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Chromosome Segment Duplications in *Neurospora crassa* and Their Effects on Repeat-Induced Point Mutation and Meiotic Silencing by Unpaired DNA

Meenal Vyas, C. Ravindran and Durgadas P. Kasbekar[1]

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

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ABSTRACT

The size and extent of four *Neurospora crassa* duplications, *Dp(AR17)*, *Dp(IBj5)*, *Dp(OY329)*, and *Dp(B362i)*, was determined by testing the coverage of RFLP markers. The first three duplications were all >∼350 kb and have been shown in earlier studies to act as dominant suppressors of repeat-induced point mutation (RIP) in gene-sized duplications, possibly via titration of the RIP machinery. *Dp(B362i)*, which is only ∼117 kb long, failed to suppress RIP. RIP suppression in gene-sized duplications by large duplications was demonstrated using another test gene, *dow*, and supposedly applies generally. Crosses homozygous for *Dp(AR17)* or *Dp(IBj5)* were as barren as heterozygous crosses. Barrenness of the heterozygous but not the homozygous crosses was suppressible by *Sad-1*, a semidominant suppressor of RNAi-dependent meiotic silencing by unpaired DNA. A model is proposed in which large duplications recessively suppress semidominant *Sad-1* mutations. The wild-isolated Sugartown strain is hypothesized to contain a duplication that confers not only dominant suppression of RIP but also a barren phenotype, which is linked (9%) to supercontig 7.118 in LG VII.

IN the fungus *Neurospora crassa*, crosses heterozygous for a chromosome segment duplication are generally barren; that is, they make normal-looking perithecia but yield very few ascospores (see Perkins 1997 for a review). Barrenness is caused by a recently discovered gene-silencing process called "meiotic silencing" by unpaired DNA (also known as MSUD, but since this is also the acronym for "maple syrup urine disease," the term "meiotic silencing" is preferred), an RNA interference-based mechanism that silences genes that are unpaired in meiosis (Shiu *et al.* 2001; Shiu and Metzenberg 2002). Consequently, it also silences all homologs of the unpaired genes, regardless of whether or not the homologs are themselves paired. Since in a duplication-heterozygous cross one copy of each duplication-borne gene is unpaired in meiosis, all the duplication-borne genes, including any required for ascus development and meiosis, are silenced and the cross is rendered barren. The genes *Sad-1*, *Sms-2*, *RecQ-2*, and *Sms-3* encode the RNA-dependent RNA polymerase, argonaute, RecQ DNA helicase, and dicer enzymes used in meiotic silencing. Semidominant mutants of *Sad-1* and *Sms-2* suppress meiotic silencing and can increase the productivity of duplication-heterozygous crosses (Shiu *et al.* 2001). The semidominant alleles are either complete or partial deletions of the *sad-1* or *sms-2* genes, or their sequence is so severely altered by point mutations that they probably fail to pair with their wild-type homologs and thus induce self-silencing (Shiu and Metzenberg 2002; Lee *et al.* 2004). Homozygosity for *sad-1* (or *sms-2*) mutants causes infertility.

Repeat-induced point mutation (RIP) is a silencing process that protects the Neurospora genome against the proliferation of transposable elements and other parasitic DNA sequences (for a recent review, see Galagan and Selker 2004). RIP occurs in the premeiosis of a sexual cross and induces hypermutation and methylation of any sizeable DNA sequence that is duplicated in an otherwise haploid genome (Selker 1990). The *RIP-defective* (*rid*) gene encodes a cytosine methyltransferase homolog essential for RIP, and RIP does not occur in crosses homozygous mutant for *rid* (Freitag *et al.* 2002). The efficacy of RIP is attested by the fact that the *N. crassa* genome contains no active transposable elements, although it does contain RIP-inactivated relics of such elements (Galagan *et al.* 2003).

Previous work from our laboratory showed that 1 or 2% of wild-isolated *N. crassa* strains display a dominant RIP suppressor phenotype (Noubissi *et al.* 2000, 2001; Bhat *et al.* 2003). One dominant RIP suppressor strain (Adiopodoume) is the only Neurospora strain known to harbor an active transposable element, called *Tad* (Kinsey 1989; Kinsey and Helber 1989). Spread of *Tad* into other strains is apparently restricted by RIP. In another suppressor strain (Sugartown), the suppressor phenotype was linked to a barren phenotype in crosses, suggesting that the Sugartown strain might contain a

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[1]*Corresponding author:* Centre for Cellular and Molecular Biology, Uppal Rd., Hyderabad 500 007, India. E-mail: kas@ccmb.res.in

naturally occurring duplication that causes both suppression of RIP and barrenness. The barren phenotype segregated independently of markers on all linkage groups (LGs) except LG VII (Bhat *et al.* 2003). We have now tested for linkage of the barren phenotype with molecular markers in LG VII.

Strains bearing chromosome segment duplications can be obtained in the laboratory as segregants from crosses between strains that are heterozygous for rearrangements such as insertional translocations (Perkins 1997). We have demonstrated that large segmental duplications are capable of dominantly suppressing RIP of genes in a smaller duplication, presumably by titrating out the RIP machinery (Bhat and Kasbekar 2001; Fehmer *et al.* 2001). How large must a duplication be to function as a dominant RIP suppressor? The size of one *N. crassa* duplication, *Dp(AR18)*, was estimated from orthogonal field agarose-gel electrophoresis studies to be 270 ± 90 kb (Smith and Glass 1996). Here we show that *Dp(AR18)* is capable of dominant RIP suppression. Two developments have now made it possible to determine the size and extent of any *N. crassa* duplication with an unprecedented precision. First, the sequencing of the *N. crassa* genome makes it easy to amplify practically any genomic segment by PCR. Second, the collection of *N. crassa* wild isolates from all over the world (>400 available from the Fungal Genetics Stock Center; Perkins and Turner 1988; Turner *et al.* 2001) offers an excellent resource for identifying DNA sequence polymorphisms (*e.g.*, RFLPs) with the amplified DNA as probe. Duplication progeny from crosses between the translocation and the wild-isolated strains are expected to display both parental alleles of any RFLP that lies within the translocated segment. Whereas for RFLPs that map just outside the translocated segment, the duplication progeny should exhibit only the allele from the wild-isolated parent. Thus the extent of duplication coverage can be determined by analyzing a series of RFLPs that correspond to linked genomic segments. Using this approach we characterized four duplications, *Dp(AR17)*, *Dp(B362i)*, *Dp(IBj5)*, and *Dp(OY329)*. Three of them were shown previously to act as dominant suppressors of RIP (Bhat and Kasbekar 2001; Fehmer *et al.* 2001). The fourth, *Dp(B362i)*, was tested in this work.

