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Dominant Suppression of Repeat-Induced Point Mutation in *Neurospora crassa* by a Variant Catalytic Subunit of DNA Polymerase-ζ

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ABSTRACT

Crosses involving the Adiopodoumé strain of *Neurospora crassa* are defective for repeat-induced point mutation (RIP), a genome defense mechanism of fungi. We show here that the Adiopodoumé strain possesses an incompletely penetrant and variably expressive dominant suppressor of RIP (*Srp*) that maps to an ~34-kbp genome segment that is ~26 kbp proximal to *mat* on linkage group IL. Gene disruption experiments revealed that *Srp* is the *upr-1* allele of Adiopodoumé (*upr-1*^{Ad}) that is contained within this segment. The *upr-1* gene codes for the catalytic subunit of the translesion DNA polymerase-ζ (Pol-ζ) and it is unusually polymorphic in Neurospora. That the *upr-1* gene contains upstream ORFs that overlap with the main ORF is potentially relevant to the incomplete penetrance and variable expressivity of the suppressor. Crosses between heterokaryons that contain *upr-1*^{Ad} and strains that prevent mating events involving nuclei that contain *upr-1*^{Ad} yielded no progeny in which RIP had occurred, consistent with the idea that the suppressor encoded by *upr-1*^{Ad} is diffusible. The potential involvement of the Pol-ζ subunit in two functions, translesion DNA synthesis and RIP regulation, might account for the rapid evolution of its gene in Neurospora.

An unusual strain of *Neurospora crassa* was isolated in the 1950s from Adiopodoumé (a village and research station near Abidjan, Cote D'Ivoire, West Africa), in that it was the only wild-isolated Neurospora strain found harboring active copies of a LINE-like transposable element named *Tad* (Kinsey and Helber 1989). All the other wild isolates of Neurospora examined (including those of species other than *N. crassa*) contained only mutation-inactivated relics of *Tad* (Kinsey 1989; Kinsey *et al.* 1994). No other active transposable element has since been found in Neurospora. Subsequently, the Adiopodoumé strain was identified as one of 7 wild-isolated *N. crassa* strains (of ~450 screened) possessing the ability to dominantly suppress repeat-induced point mutation (RIP) (Noubissi *et al.* 2000, 2001; Bhat *et al.* 2003). RIP is a genome defense process of fungi that occurs during the premeiosis of a sexual cross and hypermutates and methylates duplicated DNA sequences, thereby protecting the genome against the proliferation of transposable elements and other parasitic DNA (Cambareri *et al.* 1989; Selker 1990; Galagan *et al.* 2003; Galagan and Selker 2004). Presumably the dominant RIP suppressor phenotype facilitated *Tad*'s survival in the Adiopodoumé strain. Screening for the dominant RIP suppressors was done by crossing the wild isolates with strains of the opposite mating type bearing the transgene *Dp(erg-3)*, which duplicates a segment of the *erg-3* gene. Crosses of the *Dp(erg-3)* strains with most wild isolates yielded RIP-induced *erg-3* mutant progeny at frequencies in the 2–25% range, but in the crosses with the dominant RIP suppressors (and with a derivative of the Adiopodoumé *A* strain called T-430-Hyg^r *a*) this frequency was consistently <0.5%. The RIP suppressor phenotype also extended to a duplication of the *downy* gene (Vyas *et al.* 2006). Presumably it applies generally. Initial mapping studies suggested that the Adiopodoumé strain contains a dominant RIP suppressor (hereafter referred to as *Srp* for "*Suppressor of RIP*") linked to the mating-type locus (*mat*) on linkage group (LG) IL (Bhat *et al.* 2003). Here we show that *Srp* is the Adiopodoumé allele of the *ultraviolet photoreactivation-1* (*upr-1*) gene. This gene encodes the catalytic subunit of the translesion DNA polymerase-ζ (Pol-ζ) (Sakai *et al.* 2002). This finding revealed that at least a subset of *upr-1* alleles can act as potential regulators of RIP. A role for *upr-1* in RIP regulation could have driven its rapid evolution, as evidenced by the unusually high polymorphism exhibited by this gene in *N. crassa* (Tamuli *et al.* 2006).

We dedicate this article to the memory of David D. Perkins (1919–2007).

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MATERIALS AND METHODS

Gene and phenotype symbols: Gene symbols are italicized, while phenotype symbols are not. For example, the nonitalicized

