A Methylated Neurospora 5S rRNA Pseudogene Contains a Transposable Element Inactivated by Repeat-Induced Point Mutation

Brian S. Margolin,* Phillip W. Garrett-Engele,* Judith N. Stevens,† Deborah Y. Fritz,* Carrie Garrett-Engele,* Robert L. Metzenberg† and Eric U. Selker*,†

**Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403 and †Department of Biomolecular Chemistry, University of Wisconsin Medical School, Madison, Wisconsin 53706*

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ABSTRACT

In an analysis of 22 of the roughly 100 dispersed 5S rRNA genes in *Neurospora crassa*, a methylated 5S rRNA pseudogene, Ψ_{63}, was identified. We characterized the Ψ_{63} region to better understand the control and function of DNA methylation. The 120-bp 5S rRNA-like region of Ψ_{63} is interrupted by a 1.9-kb insertion that has characteristics of sequences that have been modified by repeat-induced point mutation (RIP). We found sequences related to this insertion in wild-type strains of *N. crassa* and other Neurospora species. Most showed evidence of RIP; but one, isolated from the *N. crassa* host of Ψ_{63}, showed no evidence of RIP. A deletion from near the center of this sequence apparently rendered it incapable of participating in RIP with the related full-length copies. The Ψ_{63} insertion and the related sequences have features of transposons and are related to the Fot1 class of fungal transposable elements. Apparently Ψ_{63} was generated by insertion of a previously unrecognized Neurospora transposable element into a 5S rRNA gene, followed by RIP. We name the resulting inactivated Neurospora transposon *Punt*^{RIP1} and the related sequence showing no evidence of RIP, but harboring a deletion that presumably rendered it defective for transposition, *dPunt*.

LIFE as we know it depends on the mutability and plasticity of genomes. A dynamic genome fosters diversity within a species, increasing the chances of survival through environmental changes and, at the same time, facilitates speciation. Many processes capable of changing a genome have developed, the most dramatic example being the evolution of sex. Although some level of genome plasticity is thought to be beneficial to a species, too high a level may be detrimental. Transposable elements can exert a huge dynamic force on a genome by disrupting genes, altering the expression of nearby genes (Cambareri *et al.* 1996; Martienssen 1996), leaving behind mutations after excision and facilitating gross chromosomal rearrangements. Considering the potential negative effects of transposable elements, it is not surprising that mechanisms have evolved to control them. It has been proposed that plants, fungi, and mammals all use cytosine methylation to control transposable elements (Fedoroff *et al.* 1995; Selker 1997; Yoder *et al.* 1997).

Active transposable elements are rare in the filamentous fungus *Neurospora crassa* (Kinsey and Helber 1989) and this may reflect the operation of a process that is tailor-made to counter transposable elements. Repeat-induced point mutation (RIP) causes numerous G:C to A:T point mutations in duplicated sequences during the *N. crassa* sexual cycle (Selker 1990). Indeed, the only previously reported active transposable element in Neurospora, *Tad*, is readily inactivated by RIP (Kinsey *et al.* 1994). Other relics of transposable elements that have been found in Neurospora also show hallmarks of RIP (E. Cambareri, personal communication; Schechtman 1990).

Sequences that have been modified by RIP are usually, although not invariably, signals for DNA methylation (Selker *et al.* 1987a; Selker and Garrett 1988; Singer *et al.* 1995). In some cases, sequences that have been mutated by RIP can still be transcribed, although they may produce abnormal-length transcripts (Rountree and Selker 1997). An abundance of unnecessary transcription may be detrimental to Neurospora, either because of the generation of harmful aberrant transcripts (Cogoni *et al.* 1996), or simply because the transcriptional machinery is taxed. RIP-associated methylation can inhibit the transcription of genes mutated by RIP (Irelan and Selker 1997; Rountree and Selker 1997). It seems plausible therefore that a function of methylation in Neurospora is to quiet the transcription of RIP-modified sequences (Selker 1997).

Approximately 1.5% of the cytosines in the 4×10^7 base pair *N. crassa* genome are methylated (Foss *et al.* 1993). To date, most of the methylation in Neurospora has been found in small, heavily methylated patches (Selker *et al.* 1987b; Selker 1993; Miao *et al.* 1994; E. U. Selker, N. Tauntas, B. S. Margolin, S. H. Cross and A. P. Bird, unpublished results) suggesting that

Corresponding author: Eric U. Selker, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.
E-mail: selker@molbio.uoregon.edu

there are roughly 500 heavily methylated gene-sized regions in the genome. Nevertheless, only four methylated regions of wild-type strains have been characterized. One is the ζ-η region, which resulted from RIP operating on an ancestral tandem duplication of a 0.8-kb segment including a 5S rRNA gene (Selker and Stevens 1985, 1987; Grayburn and Selker 1989). The second is the tandemly repeated rDNA found in the nucleolus (Russell *et al.* 1985). The third example is a region around the 5′ end of a copy of the LINE-like transposable element *Tad* inserted upstream of the *am* gene (Kinsey *et al.* 1994; Cambareri *et al.* 1996). The fourth methylated region, Ψ_{63} (Metzenberg *et al.* 1985; Foss *et al.* 1993; Miao *et al.* 1994), is the focus of this study. We report here that Ψ_{63} resulted from insertion of a transposable element into a 5S rRNA gene. The sequence composition of the transposable element and the 5S rRNA pseudogene suggests that the region was subjected to RIP after the insertion event.