The dominant RIP suppressor phenotypes were identified in screens that tested for the suppression of RIP in a small fragment carrying the *erg-3* gene (Noubissi *et al.* 2000, 2001; Bhat and Kasbekar 2001; Fehmer *et al.* 2001; Bhat *et al.* 2003). In these screens, the frequency of RIP-induced *erg-3* mutant progeny was scored in crosses that were heterozygous for the duplicated *erg-3* gene fragment and that did or did not carry the larger duplication being tested for ability to suppress RIP. Crosses with nonsuppressor strains typically yield RIP-induced *erg-3* mutant progeny at frequencies in the 2–25% range, but in the crosses with the suppressing duplications the frequency of RIP-induced mutant progeny was <0.5%. We have now verified that the low RIP frequencies are not peculiar to the *erg-3* duplication but also extend to other duplicated sequences, specifically, to a duplicated fragment of the *downy* (*dow*) gene.

Relatively few reports have described crosses homozygous for chromosome segment duplications. In her Ph.D thesis, Patricia St. Lawrence examined *T(IR;II;IVR;VL)R55*, a complex rearrangement involving four chromosomes and found that crosses homozygous for *Dp(R55)* were at least as barren as crosses heterozygous for the duplication (St. Lawrence 1953; summarized by Perkins 1997 and Raju and Perkins 1978). Another complex rearrangement, *T(IVR>VIIL;IL;IIR;IVR)S1229, arg-14*, was studied by Edward Barry in his Ph.D. thesis; he found that crosses homozygous for *Dp(S1229)* were as barren as the heterozygous crosses. Very few progeny were produced, but these were barren like their parents, indicating that the duplication was stable and was transmitted through meiosis. (Barry 1960, summarized by Raju and Perkins 1978). Raju and Perkins (1978) examined homozygous crosses of *Dp(IVR>IIIR)S4342* and *Dp(VL>IVL)AR33*. These were barren as were the corresponding heterozygous crosses to wild type. More recently, Bhat and Kasbekar (2004) reported that homozygosity for *Dp(AR17)* caused a barren phenotype. *A priori* the duplication-borne genes were not expected to be unpaired in any of these crosses. Moreover, the barrenness of the *Dp(AR17)* homozygous duplication cross differed from that of the heterozygous cross in that it was not suppressible by a semidominant *Sad-1* mutation. Shiu *et al.* (2001) also noted that the *Sad-1* mutation fails to confer fertility to duplication-homozygous crosses. We report here that homozygosity for *Dp(IBj5)* or *Dp(B362i)* also results in a barrenness that is not effectively suppressed by *Sad-1*.

MATERIALS AND METHODS

Strains from other collections: The following *N. crassa* strains were obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, Missouri: the standard wild types 74-OR23-1 A (FGSC 987) and OR8-1 a (FGSC 988) and the mutant strains *erg-3 a* (FGSC 2725), *erg-3 A* (FGSC 3439), and *dow a* (FGSC 4052), all in Oak Ridge (OR) background.

The wild-isolated strains Adiopodoumé A (FGSC 430) and its derivative T-430-Hyg^r *a* (FGSC 8609), Adiopodoumé -7 (P4305), Bayan Lepas (P2663), Bichpuri-1 (P0753), Coon (P0881), Franklin (P4467), Franklin (P4490), Fred (P1138), Lankala Koderu-1 (P1110), Mughalsarai-2 (P0736), and Sugartown (P0854). The T-430-Hyg^r a strain was derived from Adiopodoumé A by replacement of the *mat A* idiomorph by transformation with DNA of the *mat a* idiomorph (Anderson *et al.* 2001). The Adiopodoumé A and T-430-Hyg^r *a* display a dominant RIP-suppressor phenotype linked to *mat* on LG IL (Bhat *et al.* 2003). The Adiopodoumé -7, Bayan Lepas, Coon, Fred, and Sugartown strains also have a dominant RIP-suppressor phenotype (Noubissi *et al.* 2000, 2001; Bhat *et al.* 2003).

The translocation strains *T(IIIR>[IR;IIR]) AR17 A* (FGSC 2442), *T(IIIR>[IR;IIR]) AR17 a* (FGSC 1463), *T(IIL>IIIR) AR18 A* (FGSC 2643), *T(IIL>IIIR) AR18 a* (FGSC 2644), *T(IVR>I)*

B362i A (FGSC 2935), *T(IVR>I)B362i a* (FGSC 2988), *T(VIL>IR)IBj5, cpc-1 A* (FGSC 4433), *T(VIL>IR)IBj5, cpc-1 a* (FGSC 4434), *T(VIR>IIIR) OY329 A* (FGSC 3670), and *T(VIR>IIIR) OY329 a* (FGSC 3671) and the duplication strains *Dp(IVR>VII) S1229 A* (FGSC 264) and *Dp(IVR>VII) S1229 a* (FGSC 265). Translocations *T(IIIR>[IR;IIR]) AR17*, *T(IIL>IIIR) AR18*, *T(IVR>I) B362i*, *T(VIL>IR)IBj5*, and *T(VIR>IIIR) OY329* will henceforth be referred to as *T(AR17)*, *T(AR18)*, *T(B362i)*, *T(IBj5)*, and *T(OY329)*, respectively, and duplications obtained as progeny from them will be symbolized as *Dp(AR17)*, *Dp(AR18)*, etc. The translocations and duplications have been described (Perkins 1997).