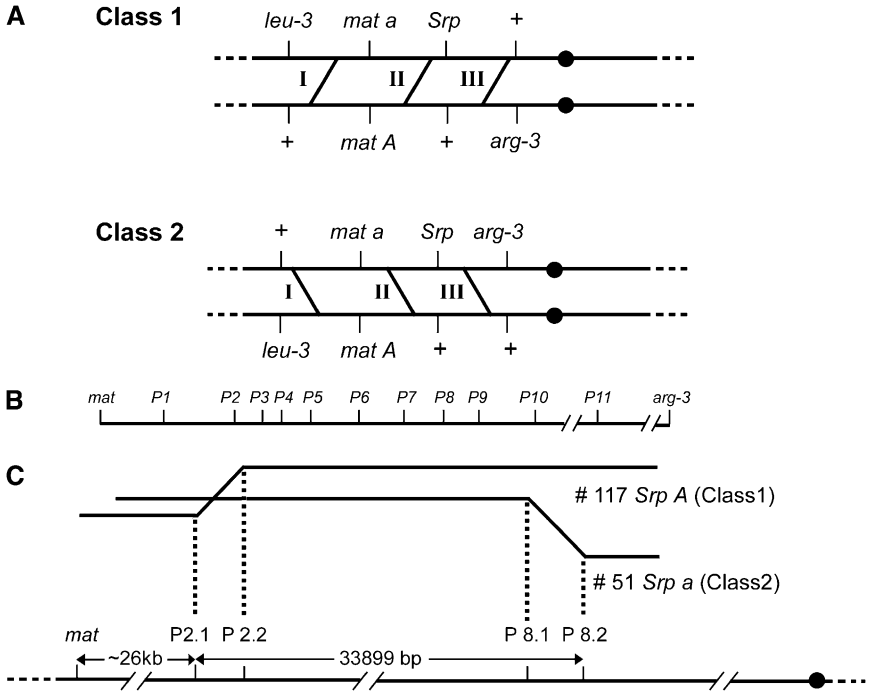

FIGURE 1.—(A) Prototrophic progeny produced by crossover if *Srp* is proximal to *mat*. For class 1 prototrophs: (I) Srp *a*, (II) Srp *A*, and (III) Srp^+ *A*. For class 2 prototrophs: (I) Srp^+ *A*, (II) Srp^+ *a*, and (III) Srp *a*. If, instead, *Srp* is distal to *mat*, then crossovers in II yield Srp^+ *a* in class 1 and Srp *A* in class 2 but the products of crossover in I and III remain unaffected. (B) Molecular markers in the *mat*-proximal region. The distances (kilobase pairs) between consecutive pairs of markers from the left (*mat*) are 12.1, 12.8, 4.1, 2.7, 4.9, 7.0, 7.0, 5.9, 5.6, 8.5, 66.7, and >1042. (C) Localization of *Srp* to an ~34-kbp region proximal to *mat*. Locations of the crossovers that produced the class 1 Srp *A* prototroph no. 117 and the class 2 Srp *a* prototroph no. 51 are shown. P2.1 and P2.2 are single-nucleotide polymorphisms (SNPs) in the interval P2–P3 and P8.1 and P8.2 are SNPs in the interval P8–P9.

symbol Srp^+ signifies nonsuppressor phenotype, nonitalicized $Srp^{+/-}$ signifies intermediate phenotype, and nonitalicized Srp^- signifies suppressor phenotype.

General genetic manipulation of *N. crassa* and the assay for RIP: Neurospora genetic analysis was essentially as described by DAVIS and DE SERRES (1970). The frequency of RIP-induced *erg-3* mutant progeny among ascospores harvested at 31 days was used as a measure of RIP efficiency and determined as the proportion of colonies with the *erg-3* mutant morphology, as seen under a dissection microscope.

General strains: Unless otherwise stated all *N. crassa* strains were obtained from the Fungal Genetics Stock Center (FGSC, University of Missouri, Kansas City). They included the standard Oak Ridge (OR) strains 74-OR23-1 *A* (FGSC 987) and OR8-1 *a* (FGSC 988) and the mutants *erg-3 a* (FGSC 2725) and *erg-3 A* (FGSC 3439). The *erg-3* mutation confers a female-sterile phenotype. The *Round spore* (*R*) strains *R A* (FGSC 4022) and *R a* (FGSC 4023) contain an ascus-dominant 20- to 30-kbp deletion that when crossed with the wild type causes unpairing of the R^+ allele and its consequent meiotic silencing; consequently, all eight ascospores become round rather than ellipsoid. *R* also confers a female-sterile phenotype. The auxotrophic strains are *arg-1 A* (FGSC 325), *arg-3 A* (FGSC 1068), *leu-3 A* (FGSC 4002), *leu-3 a* (FGSC 4003), *his-3 A* (FGSC 6103), *his-3 a* (FGSC 7088), *his-1 A* (FGSC 401), *his-1 a* (FGSC 402), *trp-1 A* (FGSC 2038), and *trp-1 a* (FGSC 2039) and the wild-isolated strain Adiopodoumé *A* (FGSC 430) and its derivativeT-430-Hyg^r *a* (FGSC 8609) that 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). Both Adiopodoumé *A* and T-430-Hyg^r *a* display the dominant RIP suppressor phenotype linked to *mat* on LG IL (BHAT *et al.* 2003). The translocation strains are *T(VIL→IR)IBj5 cpc-1 A* (FGSC 4433) and *T(VIL→IR)IBj5 cpc-1 a* (FGSC 4434), in which a segment of VIL, extending from *cpc-1* through *ylo-1*, is translocated to IR. One-third of the viable progeny from a cross of the translocation with a normal sequence contain a duplication of the translocated segment, designated *Dp(VIL→IR)IBj5*. The duplication is stably barren in crosses. The duplication is ~400 kbp in size and serves as an effective dominant suppressor of RIP (VYAS *et al.* 2006).

The *Dp(erg-3) A* and *a* strains, described by BHAT *et al.* (2003) and PRAKASH *et al.* (1999), contain the transgene *Dp(erg-3)* that duplicates a fragment of the LG IIIR gene *ergosterol-3* (*erg-3*) tagged with the bacterial *hph* gene for resistance to hygromycin. The ectopically duplicated fragment serves to target RIP to *erg-3*. The resulting RIP-induced *erg-3* mutant progeny can be scored under a dissection microscope by their distinct colony morphology on Vogel's-sorbose agar medium (NOUBISSI *et al.* 2000). The *Dp(dow) A* and *a* strains were previously referred to as *Dp1.5dow*^{rc} *A* and *a* (VYAS *et al.* 2006). They contain the *hph*-tagged transgene *Dp(dow)* that duplicates a fragment of the LG IIIR gene *downy* (*dow*) and targets RIP to *dow*. The *mus-26* (*NcRev7*) mutant strains were constructed in the laboratory and have been described by TAMULI *et al.* (2006).