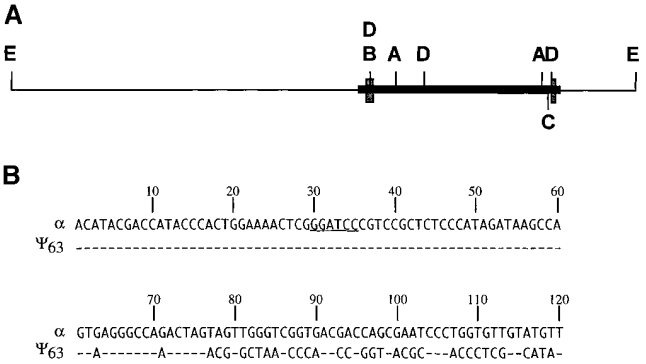

Figure 1.—(A) Map of pJS63 6.4-kb insert. The sequenced region is indicated by a bold line. *Eco*RI (E), *Bam*HI (B), *Ava*II (A), *Cla*I (C), and *Sau*3AI/*Dpn*II (D) sites are indicated. Map information for *Ava*II and *Dpn*II is complete only for the sequenced region. The interrupted 5S pseudogene (Ψ_{63}) is shown by gray rectangles. (B) Sequence of Ψ_{63} aligned with an α-type 5S rRNA gene. The sequence of the α-type 5S rRNA gene is shown on the top; differences in the Ψ_{63} sequence are shown below. Positions where the Ψ_{63} sequence is identical to the α-type gene are indicated by dashes. The *Bam*HI site is underlined.

MATERIALS AND METHODS

Strains, culturing of *N. crassa*, DNA isolation, and Southern hybridization: *N. crassa* was cultured by standard methods (Davis and De Serres 1970). Strains were obtained from the Fungal Genetics Stock Center (FGSC) or from the collection of R.L.M. The following strains were used: Oak Ridge (FGSC #2489), Abbott 4A (FGSC #1757), Abbott 12A (FGSC #351), Mauriceville (RLM 22-11), Adiopodoumé (FGSC #430), and *N. sitophila* (FGSC #2216). DNA isolation and Southern hybridization were performed as previously described (Foss *et al.* 1993) with the following modifications. After hybridization with the radioactive probe, Southern blots were washed several times with 50 mm NaCl, 20 mm sodium phosphate (pH 6.8), 1 mm EDTA, 0.1% sodium dodecyl sulfate. Washes were performed at room temperature to retain imperfect hybrids or at 65° when imperfect hybrids were not desired.

Plasmids and sequencing: Plasmid pJS63 (Metzenberg *et al.* 1985) consists of a 6.4-kb *Eco*RI Ψ_{63} fragment cloned in pBR322 (Figure 1A). Restriction fragments of plasmids pJS63 and pDY1 were subcloned by standard procedures (Sambrook *et al.* 1989) into plasmid pBluescript SK(+) (Stratagene, La Jolla, CA) and sequenced with an ABI 377 automated sequencer at The University of Oregon Biotech Facility using standard sequencing primers (T3 and T7).

Library screening: Approximately 25,000 plaques from an *N. crassa* lambda genomic library, obtained from FGSC and generated using a partial digest with *Sau*3AI, were probed with the 1.2-kb *Sau*3AI/*Cla*I fragment from pJS63 (Metzenberg *et al.* 1985) (see Figure 1A). DNA from hybridizing plaques was isolated as previously described (Sambrook *et al.* 1989) and characterized by digestion with various restriction enzymes and hybridization to a pJS63 probe. A 6.1-kb *Eco*RI fragment from lambda clone 5 that was detected by probing with pJS63 was subcloned into pBluescript SK(+), creating pDY1.

Generation of RIP indices: A total of 235 *N. crassa* sequences from the GenBank database were assembled into a concatenated sequence of 623,143 nt. Duplicate, rDNA, mitochondrial, and known RIP-modified sequences were not included. The number of TpA, ApT, CpA, TpG, ApC, and GpT dinucleotides in 500-nt windows was tabulated for the concatenated sequence using Window (Genetics Computer Group 1994). After each tabulation the 500-nt window was shifted 25 nt. The RIP indices for each data point and the means and standard deviations were calculated.