Mutations in the *RIP-defective* (*rid*) gene cause a recessive defect for RIP and the strains *rid-1 A* (N1977) and *rid-1 a*, hyg^r (N2148) were kind gifts from Eric U. Selker. The semidominant suppressor of meiotic silencing by unpaired DNA *Sad-1* (Shiu *et al.* 2001; Shiu and Metzenberg 2002) was used to overcome the barren phenotype of crosses involving duplication strains. The strains *Sad-1 A* (FGSC 8740), *Sad-1 a* (FGSC 8741), *Sad-1 rid A* (33-10), and *Sad-1 rid a* (33-11) were kindly provided by Robert L. Metzenberg.

Strains made previously in our laboratory: The *Dp1.3^{ec} hph A* and *a* strains have been described (Prakash *et al.* 1999; Bhat *et al.* 2003). The transgene *Dp1.3^{ec} hph* contains a 1.3-kb *Hin*dIII fragment of the LG IIIR gene *ergosterol-3* (*erg-3*; encoding the sterol biosynthetic enzyme sterol C-14 reductase) and is tagged with the bacterial *hph* gene, which confers resistance to hygromycin. The transgenic fragment does not encode a functional enzyme but serves to target RIP during a cross. The resulting RIP-induced *erg-3* mutant progeny can be easily scored under a dissection microscope by their distinct colony morphology on Vogel's–sorbose agar medium (Noubissi *et al.* 2000). The *Dp1.3^{ec} hph A* and *a* strains were crossed with other strains listed above to construct the following strains in both mating types: *rid; Dp1.3^{ec} hph*, *Sad-1; Dp1.3^{ec} hph*, and *Sad-1 rid; Dp1.3^{ec} hph*. Strain 22, a *Sad-1 a* segregant from the cross Sugartown A × *Sad-1; Dp1.3^{ec} hph a*, was confirmed to have inherited the dominant RIP-suppressor phenotype of its Sugartown parent.

The duplication strains *Dp(AR17)*, *Dp(AR18)*, *Dp(B362i)*, *Dp(IBj5)*, and *Dp(OY329)* were obtained from crosses between the translocation strains and normal sequence strains (OR, *dow*, or the wild isolates] whereas the *Dp(S1229)* strains were obtained from the FGSC. The duplication strains were barren in crosses with euploid strains of the opposite mating type. The construction of *Dp(AR17), dow$^+$/dow, rid-1; Dp(AR17), dow$^+$/dow* and *Sad-1*; *Dp(AR17), dow$^+$/dow* in both mating types is described by Bhat (2004). The *Sad-1 rid*; *Dp(AR17), dow$^+$/dow* strains were constructed in an analogous manner.

Growth, crosses, ascospore collection, and scoring of RIP frequencies: Crossing and maintenance of Neurospora strains was essentially as described by Davis and De Serres (1970). Crosses were performed by confrontation between mycelia inoculated as plugs on synthetic crossing medium in petri dishes. Ascospores began to be shot within 16–18 days and were harvested by washing the lids with ~1 ml water. The frequency of *erg-3* mutant progeny was scored under a dissection microscope as the proportion of colonies with the mutant morphology. In this article the frequency of *erg-3* mutant progeny is used as a measure of RIP efficiency. It is known that the frequency of RIP increases with the age of the cross (Singer *et al.* 1995). Therefore the *erg-3* mutation frequencies were determined in ascospores harvested at 31 days by which time RIP frequencies have plateaued.

PCR amplification, other molecular methods, and transfomation: PCRs were performed using custom oligonucleotide primers purchased from Bioserve (Hyderabad, India). The reaction conditions used and other molecular methods and transformation protocols were essentially the same as previously described (Bhat *et al.* 2004).

Construction of *Dp1.5dow^{ec} hph* strains: A 4921-bp gene encoding the hypothetical protein, designated NCU08565.1, had been identified in contig 3.520 (in distal LG IIIR) of the *N. crassa* genome sequence (release 3). This gene is flanked by the markers *ropy-11* (*ro-11*) and *sulfur control-2* (*scon-2*), both of which are covered by the duplication *Dp(AR17)*. The *dow* locus also maps to this region; therefore we tested whether NCU08565.1 was in fact the *dow* gene. A 1421-bp fragment of NCU08565.1 was amplified by PCR using the primers MV1 (5′-CATTCAG CTTCGACAGGACA) and MV2 (5′-CTGGCGGTATCTTCTT CAGC). The 5′ terminal nucleotide of MV1 is base 13,822 of the contig sequence and the 5′ terminal nucleotide of MV2 is the complement of base 15,243. The amplified DNA was cloned into the plasmid vector pCSN44 and transformed by electroporation into conidia of the strain *erg-3 a*. Crosses made with the transformant strains produced many *dow* mutants among the progeny and Southern analysis revealed that in many of the *dow* mutants the NCU08565.1 gene sequence had suffered both mutation and cytosine methylation as would be expected from RIP mutagenesis (data not shown). These results showed that NCU08565.1 is indeed the *dow* gene. A transgene from one of the transformants that was unlinked to *mat* or *erg-3* was designated *Dp1.5dow^{ec} hph*. This transgene was used to obtain *Dp1.5dow^{ec} hph* or *Dp1.5dow^{ec} hph; erg-3* segregants in both mating types. We also used it to construct the *Sad-1; erg-3; Dp1.5dow^{ec} hph A* strain.