Strains *Sad-2* (RIP32) *A* (RLM 30-12) and *Sad-2* (RIP32) *a* (RLM33-12) (SHIU *et al.* 2006) were kindly provided by Robert L. Metzenberg (Department of Biology, California State University, Northridge, CA). They were used to construct the heterokaryons [(*leu-3 Srp; Sad-2 a*) + (*erg-3; his-1 a*)] and [(*Dp(IBj5); trp-1; Sad-2 a*) + (*erg-3; his-1 a*)]. These heterokaryons were crossed with the strain *R; Sad-2; Dp(dow) A*.

PCR amplification, molecular markers, and transformation: Oligonucleotide primers for PCRs were custom purchased from Bioserve (Hyderabad, India). The reaction conditions, other molecular methods, and transformation protocols were as described in BHAT *et al.* (2004). PCR-based RFLPs were identified to distinguish between the Adiopodoumé and OR genomes in the *mat*-proximal region of LG IL (Figure 1B). Supplemental Table 1 lists the primers used to amplify 11 genomic segments (P1–P11) from the *mat*-proximal sequence of super-contig 7.3, as well as the restriction enzymes that enable one to distinguish between the Adiopodoumé and OR alleles of the amplified fragment.

An OR A-specific DNA insert in molecular marker P7: The molecular marker P7 is a 1658-bp PCR amplicon when OR *A* DNA was used as the template, but a smaller fragment was amplified when the template DNA was from the Adiopo-

TABLE 1

Strains used to perform *Srp*-homozygous crosses

Cross	*erg-3* mutants/progeny examined	% *erg-3*
1. 117-11 *A* × *Dp(erg-3) a*	0/560	<0.2
2. 117-44 *A* × *Dp(erg-3) a*	0/200	<0.5
3. 117-66 *A* × *Dp(erg-3) a*	1/332	0.3
4. 117-25 *A* × *Dp(erg-3) a*	50/400	12.5
5. 82-46 *a* × *Dp(erg-3) A*	0/262	<0.4
6. 82-51 *a* × *Dp(erg-3) A*	0/512	<0.2
7. 82-51-6 *a* × *Dp(erg-3) A*	8/900	0.9

doumé strain, OR *a*, or any of several other wild-isolated *N. crassa mat A* and *mat a* strains, or from the *N. tetrasperma* strain 85 *A*. A 369-bp sequence (contig 7.3, bases 345,297–345,665) is present in the fragment amplified from OR *A*, but is missing from all the other strains examined. The OR *A*-specific "insert" is a composite of three smaller sequences: an ~133-bp "left" segment that is repeated at 21 other genomic locations in the sequenced genome, an ~45-bp "middle" segment repeated at one other location, and an ~171-bp "right" segment that is repeated at 24 other locations. These three segments (or any two) are not found together at any other location in the *N. crassa* genome sequence. The 369-bp sequence showed limited homology (supplemental Table 2) with the *In(het-6) brk1* RS element described by Micali and Smith (2006).

Strains used to make *Srp*-homozygous crosses: Four *leu-3 Srp a* segregants with the Srp$^-$ phenotype (398 *a*, 475 *a*, 514 *a*, and 603 *a*) were obtained from the cross *leu-3 A* × T-430-Hyg^r *a*. Segregant 475 *a* was crossed with *arg-1 A* and a prototrophic Srp$^-$ *A* progeny was designated 117 *A*. From 117 *A* × *Dp(erg-3) a* we obtained three *mat A* segregants, 117-11 *A*, 117-44 *A*, and 117-66 *A*, that were Srp$^-$, and one *mat A* segregant, 117-25 *A*, that was a nonsuppressor (Table 1). Four *Srp arg-3 a* segregants were obtained from either the cross *arg-3 A* × T-430-Hyg^r *a* or *arg-3 A* × 475 *a*. Of 393 F_1 segregants from the first cross, 317 were arg-3$^+$ (215 *a* and 102 *A*) and 76 were arg-3$^-$ (3 *a* and 73 *A*). Of 49 leu-3$^+$ segregants examined from the second cross, 34 were *arg-3*$^+$ *A*, 10 were *arg-3*$^+$ *a*, 4 were *arg-3*$^-$ *A*, and 1 was *arg-3*$^-$ *a*. The four *Srp arg-3 a* segregants from these two crosses were confirmed to possess the Srp$^-$ phenotype and designated 33 *a*, 82 *a*, 112 *a*, and 114 *a*. Two prototrophic Srp$^-$ *a* segregants from 82 *a* × *leu-3 A* were designated 82-46 *a* and 82-51 *a* (Table 1). A *mat a* segregant was obtained from 117-11 *A* × 82-51 *a*, which showed an intermediate phenotype (Srp$^{+/-}$) and was designated 82-51-6 *a*. In sum, the strains 117-11 *A*, 117-44 *A*, 117-66 *A*, 82-46 *a*, and 82-51 *a* had the Srp$^-$ phenotype; strain 117-25 *A* was Srp$^+$ and 82-51-6 *a* was Srp$^{+/-}$ (Table 1). In all seven strains the genomic region around *mat* was derived from the Adiopodoumé strain (data not shown), which suggested that the Srp$^+$ and Srp$^{+/-}$ phenotypes of 117-25 *A* and 82-51-6 *a* were not due to crossing over in this region. Crosses among all these strains were homozygous for *Srp* (Table 1).