Isolation of *Punt* homologues: *Punt* sequences were amplified from genomic DNA by PCR in 50-μl reactions containing the following: 1× Promega *Taq* polymerase buffer, 1.5 mm $MgCl_2$, 200 μm of each nucleotide triphosphate (dATP, dGTP, dCTP, and TTP), 175 ng each primer, and 1 unit *Taq* polymerase (Promega, Madison, WI). The sequences of primers were 5′GGAATTCAAGAAGATTCTTRGGCGGGGGA3′ and 5′CGGGATCCACGTCGCGACCCYAACCCCGGT3′. A *Bam*HI site was included on the 5′ end of one primer and an *Eco*RI site was included on the 5′ end of the other primer to facilitate subsequent cloning. Samples were initially heated to 94° in a Hybaid Omnigene thermocycler for 4 min and then subjected to 30 cycles of the following regime with the machine set to tube control: 2 sec at 94°, 10 sec at 50°, 20 sec at 72°. The samples were finally heated to 72° for 5 min. Following amplification, the PCR reactions were digested with *Bam*HI and *Eco*RI, fractionated by gel electrophoresis, gel purified, and cloned into pBluescript SK(+) (Stratagene).

RESULTS

Ψ_{63}, a methylated 5S rRNA pseudogene: Discovery of the methylated ζ-η region in some strains of *N. crassa* (Selker and Stevens 1987) motivated a survey of other 5S rRNA genes and pseudogenes. In an initial survey, none of seven 5S rRNA genes and one 5S rRNA pseudogene examined showed methylation at the *Bam*HI site (position 30 in the 5S rRNA region) where the ζ and η pseudogenes show heavy methylation (Selker *et al.* 1985). We were therefore surprised and interested when we found evidence of methylation in a new 5S rRNA pseudogene, Ψ_{63}. This pseudogene is contained in a 6.4-kb *Eco*RI fragment with a single *Bam*HI site (2.7 kb from one end; Figure 1A) located in the 5S rRNA-like sequence. DNA isolated from the Oak Ridge wild-

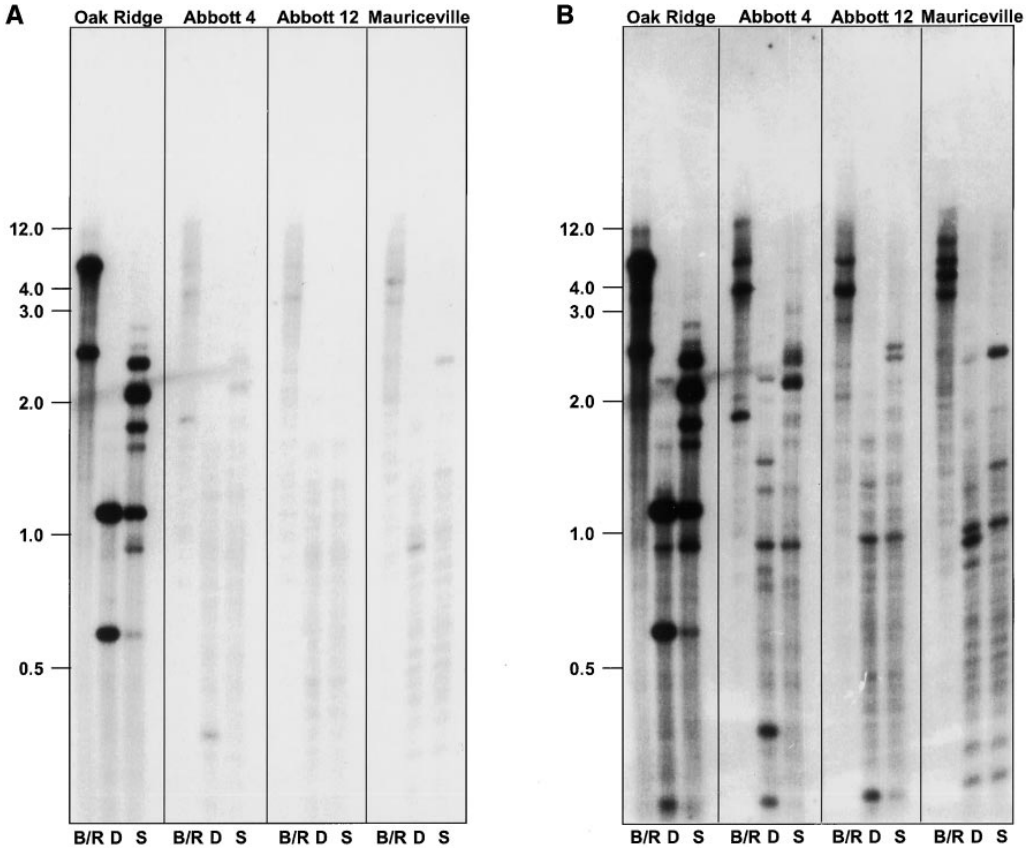

Figure 2.—Southern hybridizations of *Punt* to genomic DNA of various wild-type *N. crassa* strains. Genomic DNA from the indicated strains was digested with *Bam*HI and *Eco*RI (B/R), *Dpn*II (D), or *Sau*3-AI (S) and probed with the *Ava*II fragment of *Punt*^{RIP1}. The blot was initially washed at low stringency (B), exposed to film and subsequently washed at high stringency (A). The positions of size markers (kb) are indicated.