Localization of breakpoints: The markers *downy* (*dow*), *methionine-1* (*met-1*), *yellow-1* (*ylo-1*), and *tryptophan-2* (*trp-2*) are contained, respectively, within the duplicated segments of *Dp(AR17)*, *Dp(B362i)*, *Dp(IBj5)*, and *Dp(OY329)* (Perkins 1997; Perkins *et al.* 2001), and the gene sequence for these markers is known. The oligonucleotide primers used for PCR amplification of ~2- to 3-kb segments of each marker gene are listed in supplemental Table 1 at http://www.genetics.org/supplemental/. The amplified segments were used to probe Southern blots of restriction-digested genomic DNA from the relevant translocation (T) strains [*i.e.*, *T(AR17)*, *T(B362i)*, *T(IBj5)*, and *T(OY329)*] and wild-isolated (W) strains to identify RFLPs that molecularly distinguish between the marker alleles in T and W. Duplication (Dp) progeny from T × W were identified by their barren phenotype in crosses with OR strains and Southern analyses was done to confirm that Dp strains contained both the T and the W allele of the relevant RFLPs identified above. In this way, for each Dp we established molecular evidence for an "initial duplicated marker" within the duplicated segment. Next, we identified additional RFLPs between T and W at distances of, say, ~100 kb from the initial duplicated marker. The presence in the Dp of both alleles (T and W) of the new RFLP indicated that the duplication extended to the genomic segment marked by that RFLP, but if the Dp exhibited only one allele (W, in the absence of crossovers), then the duplication did not extend to the RFLP. In this way, each duplication breakpoint could be localized to a genomic interval bracketed by RFLP markers at most ~100 kb apart. Subsequent iterations of this approach with additional RFLPs subdivided the ~100-kb interval into progressively narrower intervals until each breakpoint was localized to an interval bracketed by RFLP markers that were only ~3–5 kb apart.

Figure 1 presents a schematic of this approach. The solid box represents the initial duplicated marker used to define each duplication [*i.e.*, *dow/Dp(AR17)*, *met-1/Dp(B362i)*, *ylo-1/Dp(IBj5)*, and *trp-2/Dp(OY329)*]. "*D*" represents the distance from the first nucleotide of the start codon to the third nucleotide of the stop codon. Two hatched boxes represent the genomic segments (designated inner and outer probes) that bracket each breakpoint. The inner probes detect the farthest RFLPs that are covered by the duplication whereas the outer probes detect the closest RFLPs that are not covered. The

primers to amplify these probes and the enzymes and W strains used to detect the RFLPs are summarized in supplemental Table 1 at http://www.genetics.org/supplemental/. A^L and A^R denote the distances from the marker gene ORF to the farthest nucleotides of the inner probes and B^L and B^R to the closest nucleotides of the outer probes. The minimum size of the duplication can be defined by ($A^L + D + A^R$) and the maximum size by ($B^L + D + B^R$). Southern analysis was done to verify that the T and the OR strain from which the translocations were derived indeed displayed an RFLP in the identified interval. This RFLP was presumably caused by the duplication breakpoint.

Mapping of the Sugartown barren phenotype: The barren phenotype of the Sugartown strain was previously shown to segregate with markers on LG VII (Bhat *et al.* 2003); therefore we tested its linkage with molecular markers in the LG VII genome sequence that showed polymorphisms between the Sugartown and OR strains. In release 7 of the *N. crassa* genome sequence (http://www.broad.mit.edu/cgi-bin/annotation/fungi/neurospora_crassa_7), seven supercontigs were assigned to LG VII. Four supercontigs (7.21, 7.55, 7.10, and 7.52) are well ordered and contain 2.05 Mb of sequence whereas three (7.23, 7.73, and 7.118) are not well ordered and contain 0.69 Mb. Well-ordered supercontigs have their relative order assigned with respect to the genetic map. The not-well-ordered supercontigs do not contain any well-ordered markers to make such assignments. The oligonucleotide primers used for PCR amplification of 19 different genomic segments from these supercontigs as well as the restriction enzyme used to distinguish between the Sugartown (S) and OR (O) alleles of the amplified fragment are listed in supplemental Table 2 at http://www.genetics.org/supplemental/. The segregation of these alleles was examined among 124 progeny from the Sugartown × OR a cross for linkage with the barren phenotype. Those with the S allele and barren phenotype or O allele and a fertile phenotype were the parental types, whereas those with the O allele and barren phenotype or S allele and fertile phenotype were the crossovers. Supplemental Table 2 at http://www.genetics.org/supplemental/ also lists the frequency of crossover types obtained for each molecular marker.

Construction of *Dp(IBj5)* strains: *Dp(IBj5)* strains of both mating types were obtained from the crosses *T(IBj5) A* × OR a or *T(IBj5) a* × OR A. They were identified by their barren phenotype in crosses with OR strains and subsequently confirmed by Southern analysis with a fragment from the *cpc-1* gene that straddles the breakpoint as probe. *rid; Dp(IBj5)* strains in both mating types and the *Sad-1 rid; Dp(IBj5) A* strain were obtained from *T(IBj5) A* × *rid a*, *T(IBj5) a* × *rid A*, and *T(IBj5) a* × *Sad-1 rid A* and identifying segregants with the mating type of the nontranslocation parent. The duplication in *rid; Dp(IBj5)* strains was confirmed both by its barren phenotype and by Southern analysis. The presence of *rid* was confirmed by recovering the mutant allele in a subset of nonduplication progeny from crosses with these strains and then confirming that crosses with *rid; Dp1.3^{ec} hph* strains produce no *erg-3* mutant progeny. The presence of *Sad-1* in the *Sad-1 rid; Dp(IBj5) A* strain was confirmed by verifying infertility in a cross with *Sad-1 a*. The presence of *Dp(IBj5)* was confirmed by Southern analysis and the presence of *rid* was confirmed as for the *rid; Dp(IBj5)* strains.