***upr-1* gene disruptions:** Oligonucleotide primers 1F 5′ TGG TTG GAC GAG AGT TCT GC and 2R 5′ TTT CTT GGC TTG ACA CCG GC 3′, and 10F 5′ ACT GCT GAA TAA CCG GCA GC and P2R 5′ TCC TGA GGA AAG CCA TGA CC, were used to PCR amplify 1410- and 2186-bp segments, respectively, from the 5′ and 3′ of *upr-1*^{Ad}. These segments were cloned in the same orientation into the *Kpn*I and *Eco*RV sites, respectively, of the vector pCSN44, to generate the plasmid pRT1. The *Kpn*I and *Eco*RV sites of pCSN44 flank the bacterial *hph* gene for resistance to hygromycin. Using pRT1 as template and the primers 1F (above) and 11R 5′ CCT GCA TGT CGA CCA TAA GC, we amplified an ~6-kbp DNA fragment that contained the *hph* gene flanked by 1410 and 1313 bp, respectively, of the *upr-1*^{Ad} 5′ and 3′ sequences. The amplified DNA was gel purified and transformed by electroporation into the Adiopodoumé strain (FGSC 430). Of 12 hygromycin-resistant transformants examined, the genomic DNA of 3 (nos. 9, 21, and 24) could template the PCR amplification of a 2312-bp fragment with the primers AD62R 5′ TGT GCG GCA ATA CCA TGT GC (which anneals to a sequence 150 bp upstream of the 5′ end of primer 2R and is therefore absent from the transforming DNA) and AD63F 5′ GTC GAC GGT ATC GAT AAG CTT G (which anneals to the pCSN44 multiple-cloning site present only in the transforming DNA). A PCR product is obtained only if the template DNA includes a *upr-1* allele disrupted by homologous integration of the transforming DNA. Consequently these primers are "disruption specific." In contrast, the primers 3F 5′ CGC TTT GTG CAA CCA GTT CC and 8R 5′ GCT ATC CTG AAC ATG ATG GAC amplify a 2519-bp fragment only from an intact *upr-1*^{Ad} allele but not from the disrupted allele and hence are "intact allele specific." Transformants 9, 21, and 24 gave PCR products with both the disruption- and intact allele-specific primers, thereby showing that they were heterokaryons that also contained both transformed and untransformed nuclei. These heterokaryons were crossed with OR *a*. Hygromycin-resistant *mat A* progeny from these crosses are homokaryons that inherit the disrupted allele, whereas the hygromycin-sensitive *mat A* progeny inherit the intact *upr-1*^{Ad} allele. We could also verify the gene disruption by Southern hybridization because a *Kpn*I restriction site present in the intact allele is absent from the disrupted allele (Figure 2). Disruption of *upr-1*^{Ad} conferred an increased sensitivity to UV, a characteristic of *upr-1* null mutants (R. Tamuli and D. P. Kasbekar, unpublished results), thus providing evidence that *upr-1*^{Ad} indeed encodes an active Pol-ζ.

RESULTS AND DISCUSSION

***Srp* shows incomplete penetrance and variable expressivity:** Segregation of the Srp phenotype was examined in 120 F_1 progeny from the cross T-430-Hyg^r *a* × 74-OR23-1 *A* by crossing them to *Dp(erg-3)* strains of the opposite mating type and determining the frequency of *erg-3* mutant progeny in the F_2. All 65 *mat A* progeny were Srp$^+$ (*erg-3* mutation frequency >1.0%), whereas 51 of the 55 *mat a* progeny were Srp$^-$ (*erg-3* mutation frequency <0.5%) and 4 had an intermediate (Srp$^{+/-}$) phenotype (*erg-3* mutation frequency 0.5–1.0%). These results confirmed the linkage of *Srp* to *mat*. That 4/55 *mat a* but 0/65 *mat A* progeny showed an intermediate phenotype alerted us to the possibility that occasionally (here, ~7%) *Srp* might show variable expressivity and confer an Srp$^{+/-}$ rather than an Srp$^-$ phenotype.

Of 733 F_1 progeny examined from the cross T-430-Hyg^r *a* × *leu-3 A*, 673 were parental type with respect to *leu-3* and *mat* (452 *leu*$^+$ *a* and 221 *leu-3 A*) and 60 (8.2%) were crossover types (32 *leu*$^+$ *A* and 28 *leu-3 a*). All the crossover progeny and 42 *leu-3 A* and 54 *leu*$^+$ *a* progeny were tested for their Srp phenotype. The 74 *mat A* progeny were all Srp$^+$, whereas 64 of the 82 *mat a* progeny were Srp$^-$, 8 were Srp$^{+/-}$, and 10 were Srp$^+$.