type strain is resistant to cleavage by *Bam*HI at this site due to DNA methylation, as with the ζ and η pseudogenes (Foss *et al.* 1993). DNA sequencing in both directions from the Ψ_{63} *Bam*HI site revealed a 76-bp segment nearly identical to the first 76 bp of the most common 5S rRNA gene type (Selker *et al.* 1981; Figure 1B). Ψ_{63} has only two point differences relative to an α-type sequence in this segment, both G to A transition mutations (positions 63 and 71). The nucleotide sequence immediately following position 76 shows no obvious similarities to 5S rRNA genes. It was unclear if this breakpoint was due to a deletion, translocation, inversion, or insertion.

To explore the possibility that a rearrangement had occurred relatively recently, we analyzed a number of progenitors of the Oak Ridge strain and other wild-type strains. Results of Southern hybridizations revealed that the Ψ_{63} *Bam*HI site is not methylated in all strains and revealed RFLPs suggestive of an ~1.8-kb insertion associated with the methylated Ψ_{63} allele (data not shown; Figures 2 and 3). Two progenitors of the Oak Ridge strain, Abbott 4A and Abbott 12a, and the Mauriceville wild-type strain showed no evidence of methylation, in contrast to several other progenitors. In principle, the methylation at the Ψ_{63} locus of the Oak Ridge strain could be due to a *trans*-acting factor (*i.e.*, not present in the Abbott and Mauriceville strains) or a *cis*-acting difference (*i.e.*, a methylation signal associated with the Oak Ridge Ψ_{63} allele). Results of two experiments led us to conclude that the difference is due to a *cis*-acting difference: (1) In a cross of Oak Ridge and Mauriceville strains, all progeny that inherited the Oak Ridge allele, but none that inherited the Mauriceville allele showed methylation (data not shown); (2) an ~3-kb segment of the Oak Ridge Ψ_{63} region served as a methylation signal in transformation experiments (Miao *et al.* 1994).

Characterization of putative insertion in Ψ_{63}: It

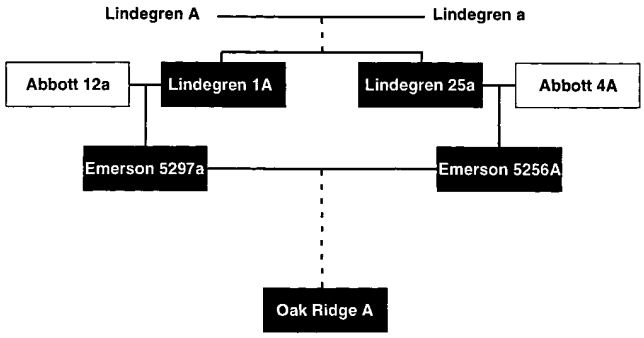

Figure 3.—Pedigree of Oak Ridge strains of *N. crassa* (Newmeyer *et al.* 1987). Strain names and mating type are shown. Strains that have been tested for the presence of Ψ_{63} are listed in boxes. A black box with white letters indicates that the strain contains Ψ_{63}; a white box with black letters indicates that the strain lacks Ψ_{63}. Dashed lines indicate that two or more intercrosses separate the strains.

seemed likely that the methylation of Ψ_{63} was related to the putative insertion event in the Oak Ridge allele and/or to the sequence differences near the breakpoint (Figure 1B). We therefore checked for these sequence features in the sequence present at the unmethylated locus (*Fsr63*) of Abbott 4A. An Abbott 4A genomic lambda library was prepared from *Bgl*II-digested genomic DNA and a potential homologue was identified by probing with the pJS63 3.7-kb *Bam*HI/*Eco*RI fragment, represented in both Oak Ridge and Abbott strains. The 3.7- and 0.5-kb *Bam*HI-*Eco*RI fragments from this clone were subcloned and sequenced from the *Bam*HI site. The sequence data indicated that the *Fsr63* homologue of Ψ_{63} from Abbott 4A is a perfect α-type 5S rRNA gene (Figure 1B). Both of the point differences in the homologous regions of the Abbott 4A and Oak Ridge alleles are G to A changes, suggesting that they may have been caused by RIP. Indeed, the two differences are at the dinucleotides CpA and CpT, preferred targets of RIP (Selker 1990).