RESULTS

Dominant suppression of RIP in *dow*: The wild-isolated strains Adiopodoumé *A* (FGSC 430) and its derivative strains T-430-Hyg^r a (FGSC 8609), Sugartown A (P0854), Adiopodoumé -7 A (P4305), Fred a (P0833), Coon a (P0881), and Bayan Lepas a (P2663) were shown previously to dominantly suppress RIP in a duplication of the *erg-3* gene fragment (Noubissi *et al.* 2000, 2001; Bhat *et al.* 2003). A similar dominant RIP-suppressor phenotype was also displayed by segregants bearing the chromosome segment duplications *Dp(AR17)*, *Dp(IBj5)*, *Dp(OY329)*, or *Dp(S1229)* (Bhat and Kasbekar 2001; Fehmer *et al.* 2001). We wanted to test whether these dominant RIP-suppressor phenotypes also extend to other duplicated sequences, specifically, to a duplicated fragment of the *dow* gene. For this, each suppressor strain was crossed with *Dp1.5dow^{ec} hph; erg-3* strains of the opposite mating type. *Dp1.5dow^{ec} hph* is a tagged duplication that targets RIP to *dow* (see materials and methods). The *dow* locus is linked to *erg-3* (~10%); therefore any *dow* mutants induced by RIP in these crosses would be recovered among the *erg-3* progeny. For controls, the *Dp1.5dow^{ec} hph; erg-3* strains were crossed with the OR strains 74-OR23-1 A and OR8-1 a. The RIP suppressor of the Sugartown strain was tested using a *Sad-1* segregant (strain 22) from a cross between Sugartown and a *Sad-1* mutant strain (see materials and methods). The *Sad-1* mutation overcomes the barren phenotype characteristic of crosses with the Sugartown strain.

The frequencies of RIP-induced *dow* mutants recovered in the *erg-3* progeny from these crosses are summarized in Table 1. As can be seen in the Table 1, a sizable fraction of *erg-3* segregants from the control crosses were mutant in *dow*. In contrast, no *dow* mutants were detected among the *erg-3* progeny examined from the crosses involving Adiopodoumé A, T-430-Hyg^r a, strain 22, Fred, and Coon. On the basis of these results we can conclude that the dominant RIP suppressors of the Adiopodoumé, Sugartown, Fred, and Coon strains are capable of suppressing RIP in any duplication. However, the crosses with the Adiopodoumé -7 and Bayan Lepas strains did produce some *dow* mutant progeny. It is possible that these two wild-isolated strains do not suppress RIP as effectively as the other four wild isolates do.

No *dow* mutants were found among the *erg-3* segregants from the crosses heterozygous for *Dp(AR17)*, *Dp(IBj5)*, *Dp(OY329)*, or *Dp(S1229)*. This confirmed that the large duplications, as well as fairly small ones, also are capable of dominantly suppressing RIP in many, and possibly all, gene-sized duplications. The *Sad-1; Dp(AR17)* × *Dp1.5dow^{ec} hph; erg-3* cross presented an interesting case. If we disregard crossovers, *dow* mutants generated by RIP in *Dp(AR17)* are recoverable among the erg$^+$ progeny, whereas those generated by RIP in the small duplication are recoverable among the *erg-3* progeny. As can be seen in Table 1, no *dow* mutants were generated in the small duplication but they were indeed produced by RIP in *Dp(AR17)*. This confirms that *Dp(AR17)* suppresses RIP in *Dp1.5dow^{ec}*.

The size and extent of duplications: The proximal and distal breakpoints of the duplications *Dp(AR17)*, *Dp(B362i)*, *Dp(IBj5)*, and *Dp(OY329)* were localized as

TABLE 1

RIP frequencies in crosses with *Dp1.5dow^{ec} hph; erg-3* strains

Cross	*dow erg-3/ erg-3*[a]
1. OR A × *Dp1.5dow^{ec} hph; erg-3 a*	9/68
2. OR a × *Dp1.5dow^{ec} hph; erg-3 A*	11/200
3. Adiopodoume A × *Dp1.5dow^{ec} hph; erg-3 a*	0/124
4. T-430-Hyg^{r} a × *Dp1.5dow^{ec} hph; erg-3 A*	0/101
5. Adiopodoume -7 A × *Dp1.5dow^{ec} hph; erg-3 a*	2/76
6. Strain 22 a[b] × *Dp1.5dow^{ec} hph; erg-3 A*	0/138
7. Fred a × *Dp1.5dow^{ec} hph; erg-3 A*	0/120
8. Coon a × *Dp1.5dow^{ec} hph; erg-3 A*	0/124
9. Bayan Lepas a × *Dp1.5dow^{ec} hph; erg-3 A*	3/120
10. *Sad-1 rid; Dp(IBj5) A* × *Dp1.5dow^{ec} hph; erg-3 a*	0/105
11. *Sad-1; Dp(AR17) A* × *Dp1.5dow^{ec} hph; erg-3 a*	0/86 (*erg-3* progeny) 4/98 (erg^{+} progeny)[c]
12. *Sad-1; erg-3; Dp1.5dow^{ec} hph A* × *Dp(OY329) a*	0/125
13. *Sad-1; erg-3; Dp1.5dow^{ec} hph A* × *Dp(S1229) a*	0/119

[a] *dow* mutants generated by RIP in the *Dp1.5dow^{ec} hph; erg-3* nucleus are linked to *erg-3*. Thus the frequency of *dow erg-3/ erg-3* progeny provides a measure of RIP efficiency in *Dp1.5dow^{ec} hph*. The *erg-3* segregants can be recognized by their colony morphology on Vogel's–sorbose medium.

[b] Strain 22 is a *Sad-1* segregant with the dominant RIP suppressor of the Sugartown strain.

[c] *dow* mutants among the erg^{+} progency are induced by RIP in *Dp(AR17)*.

outlined in MATERIALS AND METHODS and a summary of these experiments is presented in Table 2. The results showed that the duplications were, respectively, 351–357, 116–119, 402–405, and 703–707 kb. This work also revealed the actual genomic segment that is covered by the duplications. The localization of the proximal (right) breakpoint of *Dp(IBj5)* in Table 2 was consistent with the one made by PALUH *et al.* (1990).

More precise localizations could be achieved using oligonucleotides from within the C^{L} and C^{R} genomic intervals (see Figure 1). If the oligonucleotides can prime PCR amplifications from both the OR and the translocation strains, it follows that they do not bracket the breakpoint. Conversely, if the primers fail to amplify from the translocation but do so from OR, it would suggest that they bracket the translocation breakpoint. The oligonucleotides 5′-agaaaggctcttacacaaggtag and 5′-gta gcgaagtccaaatcatgaac from the C^{L} interval of *Dp(AR17)* could prime the amplification of a 581-bp fragment using OR DNA as template, but no product was obtained with DNA from *T(AR17)* strains (data not shown). Thus the proximal break of *Dp(AR17)* appears to be in this 581-bp segment. In a like manner, the distal break of *Dp(AR17)* was localized to a 90-bp segment that is defined by the oligonucleotides 5′-caagtgaaaagcaaaagattggt and 5′-attc ttccacaactccatccttga from the C^{R} interval of *Dp(AR17)*. These results narrow down the range of *Dp(AR17)*'s size to 354,651–355,230 bp.