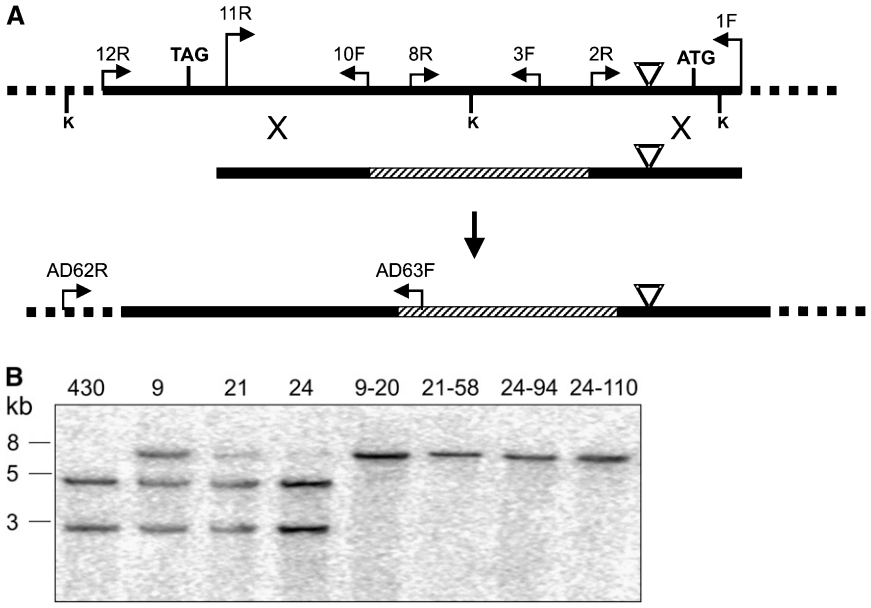

FIGURE 2.—Disruption of *upr-1*^{Ad}. (A) The top line is a schematic of the target *upr-1*^{Ad} allele, and the middle line represents the transforming DNA in which the *hph* gene (hatched segment) is flanked by 5′ and 3′ *upr-1*^{Ad} sequences (respectively, on the right and left of *hph*). The bottom line shows the disrupted allele resulting from homologous crossovers in the flanking *upr-1*^{Ad} sequences. The bent arrows represent the primers used to construct the transforming DNA (see text for details) as well as the "disruption-specific" primers AD62R and AD63F and the "intact-specific" primers 8R and 3F. The inverted triangle represents the intron. K, *Kpn*I restriction sites. Note the loss of the middle *Kpn*I site from the disruption allele. (B) Confirmation of *upr-1*^{Ad} disruption by Southern analysis. Genomic DNA of the Adiopodoumé strain (430), of the three primary transformants (9, 21, and 24), and of four of their hygromycin-resistant *mat A* homokaryotic progeny (9-20, 21-58, 24-94, and 24-110) was digested with *Kpn*I and probed with the *upr-1*^{Ad} allele obtained by PCR amplification with the primers 1F and 12R. Note that the 2812- and >3460-bp fragments resulting, respectively, from digestion at the right and middle and the middle and left *Kpn*I sites of the intact *upr-1*^{Ad} allele are replaced in the disruption progeny strains by a single fragment of ~6 kbp due to loss of the middle site from the disrupted allele. A 143-bp fragment expected from the N-terminal end of *upr-1*^{Ad} is not visible in this blot. Note also that the primary transformants contain both the intact and the disrupted allele as expected for heterokaryons. Although the disruption band is not seen as prominently in the DNA from no. 24, it is seen in its hygromycin-resistant *mat A* progeny 24-94 and 24-110.

Although the 10 Srp$^+$ *a* progeny could, in principle, have arisen by crossover between *Srp* and *mat*, the absence of the complementary Srp$^-$ *A* progeny led us to postulate that they might not be products of crossover but might reflect an extreme version of the Srp$^{+/-}$ phenotype. That is, *Srp* might also be incompletely penetrant.

To confirm the variable expressivity and incomplete penetrance of *Srp*, we tested *Srp*-homozygous crosses for the production of non-Srp$^-$ progeny. The cross T-430-Hyg^r *a* × Adiopodoumé *A* is infertile (ANDERSON *et al.* 2001; BHAT *et al.* 2003), but crosses of the *Srp a* strains T-430-Hyg^r *a*, 82-46 *a*, and 82-51 *a* with the *Srp A* strains 117-11 *A*, 117-44 *A*, and 117-66 *A* were fertile (see MATERIALS AND METHODS for strain descriptions). Five of 30 and 10 of 18 progeny examined, respectively, from crosses involving T-430-Hyg^r *a*, or crosses of 117-11 *A* with 82-46 *a* or 82-51 *a*, showed the Srp$^{+/-}$ or Srp$^+$ phenotypes (Table 2). Confirmation of *Srp*'s incomplete penetrance signaled that the Srp$^+$ segregants from a *Srp* × *Srp*$^+$ cross need not necessarily be of the *Srp*$^+$ genotype and cautioned us to use only the Srp$^-$ segregants (whose genotype is necessarily *Srp*) for mapping *Srp*.

The non-Srp$^-$ phenotypes of *Srp* strains were reversible through crosses. Strains 117-25 *A* and 82-51-6 *a* are, respectively, Srp$^+$ and Srp$^{+/-}$, but derive their LG IL region around *mat* from the Adiopodoumé background (data not shown; see MATERIALS AND METHODS for strain descriptions). Two of the 20 progeny tested from 117-25 *A* × 82-51-6 *a* showed reversion to Srp$^-$ (Table 2). Seven other progeny that inherited the *mat* locus from the Srp$^{+/-}$ parent showed a further conversion to Srp$^+$.