The methylation of the Oak Ridge allele and the suggestion of RIP prompted us to examine the possibility that Ψ_{63} resulted from RIP operating upon a repeated element inserted into a 5S rRNA gene. To determine if the sequences downstream of the truncation point in Ψ_{63} are repeated, we used these sequences as probes for Southern hybridizations with genomic DNA from Oak Ridge, Abbott 4A, and other Neurospora wild-type strains. High stringency probings of genomic digests of Oak Ridge DNA showed only the bands expected from Ψ_{63} (Figure 2A; Foss *et al.* 1993). Both *Sau*3AI and *Dpn*II recognize the sequence GATC, but *Dpn*II cuts whether or not the cytosine is methylated, whereas *Sau*3AI only cuts when the cytosine is not methylated (Nelson *et al.* 1993). The *Dpn*II digest of Oak Ridge showed strongly hybridizing 0.6- and 1.2-kb bands consistent with the map of pJS63. Both bands were also seen in a *Sau*3AI digest of the same strain although they are lighter presumably due to methylation. Additional strong bands representing higher molecular weight fragments were also detected, consistent with other experiments indicating that Ψ_{63} is methylated (Foss *et al.* 1993; Miao *et al.* 1994). No additional bands indicative of repetitive DNA downstream of the truncation point were detected in this probing. This result ruled out the existence of perfect repeats but did not eliminate the possibility of divergent sequences, such as would be expected after the operation of RIP on a repeated sequence (Cambareri *et al.* 1989, 1991; Selker 1990; Kinsey *et al.* 1994). We therefore probed genomic digests with the *Ava*II fragment of pJS63 and washed the blots at low stringency (Figure 2B). DNA from each strain was digested with *Bam*HI plus *Eco*RI to look for the characteristic 2.7- and 6.4-kb bands of Ψ_{63}, as well as with *Sau*3AI or *Dpn*II to assay additional sites for methylation (Nelson *et al.* 1993). The probe showed multiple bands in all strains tested (Figure 2B). In addition to several strong bands matching the fragments detected in the high stringency probing, digests of Oak Ridge DNA revealed many weaker bands that were apparently lost by washing at the higher temperature. The presence of additional light bands in hybridizations with all four strains suggested that sequences related to Ψ_{63} are repeated in these strains, consistent with the idea that the pseudogene resulted from insertion of a repeated sequence. To determine if the truncation of the 5S rRNA gene at nucleotide 76 was indeed due to an insertion event, we sequenced about 2 kb beyond the breakpoint in search of the downstream end of the gene. The 3′ portion of the 5S rRNA gene was found 1874 bp beyond the breakpoint (Figure 4).

Evidence that the 5S rRNA gene is interrupted by a transposon: Analysis of the sequence data showed that the inserted DNA had several characteristics of a DNA-type transposon. The insert has imperfect terminal inverted repeats that are 45 bp long and 87% identical (Figure 4). In addition, at either end of the inserted DNA a CTA trinucleotide is found (shown in Figure 4 as TAG on the opposite DNA strand) suggestive of a 3-bp target-site duplication. The repeated nature of the inserted sequence and these structural characteristics suggest that the insert is a DNA-type transposon. We have named the insert *Punt*.

Evidence of RIP at the Ψ_{63} region: As mentioned above, the 5S rRNA-like region of Ψ_{63} includes two mutational differences. These differences, which are seen at sites favored by RIP, suggest that RIP may have operated in this region. To explore this possibility, we analyzed the sequence of the Ψ_{63} insert. It has an A+T content of 66%, which is abnormally high for Neurospora DNA. The average A+T content of Neurospora is 41% in coding regions, 51% in noncoding regions and 46% in the genome overall (Villa and Storck 1968). Sequences mutated by RIP are typically A+T rich (Cambareri *et al.* 1989; Singer *et al.* 1995) and show skewed dinucleotide frequencies because of the sequence preference of RIP (CpA > CpT > CpG > CpC). Approximately 64, 18, 13, and 5% of the changes occur at CpA, CpT, CpG, and CpC, respectively (Selker 1990). We use two formulas to assess the likelihood that sequences have been mutated by RIP.

The simplest formula that controls for differences in overall base composition is the ratio of TpA to ApT dinucleotides. High TpA to ApT ratios should indicate sequences that have higher than normal TpA levels for their respective nucleotide compositions. We calculated the TpA/ApT ratio for 235 published Neurospora sequences presumed not to have been exposed to RIP and for 10 sequences that were known to be products of RIP. The TpA/ApT ratio for the nonmutated sequences calculated in 500-bp windows was 0.66 ± 0.23. This value differs significantly from the values calculated for sequences known to have been at least moderately altered by RIP (Table 1). The only RIP-altered sequences that fell within the range of nonaltered sequences were those that, unlike most products of RIP, are only very

Figure 4.—Sequence of the Ψ_{63} region from an Oak Ridge *N. crassa* strain. The 5S rRNA pseudogene is indicated in white letters on a black background. The putative target site duplication is indicated by a solid line above it. The inverted terminal repeats are boxed and nucleotides that do not match are shown in lower case. The sequence is numbered from the first nucleotide of the 5S rRNA pseudogene.

lightly mutated and do not trigger *de novo* methylation (*am*^{RIP2}, *am*^{RIP3}, and *am*^{RIP4}; Singer *et al.* 1995; Table 1).