Tests of *Dp(AR18)* and *Dp(B362i)* for dominant RIP suppression: The three largest duplications in the studies described above [*Dp(AR17)*, *Dp(IBj5)*, and *Dp(OY329)*] were shown previously to be capable of dominant suppression of RIP. At ~117 kb *Dp(B362i)* is significantly smaller; therefore we examined whether it, too, was a dominant RIP suppressor. A cross was made between the translocation strain *T(B362i) A* and the wild-isolated strain Lankala Koderu-1, and of 22 F_1 segregants examined, 13 were *A* and 9 were *a*. Southern analysis using the *met-1* gene fragment as probe revealed that three *mat A* segregants (3, 8, and 10) had the *met-1* RFLPs of both parental strains, thus indicating that they were genotypically *Dp(B362i)*. Consistent with this indication, crosses of these segregants with OR a were barren. The frequency of *erg-3* mutant progeny from crosses of the *Dp(B362i)* segregants with *Sad-1; Dp1.3^{ec} hph a* were, respectively, 7.3% (284), 7% (240), and 6.2% (208). (Numbers in parentheses indicate the number of F_2 progeny examined.) This showed that *Dp(B362i)* does not suppress RIP in *trans*.

Five hygromycin-resistant F_2 progeny (3-4, 3-11, 8-8, 8-26, and 10-8) from the crosses between segregants 3, 8, and 10 and *Sad-1; Dp1.3^{ec} hph a* were determined by Southern analysis to be genotypically *Dp (B362i); Dp1.3^{ec} hph A* and one (8-5) to be *Sad-1; Dp (B362i); Dp1.3^{ec} hph A*. We verified that crosses between segregants 3-4, 3-11, 8-8, 8-26, and 10-8 and OR a were barren. These five segregants were crossed with *Sad-1 a* and the frequencies of *erg-3* mutant progeny from these crosses were 5.4% (204), 5.8% (189), 7.3% (259), 5.1% (98), and 5.7% (207). These results show that *Dp(B362i)* also does not suppress RIP *in cis*. Thus we can conclude that the ~117-kb duplication fails to act as a dominant RIP suppressor.

We also examined *Dp(AR18)* for the dominant RIP-suppressor phenotype. First, to confirm that translocation *T(AR18)* itself does not possess this phenotype, we crossed *T(AR18) a* and *Sad-1; Dp1.3^{ec} hph A* (in this cross the *Sad-1* mutation is irrelevant). The frequency of *erg-3* mutants among the progeny harvested at 31 days was 2.4% ($N = 598$). Next, we crossed *T(AR18) A* and OR a

TABLE 2

Estimating the size and extent of duplications

Duplication	Initial marker	*D*	A^L	B^L	A^R	B^R	C^L	C^R	$A^L + D + A^R$	$B^L + D + B^R$
Dp(AR17)	*dow*	4920	246322	248861	100216	103453	2539	3237	351458	357234
Dp(B362i)	*met-1*	1755	45086	46411	69974	71168	1325	1194	116815	119334
Dp(IBj5)	*ylo-1*	1686	280551	282726	120636	121377	2175	741	402873	405789
Dp(OY329)	*trp-2*	1826	193690	195849	508009	509279	2159	1270	703525	706954

and several F_1 progeny from this cross were, in turn, crossed with OR and *Sad-1; Dp1.3^{ec} hph* strains of the appropriate mating type. The F_1 progeny that gave barren crosses with OR were assumed to be *Dp(AR18)*, whereas those that gave fertile crosses could be either translocation or normal-sequence euploid (E) strains. The frequencies of *erg-3* mutant progeny from six different *Dp(AR18)* × *Sad-1; Dp1.3^{ec} hph* crosses examined were all <0.2%, whereas those from the two control E × *Sad-1; Dp1.3^{ec} hph* crosses examined were 15% and 4.9%. These results show that *Dp(AR18)* functions as a dominant suppressor of RIP.

Mapping the barren phenotype of Sugartown: We examined 124 F_1 segregants from the cross Sugartown × OR a; 72 were fertile when crossed with euploid strains of the opposite mating type whereas 52 were barren. Of 59 fertile segregants that were examined for the dominant RIP-suppressor phenotype, 56 (95%) were phenotypically Sup^+ (frequency of *erg-3* mutant progeny >1.0%) and three (5%) had an intermediate phenotype (frequency of *erg-3* mutant progeny 0.5–1.0%). Of the 40 barren segregants examined in a similar manner, 32 (80%) were Sup^- (frequency of *erg-3* mutant progeny <0.5%), 7 (17.5%) were intermediate, and 1 (2.5%) was Sup^+. These results were consistent with previous studies showing linkage between the suppressor and barren phenotypes.

The barren phenotype segregates with markers on LG VII [*spco-4* (28.6%), *slo-2* (28.9%); Bhat *et al.* 2003]; therefore we tested its linkage with 19 LG VII molecular markers that were polymorphic between the Sugartown and OR strains. We prepared genomic DNA from the 124 F_1 segregants from Sugartown × OR a and analyzed them for the segregation of the molecular markers as described in materials and methods. The results of this analysis are summarized in supplemental Table 2 at http://www.genetics.org/supplemental/. As can be seen in Table 2, the tightest linkage (~9% crossovers) was observed with a marker on supercontig 7.118 and somewhat lower linkage (~20%) was seen with two markers on supercontig 7.73.