Genetic localization of *Srp*: We performed crosses between the strains *arg-1 A* or *arg-3 A* and the four *leu-3 Srp a* strains 398 *a*, 475 *a*, 514 *a*, and 603 *a* (see MATERIALS AND METHODS) and between *leu-3 A* and the four *Srp arg-3 a* strains 33 *a*, 82 *a*, 112 *a*, and 114 *a* (see MATERIALS AND METHODS). Most progeny from these crosses are auxotrophic for either leucine or arginine, but prototrophs (designated, respectively, as classes 1 and 2 from the two sets of crosses) are produced by crossover between *leu-3* and *arg-1* (or *arg-3*), which are, respectively, distal and proximal to *mat*. Since *Srp* and *mat* were closely linked, the auxotrophic markers also bracketed *Srp*, and the prototrophs were enriched for crossovers between *Srp* and *mat*. Depending on whether *Srp* is proximal or distal to *mat*, crossover between them yields class 1 and class 2 prototrophs that are, respectively, Srp$^-$ *A* and Srp$^+$ *a* (Figure 1A) or Srp$^+$ *a* and Srp$^-$ *A*. Of 81 class 2 prototrophs tested, 80 were parental types (42 Srp$^+$ *A* + 38 Srp$^-$ *a*) and one, no. 87, was the crossover type, Srp$^+$ *a* (data not shown). This was consistent with *Srp* being proximal to *mat*, but also with the alternative model that the prototrophy of no. 87 resulted from a crossover outside *mat-Srp* and that its Srp$^+$ phenotype was due to the incomplete penetrance of *Srp*.

Of 177 class 1 prototrophs (83 *mat a* and 94 *mat A*), 2 (nos. 117 and 141) were Srp$^-$ *A* (data not shown), as would be expected only if *Srp* was proximal to *mat*. The

TABLE 2

Phenotypes of progeny from *Srp*-homozygous crosses

Cross	Segregants examined	Phenotype					
		Srp$^-$		Srp$^{+/-}$		Srp$^+$	
		A	*a*	*A*	*a*	*A*	*a*
117-11 *A* (Srp$^-$) × T-430 Hyg^r *a*	19	9	8	2	0	0	0
117-44 *A* (Srp$^-$) × T-430 Hyg^r *a*	5	2	1	0	0	2	0
117-66 *A* (Srp$^-$) × T-430 Hyg^r *a*	6	2	3	1	0	0	0
117-11 *A* (Srp$^-$) × 82-46 *a* (Srp$^-$)	8	1	2	1	1	2	1
117-11 *A* (Srp$^-$) × 82-51 *a* (Srp$^-$)	10	0	5	0	1	4	0
117-25 *A* (Srp$^+$) × 82-51-6 *a* (Srp$^{+/-}$)	20	1	1	0	0	11	7

remaining 92 *mat A* class 1 prototrophs were Srp$^+$ and presumably resulted from crossovers proximal to *Srp*. Of the 83 *mat a* class 1 prototrophs, 40 were Srp$^+$ (data not shown) and were attributed to incomplete penetrance; thus penetrance can occasionally be as low as ~52%.

The genetic interval to which *Srp* maps is bounded distally by the more proximal of the two crossovers that produced the class 1 Srp$^-$ *A* prototrophs 117 and 141 and proximally by the most distal of the 38 crossovers that produced the class 2 Srp$^-$ *a* prototrophs (*i.e.*, crossovers in III). The crossovers were mapped with respect to molecular polymorphisms between Adiopodoumé and OR in the *mat*-proximal region of LG IL (Figure 1B and supplemental Table 1). Figure 1C summarizes this analysis for the crossovers that generated no. 117 and the class 2 Srp$^-$ *a* prototroph no. 51. The remaining 37 class 2 crossovers were proximal to that in no. 51 and the other class 1 crossover was distal to that in no. 117 (data not shown). *Srp* was localized to the ~34-kbp interval between the SNPs P2.1 and P8.2. Consistent with this localization, prototroph no. 87 was found to have resulted from a crossover between *mat* and P1 (data not shown).

***Srp* is identical with *upr-1*^{Ad}:** The ~34-kbp *Srp* interval contains 10 genes (or hypothetical genes) designated (proximal to distal) NCU01942.1–NCU01951.1 (http://www.broad.mit.edu/cgi-bin/annotation/fungi/neurospora_crassa_7). The sequence of the corresponding interval from the Adiopodoumé strain (accession no. DQ 387872) revealed at least one nonsynonymous difference between the Adiopodoumé and the OR alleles of five genes (NCU01942.1, NCU01946.1, NCU01947.1, NCU01950.1, and NCU01951.1; supplemental Table 3). The largest candidate gene, NCU01951.1, also known as *upr-1*, had 118 nucleotide differences between its Adiopodoumé and OR alleles (*upr-1*^{Ad} and *upr-1*^{ORA}), including 66 nonsynonymous changes. Crosses homozygous for RIP-induced mutants of *upr-1* are RIP competent (Tamuli *et al.* 2006), but this did not disqualify *upr-1*^{Ad} from being *Srp*.

