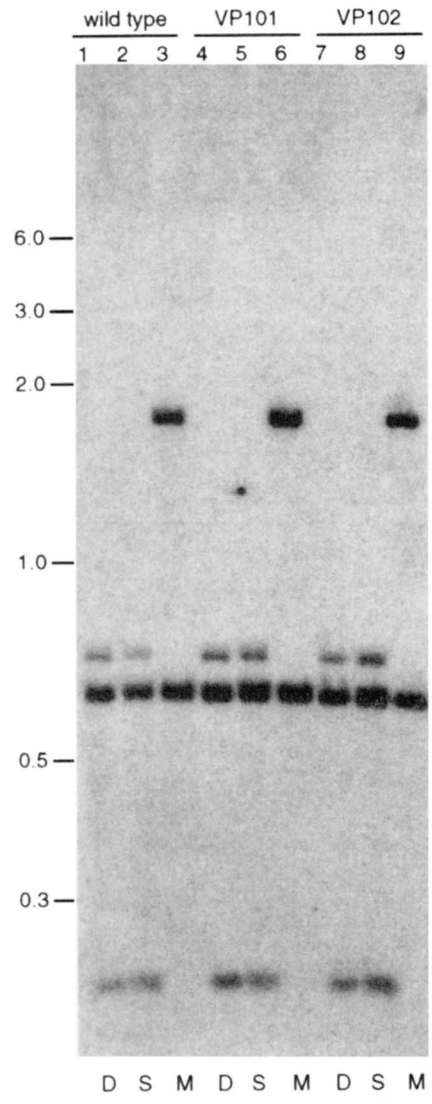

FIGURE 6.—Assay for methylation and polymorphisms at the *arg-6* locus. Genomic DNA from wild type (lanes 1–3), strain VP101 (lanes 4–6), and VP102 (lanes 7–9) were digested with *Dpn*II (D), *Sau*3AI (S), or *Mse*I (M); fractionated on a 1% agarose gel; and simultaneously probed with the three sequenced *arg-6* segments (Figure 4C). The positions of molecular weight markers are indicated in kilobase pairs at the left.

this hypothesis but fall short of demonstrating it convincingly. The hypothesis would receive strong support if a method could be found to suppress RIP and if this also increased fertility of the duplications.

Segmental duplications and gene inactivation in other fungi: Findings reported here are consistent with the hypothesis that the impaired fertility of segmental duplications is due to premeiotic RIP. This "RIP hypothesis" would predict that duplication strains would be fertile rather than infertile in a genotype that was incapable of RIP or that was deficient in the similar premeiotic gene-inactivating process in *Ascobolus immersus* MIP (methylation induced premeiotically; see ROSSIGNOL and FAUGERON 1994). This hypothesis could be tested directly if a Neurospora mutant were available that had lost the ability to carry out RIP. Attempts to find a mutant of this type have failed. Information as to whether the infertility of duplications is a correlate of RIP or MIP might be obtained, however, by examining the behavior of duplications in other fungi that are known to be capable or incapable of premeiotic gene inactivation.

Gene duplications created by transformation are not inactivated premeiotically in the homothallic species *Sordaria macrospora* (LE CHEVANTON *et al.* 1989). It has been suggested that premeiotic inactivation by RIP may reflect a regulatory function related to expression of the two nuclei of opposite mating type and that this function is required in a heterothallic species such as *N. crassa* or *Ascobolus immersus* but not in a homothallic species such as *S. macrospora* (LE CHEVANTON *et al.* 1989; SELKER 1990). Five duplication-generating chromosome rearrangements have been obtained in *S. macrospora* by ARNAISE *et al.* (1984), but the segmental duplications they produce have not been tested in crosses, and it is not known whether they would remain fully fertile, as predicted from the RIP hypothesis.

Similarly, the heterothallic species *S. brevicollis* and *A. immersus* do not provide all the information necessary for testing the RIP hypothesis. In *S. brevicollis*, duplications produce barren perithecia (BOND 1979) but whether duplicated genes undergo RIP is not known. In *A. immersus*, duplicated genes introduced ectopically by transformation are inactivated by MIP (ROSSIGNOL and FAUGERON 1994) but no segmental duplications are available for testing.

The homothallic species *Aspergillus nidulans* comes closer to providing the needed test. Duplications derived from three nonreciprocal translocations have been described as barren (ARST 1982 and personal communication; GEISER *et al.* 1996) and ascospore numbers are greatly reduced in crosses that involve a long II→I insertional duplication that was obtained following nitrosoguanidine treatment (CASTRO-PRADO *et al.* 1996). When this *Dp(II→I)* is crossed, genes within the duplication are inactivated in some of the progeny, an observation similar to that reported here for Neurospora. Reactivation occurs either spontaneously or following induction by 5-azacytidine (CASTRO-PRADO and ZUCCHI 1993, 1996; CASTRO-PRADO *et al.* 1996). Thus, the inactivation is apparently epigenetic rather than mutational, similar to MIP in *A. immersus* (ROSSIGNOL and FAUGERON 1994).

These results with *A. nidulans* suggest that premeiotic inactivation is not limited to heterothallic species. In most other published studies with *A. nidulans*, however, no indication has been given that fertility of a cross is less than normal when a long segmental duplication is present. Whether the short ectopic duplications that

result from transformation are subject to premeiotic inactivation also remains unclear.

Practical applications: We have demonstrated that translocation-generated duplications can be used for targeted mutagenesis in specific chromosome segments. Mutations with useful phenotypes can be obtained in chromosome regions previously devoid of markers or sparsely marked (*e.g., col-18* in VIR; Table 1). Markers can be put into segments that are too short for insertion by crossing over (*e.g., un-24* into *AR-18* translocation sequence; Table 2). Previously unidentified loci with novel phenotypes may be discovered (*e.g., vvd* and *ro-11*; Table 1). New alleles can be acquired at already established loci (Table 1).

Evolutionary implications: Our demonstration that genes in long translocation-generated duplications are subject to RIP not only provides a new tool for understanding the underlying mechanism but also supports the suggestion that RIP may facilitate rapid divergence of duplications during evolution (SELKER 1990). Duplications might be a significant source of new alleles in euploid genomes, even though the duplications and the rearrangements that produced them were transitory. Chromosome rearrangements must arise repeatedly in natural Mendelian populations. Most rearrangements are no doubt weeded out promptly because of their detrimental effects on fertility, but before a new rearrangement disappeared it might well have produced offspring that harbored duplications. Insertional and quasiterminal translocations, which generate viable duplication strains in this way, make up about one sixth of newly arisen rearrangements in Neurospora (PERKINS 1997). Duplication-generating rearrangements may also arise frequently in other organisms, and the duplication gametes they produced would provide a potential substrate for RIP. New mutant alleles that originated in this way might subsequently be segregated into euploid genomes and might persist in the population even though the rearrangement that produced them was eliminated.

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