Crosses homozygous for *Dp(AR17)*, *Dp(IBj5)*, or *Dp(B362i)*: Bhat and Kasbekar (2004) had shown that crosses that are either heterozygous or homozygous for *Dp(AR17)* have a barren phenotype and that in both cases the barrenness was independent of RIP. Moreover, the barrenness of the heterozygous cross was suppressible by a semidominant *Sad-1* mutation but that of the homozygous cross was not suppressible by *Sad-1*. We repeated these crosses and confirmed the earlier results. We extended this study by constructing strains of the genotype *Sad-1 rid*; *Dp(AR17), dow$^+$/dow* and used them to make crosses that were homozygous for *Dp(AR17)* and either heterozygous for *rid* and *Sad-1* or homozygous for *rid* and heterozygous for *Sad-1*. The former cross was barren whereas the latter was fertile. That homozygosity for *Dp(AR17)* and heterozygosity for *rid* and *Sad-1* causes barrenness is not surprising because the *rid* mutation is recessive, thus making these crosses essentially similar to ones that were homozygous for *Dp(AR17)* and heterozygous for *Sad-1*. However, the fertile phenotype of crosses homozygous for both *Dp(AR17)* and *rid* and heterozygous for *Sad-1* was an unexpected result.

To examine whether crosses heterozygous or homozygous for *Dp(IBj5)* show similar effects, we constructed *Dp(IBj5), rid*; *Dp(IBj5), Sad-1*; *Dp(IBj5)*, and *Sad-1 rid*; *Dp(IBj5)* strains in both mating types (see materials and methods) and performed the crosses summarized in Table 3. As can be seen in Table 3, both heterozygosity and homozygosity for *Dp(IBj5)* caused barrenness and in both cases the barrenness was independent of RIP (crosses 7 and 15). Moreover, the barrenness of the heterozygous cross was suppressible by *Sad-1* whereas that of the homozygous cross was not suppressible by *Sad-1* (compare crosses 2 and 11 with 9, 10, and 12). These results parallel those seen in equivalent crosses with *Dp(AR17)*. However, cross 13 [*Sad-1 rid*; *Dp(IBj5)* × *rid*;

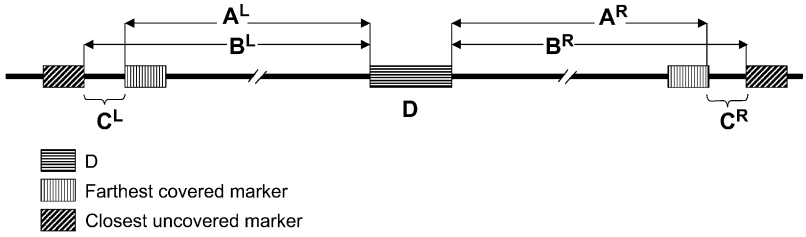

Figure 1.—Determining the size and extent of the duplications in Table 2. The initial duplicated marker ORF is represented by *D*. Boxes with vertical lines and diagonal lines represent, respectively, genome segments that define RFLP markers covered or uncovered by the duplication and thus bracket the breakpoints. Thus the minimum and maximum sizes of the duplication are, respectively, $A^L + D + A^R$ and $B^L + D + B^R$.

TABLE 3

Phenotype of crosses involving *Dp(IBj5)*

Cross	Productivity
1. *Dp(IBj5) a* × *OR A*	Barren
2. *Dp(IBj5) a* × *Sad-1 A*	Fertile
3. *Dp(IBj5) a* × *Dp(IBj5) A*	Barren
4. *rid-1; Dp(IBj5) a* × *ORA*	Barren
5. *rid-1; Dp(IBj5) a* × *Sad-1 A*	Fertile
6. *rid-1; Dp(IBj5) A* × *Dp(IBj5) a*	Barren
7. *rid-1; Dp(IBj5) a* × *rid-1; Dp(IBj5) A*	Barren
8. *Sad-1; Dp(IBj5) A* × *OR a*	Fertile
9. *Sad-1; Dp(IBj5) A* × *Dp(IBj5) a*	Barren
10. *Sad-1; Dp(IBj5) A* × *rid-1; Dp(IBj5) a*	Barren
11. *Sad-1; rid-1; Dp(IBj5) A* × *OR a*	Fertile
12. *Sad-1; rid-1; Dp(IBj5) A* × *Dp(IBj5) a*	Barren
13. *Sad-1; rid-1; Dp(IBj5) A* × *rid-1; Dp(IBj5) a*	Barren
14. *Dp(AR17) A* × *Dp(IBj5) a*	Barren
15. *rid-1; Dp(AR17) A* × *rid-1; Dp(IBj5) a*	Barren
16. *Sad-1; rid-1; Dp(AR17) A* × *Dp(IBj5) a*	Fertile

Dp(IBj5)] was barren. Thus, although the RIP defect appeared to alleviate the inability of *Sad-1* to suppress the barren phenotype of the *Dp(AR17)*-homozygous cross, it did not do so for the *Dp(IBj5)*-homozygous cross. Finally, cross 16, which was heterozygous for *Dp(AR17)*, *Dp(IBj5)*, and *Sad*-1, was fertile. The combined size of the two duplications (753–762 kb) is greater than that of *Dp(OY329)* (703–707 kb).

A cross was made between *T(B362i) a* and OR A and 10 *mat a* segregants were obtained that were barren in crosses with euploid. We confirmed by Southern analysis that these segregants were genotypically *Dp(B362i)*. Each of the 10 segregants was also crossed with a *Dp(B362i) A* strain (no. 8; see above) and a *Sad-1; Dp(B362i) A* strain (no. 8-5; see above). All 20 *Dp(B362i)*-homozygous crosses were barren. Thus crosses homozygous for *Dp(B362i)* are barren and the barren phenotype is not suppressed by *Sad-1*.

However, we noted a consistent difference in the (very limited) productivity of the crosses of the *Dp(B362i)* segregants with OR A, *Dp(B362i) A*, and *Sad-1; Dp(B362i) A*. The crosses with OR A were the least productive, those with *Dp(B362i) A* were somewhat more productive, and those with *Sad-1; Dp(B362i) A* were the most productive. In a blind test based solely on the productivity differences, we could distinguish between the three cross types reasonably successfully (the genotypes of 26 of 30 crosses could be correctly assigned). In contrast, had the assignments been made at random, only 10 crosses would be expected to be correctly genotyped.