The second formula used to assess incidence of RIP compares the frequency of the most common target dinucleotides of RIP, CpA and TpG, with the frequencies of two other dinucleotides of the same composition, ApC and GpT. We calculated (CpA+TpG)/(ApC+GpT) values for the same published Neurospora sequences presumed not to have been exposed to RIP and the same 10 sequences that were known to be products of RIP. The (CpA+TpG)/(ApC+GpT) ratio was 1.21 ± 0.18 for these presumably unaltered sequences. This value differs significantly from the values calculated for sequences known to have been at least moderately altered by RIP (Table 1). Once again, the only RIP-altered sequences that fell within the range of nonaltered sequences were those that are only very lightly mutated and do not trigger *de novo* methylation (Table 1). The TpA/ApT and (CpA+TpG)/(ApC+GpT) ratios for the Ψ_{63} insert are 1.32 and 0.56, respectively, strongly suggesting that the insert was mutated by RIP. We therefore refer to it as *Punt*^{RIP1}.

Search for other copies of *Punt*: To learn more about *Punt*, and to gain insight into possible partners of *Punt*^{RIP1} in RIP, we looked for other copies of the transposon. A lambda genomic library was probed with the *Sau*3AI/*Cla*I pJS63 fragment, which is internal to *Punt*^{RIP1}. Restriction fragments hybridizing to a Ψ_{63} probe were subcloned from positive phage and sequenced on both strands generating a complete overlapping sequence (Figure 5). The new sequence, termed *dPunt*, is 75% identical to *Punt*^{RIP1}, and shows no similarity outside of the transposon. Alignments of the two sequences showed that *Punt*^{RIP1} is 759 bp longer than *dPunt* because of either an insertion into the *Punt*^{RIP1} sequence or a deletion from the *dPunt* sequence. The ends of the transposon were inferred by comparison to *Punt*^{RIP1}. Like *Punt*^{RIP1}, *dPunt* also has 45-bp imperfect inverted terminal repeats. Unlike *Punt*^{RIP1} however, one of the *dPunt* repeats has a 4-bp tandem duplication (Figure 5). The sequence is consistent with the possibility that a 2-bp (TA) or 3-bp (CTA) target-site duplication occurred on insertion of the transposon.

***dPunt* is not altered by RIP:** In contrast with *Punt*^{RIP1}, *dPunt* does not appear to have been altered by RIP. The TpA/ApT ratio (0.65), (CpA+TpG)/(ApC+GpT) ratio (1.29), and percentage A+T (49%) of *dPunt* are all well within the values for sequences that have not been altered by RIP. Furthermore, the differences between *dPunt* and *Punt*^{RIP1} are primarily GC to AT, comparing *dPunt* to *Punt*^{RIP1}, respectively, as expected if *Punt*^{RIP1}, but not *dPunt*, had been subjected to RIP (Figure 5; Table 2). These differences were primarily at the most common target dinucleotide of RIP, CpA, or its complement, TpG (Table 2). Thus the differences between *Punt*^{RIP1} and *dPunt* suggest that *dPunt* represents a copy of the transposon *Punt* rendered defective by deletion.

Characterization of *dPunt*: To assess the distribution, copy number, and methylation state of *dPunt*, an *Ava*II fragment of *dPunt* was used to probe the same Southern

TABLE 1

Base composition and RIP indices of various *N. crassa* sequences

Sequence	Percentage A+T (%)	TpA[a] / ApT	CpA+TpG[a] / ApC+GpT
Neurospora sequences[b]	46 ± 5	0.66 ± 0.23	1.21 ± 0.18
Punt^{RIP1} (nt 74–1947)	66	**1.32**	**0.56**
dPunt (nt 50–1171)	49	0.65	1.29
Wild-type sequences			
Mating type A (Glass *et al.* 1990)	50	0.54	1.33
θ (5S rRNA gene at ζ-η locus of Abbott 4A)	53	0.86	1.03
qa gene cluster (Geever *et al.* 1989)	49	0.64	1.26
am^{+} (Kinnaird and Fincham 1983)	46	0.68	1.15
RIP-modified sequences			
am^{RIP2} (Singer *et al.* 1995)[c]	47	0.76	1.12
am^{RIP3} (Singer *et al.* 1995)[c]	48	0.91	1.03
am^{RIP4} (Singer *et al.* 1995)[c]	48	0.92	1.05
am^{RIP5} (Singer *et al.* 1995)	49	**1.06**	**0.98**
am^{RIP6} (Singer *et al.* 1995)	49	**1.02**	**1.00**
am^{RIP7} (Singer *et al.* 1995)	49	**1.13**	**0.93**
am^{RIP8} (Singer *et al.* 1995)	52	**1.18**	**0.91**
pEC25 (Cambareri *et al.* 1989)	49	**1.29**	**0.75**
pBJ20 (Cambareri *et al.* 1989)	53	**1.35**	**0.87**
ζ-η (Selker *et al.* 1993)	67	**1.29**	**0.47**