If *Srp* was identical with *upr-1*^{Ad}, disruption of *upr-1*^{Ad} should abolish the Srp$^-$ phenotype. We used PCR to amplify an ~6-kbp DNA fragment that contained the bacterial *hph* gene for resistance to hygromycin flanked by 1410 and 1313 bp of sequences from, respectively, the 5′ and 3′ of *upr-1*^{Ad} (Figure 2, also see materials and methods). The amplified DNA was transformed into the Adiopodoumé strain by electroporation and transformants were selected on hygromycin medium. In *N. crassa*, transforming DNA can integrate either by nonhomologous end joining into an ectopic site or, less frequently, by homologous integration. We identified three transformants (nos. 9, 21, and 24) in which integration had occurred by homologous recombination, thereby disrupting the *upr-1*^{Ad} allele in the transformed nucleus (Figure 2). The transformants were heterokaryons; that is, they also contained additional untransformed nuclei. The heterokaryons were crossed with OR *a*, and hygromycin-resistant *mat A* progeny from these crosses represented homokaryons that contained the disrupted allele, whereas the hygromycin-sensitive *mat A* progeny were homokaryons with the intact *upr-1*^{Ad} allele. All segregants that inherited the disrupted *upr-1*^{Ad} allele were Srp$^+$, whereas the Srp$^-$ or Srp$^{+/-}$ phenotypes were seen only among segregants that inherited the intact *upr-1*^{Ad} allele (Figure 3). Since disruption of *upr-1*^{Ad} abolished the Srp$^-$ and Srp$^{+/-}$ phenotypes, we could conclude that *Srp* is identical with *upr-1*^{Ad}.

The *mus-26* gene encodes a regulatory subunit of Polζ (Sakai *et al.* 2003). Homozygous *mus-26* mutant crosses are not ordinarily RIP defective (Tamuli *et al.* 2006). We found that RIP was suppressed in the cross *Srp*; *mus-26* (84) *a* × *mus-26* (27); *Dp(erg-3) A* (frequency of *erg-3* progeny <0.5). Therefore suppression of RIP by *upr-1*^{Ad} is independent of the regulatory subunit.

A difference in dominant suppression of RIP by *Srp* and that by large chromosome segment duplications: The heterokaryon [(*leu-3 Srp; Sad-2 a*) + (*erg-3; his-1 a*)] was isolated on the basis of the complementation between its auxotrophic components and used to make

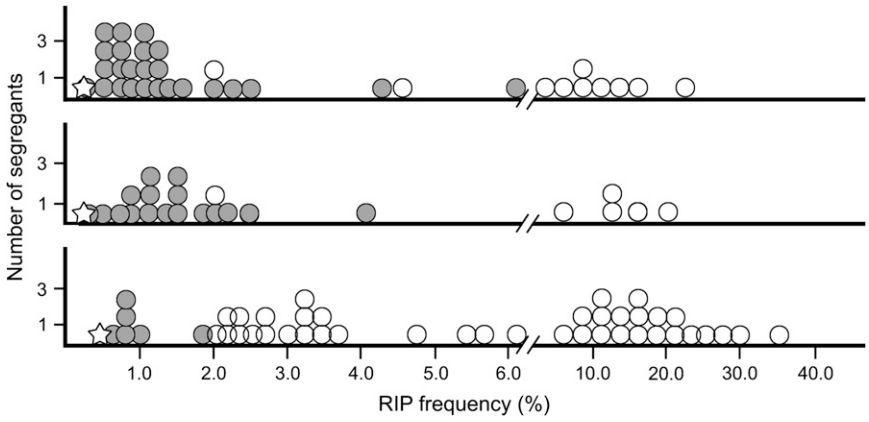

FIGURE 3.—Frequency of RIP-induced *erg-3* mutant progeny from crosses of *Dp(erg-3) a* with the primary transformants 9, 21, and 24 and with the hygromycin-sensitive (shaded circles) and hygromycin-resistant (open circles) *mat A* homokaryotic progeny. In each section the primary transformant is indicated by the star (nos. 9, 21, and 24, respectively, in the bottom, middle, and top sections) and the circles represent its progeny homokaryons from crosses with OR *a*. Note that all segregants homokaryotic for the disrupted *upr-1*^{Ad} allele (open symbols) were Srp^{+} (*erg-3* frequency >1%) whereas the Srp^{-} or Srp$^{+/-}$ phenotypes (*erg-3* frequencies <0.5% and 0.5–1.0%, respectively) were seen only among the heterokaryotic primary transformants and the homokaryotic segregants that inherited the intact *upr-1*^{Ad} allele (shaded symbols). Note also that several segregants with the intact *upr-1*^{Ad} allele show the Srp^{+} phenotype, thereby demonstrating the incomplete penetrance of *Srp*.

crosses with the strain *R; Sad-2; Dp(dow) A*. Mating of *R; Sad-2; Dp(dow) A* with the *leu-3 Srp; Sad-2 a* component is nonproductive due to homozygosity for *Sad-2* and crosses of *R; Sad-2; Dp(dow) A* with adventitiously formed *erg-3; his-1 a* homokaryons also are nonproductive because both *R* and *erg-3* confer female sterility. However, the heterokaryon is female fertile and therefore progeny can be obtained from the mating between its *erg-3; his-1 a* component and *R; Sad-2; Dp(dow) A*. We examined 120 erg^{+} progeny from this cross but none were RIP-induced *dow* mutants. In contrast, the control *Sad-2; Dp(dow) A* × *erg-3 a* crosses yielded RIP-induced *dow* mutants among the erg^{+} progeny at frequencies typically >8%. These results suggested that the Srp^{-} phenotype of *Srp* is able to spread to the *Srp*$^{+}$ × *Srp*$^{+}$ asci of the mosaic perithecia that form in [*Srp* + *Srp*$^{+}$] × *Srp*$^{+}$ crosses. (Strictly speaking, since RIP actually occurs in the premeiotic dikaryon that precedes karyogamy, it is more accurate to say the Srp^{-} phenotype can spread into the premeiotic dikaryons that go on to generate productive karyogamies. The term "asci" is used here merely as a matter of convenience.) This result is consistent with the idea that RIP suppression is due to the synthesis of a diffusible, *upr-1*^{Ad}-encoded, suppressor Pol-ζ subunit.