DISCUSSION

Duplications as dominant suppressors of RIP: Of the six duplications tested thus far [*Dp(AR17)*, *Dp(AR18)*, *Dp(B362i)*, *Dp(IBj5)*, *Dp(OY329)*, and *Dp(S1229)*], only one, *Dp(B362i)*, failed to display the dominant RIP-suppressor phenotype. The size of *Dp(S1229)* has not been determined but it is known that it covers the gene *methyltryptophan resistant* (*mtr*) and that it has a breakpoint that is inseparable from an *arginine-14* (*arg-14*) mutation (Perkins 1997). A gap of unknown size is present between the supercontigs that bear *mtr* and *arg-14*, but the known sequence between these two markers is 270,388 bp. Therefore *Dp(S1229)* is at least 270 kb. *Dp(AR18)* was estimated to be 270 ± 90 kb from the results of Smith and Glass (1996). Thus *Dp(B362i)* is the smallest of the six duplications tested for dominant RIP suppression. Our findings are consistent with the model that chromosome segment duplications in *N. crassa* can act as dominant suppressors of RIP, possibly by titrating out the RIP machinery, but with an added proviso that the titrating duplication is >117 kb. A more precise determination of the threshold size above which duplications can suppress RIP might be achievable by studying the mitotic deletion derivatives of *Dp(AR18)*. Such deletion derivatives can be obtained as "escapes" from *het-6* incompatibility in *Dp(AR18), het-6*^{OR}/*het-6*^{PA} partial diploids (Smith *et al.* 1996). The deletions reportedly range in size from ~70 kb putatively up to the entire 270-kb duplicated segment, but always including a 35-kb region in which the *het-6* incompatibility locus is located.

The barren and suppressor phenotypes of the Sugartown strain: Of the 99 segregants examined from Sugartown × OR a, 88 showed the parental phenotypes (56 fertile/Sup$^+$ + 32 barren/Sup$^-$); 10 (three fertiles + seven barrens) were intermediate and therefore could not be classified as Sup$^+$ or Sup$^-$, and 1 was barren and Sup$^+$. These results can be interpreted to support the model that both the suppressor and the barren phenotype are caused by a >117-kb duplication in the Sugartown strain, provided we disregard the single barren Sup$^+$ segregant as either an experimental artifact or a deletion derivative that reduces the size of the duplication to below the threshold required for dominant RIP suppression. An alternative model is that the two phenotypes, although closely linked, are separable by crossing over (~1%). To distinguish between these models, we undertook to map the barren phenotype as a prelude to molecular characterization. Our results showed that the barren phenotype was most tightly linked (9% crossovers) with a marker on supercontig 7.118 and somewhat less tightly linked (20%) with two markers on supercontig 7.73. These two supercontigs are, respectively, 15.6 and 539 kb. Unfortunately, they are not yet well ordered. As the sequences in supercontig 7.118 become ordered in future releases of the genome sequence, the search for more tightly linked markers can be resumed using genomic DNA from the 124 segregants. As more tightly linked markers are identified, the genomic segment that is responsible for the barren phenotype should be progressively narrowed down.

Duplication homozygosity and meiotic silencing: The results described here together with those of Bhat and Kasbekar (2004) show that crosses homozygous for *Dp(AR17)*, *Dp(B362i)*, or *Dp(IBj5)* are barren. In contrast, crosses homozygous for small gene-sized duplications were not barren even though the heterozygous cross could be barren (Shiu *et al.* 2001). Bhat and Kasbekar (2004) hypothesized that when a large duplication is homozygous, the duplication-borne genes might be able to pair in meiosis with allelic or nonallelic "partners," and this ability might even induce them to switch partners. If these genes became transiently unpaired during the switches, they might trigger meiotic silencing and thus render the homozygous cross as barren as the heterozygous one. Gene-sized duplications might not switch partners in homozygous crosses or might do so in a way that does not trigger meiotic silencing. Interestingly, although the *Dp(B362i)*-homozygous crosses were barren, they were consistently more productive than the *Dp(B362i)*-heterozygous crosses. In other words, the behavior of *Dp(B362i)* was intermediate between that of the small gene-sized duplications used by Shiu *et al.* (2001) and the much larger duplications like *Dp(AR17)* and *Dp(IBj5)*.

The semidominant *Sad-1* mutation suppressed the barrenness of the heterozygous but not the homozygous crosses. Although the possibility that the barren phenotype of the homozygous crosses might have a different provenance from that of the heterozygous crosses is not ruled out, our finding of an increased productivity of *Dp(B362i)*-homozygous crosses if they were also made heterozygous for *Sad-1* supports the idea that meiotic silencing contributes to the barrenness of the duplication-homozygous crosses. Large duplications appear to exert a recessive suppression of the semidominant *Sad-1* mutation, possibly by desensitizing the detection of unpairing at *sad-1*$^+$ and thus rendering the *Sad-1* allele recessive. Transient unpairing and repairing of multiple duplication-borne genes in the duplication-homozygous cross might increase the "noise" and thus reduce the signal-to-noise ratio below the threshold required to detect the unpairing of *sad-1*$^+$. In a like manner, large duplications might also recessively suppress other ascus-dominant mutations.

The inability of *Sad-1* to suppress the barren phenotype of the *Dp(AR17)*-homozygous cross appeared to be alleviated if the cross was also made homozygous mutant for the recessive RIP-deficient gene *rid*. We suggest that the "noise" is reduced in the *Dp(AR17)*-homozygous cross in the absence of RIP (or RIP-associated cytosine methylation), thus allowing *Sad-1* to suppress meiotic silencing. The occurrence of RIP might promote meiotic pairing between nonallelic partners, which were presumably paired for RIP already prior to meiosis. In contrast, non-RIPed sequences might tend to pair primarily with allelic partners. In other words, by tending to increase partner switching in this way, RIP would increase the noise. Presumably, noise reduction in the *Dp(IBj5)*-, *rid*-homozygous cross was insufficient to overcome the suppression of *Sad-1* semidominance by *Dp(IBj5)*.

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