[a] Boldface type indicates values that differ significantly from set of Neurospora sequences not modified by RIP.

[b] Set of Neurospora sequences not modified by RIP. The means and standard deviations for the set calculated in 500-bp windows are listed.

[c] Lightly mutated and not methylated *de novo*.

blot that had been probed with *Punt*^{RIP1}. The *Ava*II fragment used for the probe corresponds to the *Punt*^{RIP1} fragment used for Figure 2 but is smaller because of the deletion in the *dPunt* sequence. Unlike the situation with *Punt*^{RIP1}, the *dPunt* probe hybridized strongly to bands in all strains tested (Figure 6). All the *Bam*HI/*Eco*RI (B/R) digests showed a single band at approximately 4.5 kb. As there are no *Bam*HI or *Eco*RI sites within *dPunt*, these bands represent digestion in the flanking sequences. The *Dpn*II digests of all four strains show a band expected from ~200-bp internal fragments of *dPunt*. In addition, each shows a band corresponding to either a 1.0- or 1.3-kb *Dpn*II fragment, which results from digestion at nucleotide 800 within the *dPunt* sequence and a site outside of the sequenced region. The presence of only one junction fragment for each strain is consistent with the conclusion that there is only a single copy of *dPunt* present in all four strains. The difference in size of the junction fragments of the strains may reflect a polymorphism present in the flanking sequence of the strains. Alternatively, *dPunt* may occupy different locations in the genomes of the Abbott 4A and Mauriceville strains relative to the Oak Ridge strains. The bands detected in the *Sau*3AI and *Dpn*II digests are identical for each of the four strains, suggesting that *dPunt* is not methylated in any of these strains. This is consistent with the interpretation that *dPunt* was not altered by RIP.

***Punt* homologues in other fungi:** We carried out computer analyses to further explore the possibility that *dPunt* represents a transposon that was rendered defective by a deletion of its central region. An open reading frame, which could encode a 267-amino-acid polypeptide, spans the putative deletion. A search of the GenBank and EMBL databases for homologous proteins using the putative 267-amino-acid *dPunt* polypeptide identified eight similar sequences, all from filamentous fungi: Fot1 from *Fusarium oxysporum* (Daboussi *et al.* 1992), Pot2 and Pot3 from *Magnaporthe grisea* (Kachroo *et al.* 1994; Farman *et al.* 1996), Flipper from *Botryotinia fuckeliana* (*Botrytis cinerea*; Levis *et al.* 1997), Tan1 from *Aspergillus niger* (Nyyssonen *et al.* 1996), Fcc1 from *Cochliobolus carbonum* (Panaccione *et al.* 1996), Nht1 from *Nectria haematococca* (Enkerli *et al.* 1997), and a sequence from *Emericella nidulans* (*Aspergillus nidulans*; GenBank accession no. 1870209; Figure 7). All of these sequences are related to the Fot1 family of transposable elements, which are thought to transpose through a DNA intermediate (Daboussi 1996). *dPunt* is 32% identical to Fot1 at the amino acid sequence level, overall. In the region upstream of the deletion *dPunt* is 43% identical to Fot1; beyond the deletion *dPunt* is 30% identical to Fot1. The decreased similarity between *dPunt* and Fot1 in the downstream region is mirrored by low levels of similarity between Fot1 and the other transposons of the Fot1 family in this region.