The ~400-kbp duplication *Dp(IBj5)* is an effective dominant suppressor of RIP (VYAS *et al.* 2006). We constructed the heterokaryon [(*Dp(IBj5); trp-1; Sad-2 a*) + (*erg-3; his-1 a*)] and crossed it with the *R; Sad-2; Dp(dow) A* strain. Again, progeny are generated only from the mating of *R; Sad-2; Dp(dow) A* with the *erg-3; his-1 a* component of the heterokaryon. Seven of the 70 erg^{+} progeny examined were RIP-induced *dow* mutants. Thus, although the RIP suppressor phenotype of *Dp(IBj5)* is dominant within an ascus, in a mosaic perithecium it did not affect asci that do not contain the *Dp*. That is, the presumed titration of the RIP machinery requires the presence of the *Dp* in the ascus.

Conclusions: We have shown that *Srp*, the dominant RIP suppressor of the wild-isolated Adiopodoumé strain, is identical with its *upr-1* allele (*upr-1*^{Ad}). Although Pol-ζ and the other translesion polymerases (Pol-η, Pol-ι, Pol-κ, and Rev1) are dispensable for RIP (TAMULI *et al.* 2006), a RIP-suppressive Pol-ζ catalytic subunit encoded by *upr-1*^{Ad} might be well placed to interfere with the assembly of other factors essential for RIP in Neurospora. RIP suppression by *upr-1*^{Ad} did not require the Pol-ζ regulatory subunit encoded by *mus-26*. Recently, ACHARYA *et al.* (2006) established that the *Saccharomyces cerevisiae* Rev1 protein binds separately to the catalytic and regulatory subunits of Pol-ζ (*i.e.*, Rev3 and Rev7, respectively), suggesting the possibility of separate Rev1–Rev3 and Rev1–Rev7 complexes *in vivo*. Since Rev1 has been suggested to play a role in assembling Pol-η, Pol-ι, Pol-κ, and Pol-ζ at the replication fork in mouse and human cells (PRAKASH *et al.* 2005), it is possible that Rev1 could play a role in assembling the Pol-ζ catalytic subunit at replication forks separately from the regulatory subunit encoded by *mus-26*. Presumably, the association of a complex without the *mus-26*-encoded regulatory subunit would not be capable of polymerization but may be capable of inhibiting the association of other factors essential for RIP. The Neurospora Rev1 homolog, encoded by the *mus-42* gene, is located 358 kbp distal to *upr-1* on LG IL, which is why we have not yet obtained the rare *mus-42 upr-1*^{Ad} crossover type that would enable us to put this idea to a genetic test.

Both *upr-1*^{ORA} and *upr-1*^{Ad} possess out-of-frame ATG codons that initiate short open reading frames upstream of the main *upr-1* ORF (TAMULI *et al.* 2006). Similar upstream out-of-frame ORFs (uORFs) are conserved in the yeast and human homologs of *upr-1* (GIBBS *et al.* 1998). Since uORFs reduce the translational efficiency of the main ORF (MORRIS and GEBALLE 2000), it has been suggested that they serve to keep cellular levels of Pol-ζ very low (LAWRENCE 2002). It is

tempting to speculate that the uORFs might contribute to the variable expressivity and incomplete penetrance of *Srp*. That is, *Srp* strains that are otherwise genetically identical might differ in uORF expression and this might generate differences in the amount of the suppressor Pol-ζ translated from the main ORF, thus giving rise to the variable expressivity and incomplete penetrance. That is, the variable expressivity and incomplete penetrance might reflect consequences of differences in uORF activity. If the effective cellular levels of the suppressor subunit decrease to below the threshold required to suppress RIP, then a *upr-1*^{Ad} strain would mimic the *upr-1* null mutant. Incomplete penetrance of the Srp phenotype allows us to reconcile our observations of RIP suppression with the observations of RIP in other *Srp*-homozygous crosses (Anderson *et al.* 2001 and this work). Temporary loss of suppressor function and its partial or complete recovery in subsequent generations might also involve an epigenetic change reminiscent of paramutation in maize or metastable epialleles in other systems (Hollick *et al.* 1997; Poirier *et al.* 2002; Rakyan *et al.* 2002; Chandler and Stam 2004; Della Vedova and Cone 2004) or it can be due to stochastic events during development (Lalucque and Silar 2004). Although we have no experimental evidence as yet, it is conceivable that *Srp* strains are effectively heterokaryotic and express *upr-1*^{Ad} in only a subset of nuclei and epigenetically switch it off in other nuclei. Among the progeny from crosses parented by such a heterokaryon, those inheriting the epigenetically silenced *upr-1*^{Ad} gene would be phenotypically Srp^{+}. The range in *Srp*'s penetrance seen in this work (52–100%) might reflect the range in the proportion of nuclei in which *upr-1*^{Ad} is silenced. In sum, the rapidly evolving *upr-1* gene (Tamuli *et al.* 2006) might represent the key to solve the riddle of *Tad*'s survival in the Adiopodoumé strain, wrapped in the mystery of incompletely penetrant dominant RIP suppressors, inside the enigma of RIP regulation.

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