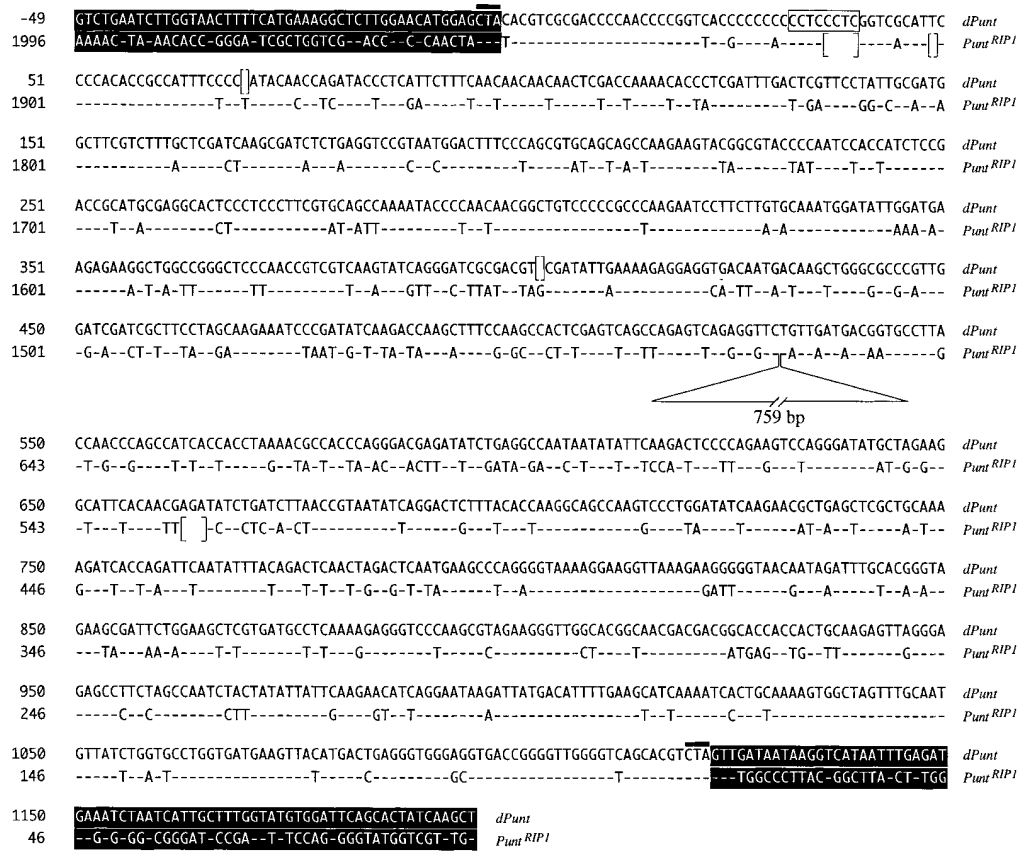

Figure 5.—Sequence comparison of *dPunt* and *Punt*^{RIP1}. Positions of identity are indicated by dashes in the *Punt*^{RIP1} sequence below the sequence of *dPunt*. Small gaps are indicated with square brackets; the 759-bp deletion of *dPunt* relative to *Punt*^{RIP1} is shown as an expansion at nucleotide 1481. Sequences flanking the transposons are indicated as white letters on a black background. A 4-bp duplication in one of the *dPunt* inverted repeats is shown boxed. The *Punt*^{RIP1} sequence is numbered starting from the first nucleotide of the 5S rRNA pseudogene. Note that the *Punt*^{RIP1} sequence is shown in reverse orientation relative to Figure 4. The *dPunt* sequence is numbered starting from the left boundary of the transposon.

In addition to the sequence similarity, *Punt* has other structural similarities to the Fot1 transposons. All are bounded by inverted terminal repeats. Additionally, these elements each apparently duplicate a short target sequence upon integration into a new genomic site. As noted above, both copies of *Punt* for which full sequence information is available appear to be integrated at a duplicated CTA site.

Analysis of other copies of *Punt* in Neurospora species: Results of Southern hybridizations suggested that there are multiple copies of sequences related to *Punt*^{RIP1} in the Oak Ridge genome but hybridizations with *dPunt*

TABLE 2

Summary of differences between *Punt* sequences and *dPunt*

	$\frac{C}{G}\rightarrow\frac{T}{A}$ transitions[a]					$\frac{T}{A}\rightarrow\frac{C}{G}$ transitions[b]					
Sequence (and origin)	CpA	CpT	CpG	CpC	Sum[c]	CpA	CpT	CpG	CpC	Sum[c]	Transversions
Punt^{RIP1} (nt 74–1947)	70	22	10	7	153	10	9	4	4	37	81
Punt^{RIP2} (Oak Ridge) (nt 1–281)	11	6	7	1	31	1	0	2	2	5	1
Punt^{RIP3} (Oak Ridge) (nt 1–280)	10	5	6	2	27	0	0	2	1	3	1
Punt-Al (Adiopodoumé) (nt 1–280)	1	0	0	1	4	1	0	1	0	2	1
Punt^{RIP4} (*N. sitophila*) (nt 1–279)	11	4	3	2	25	1	0	4	1	9	9

[a] Indicates a C→T or G→A change of the listed sequence, relative to *dPunt*.
[b] Indicates a T→C or A→G change of the listed sequence, relative to *dPunt*.
[c] The sum of the four dinucleotide classes may be lower than the indicated sum because the dinucleotide classes cannot be calculated for changes directly next to one another.

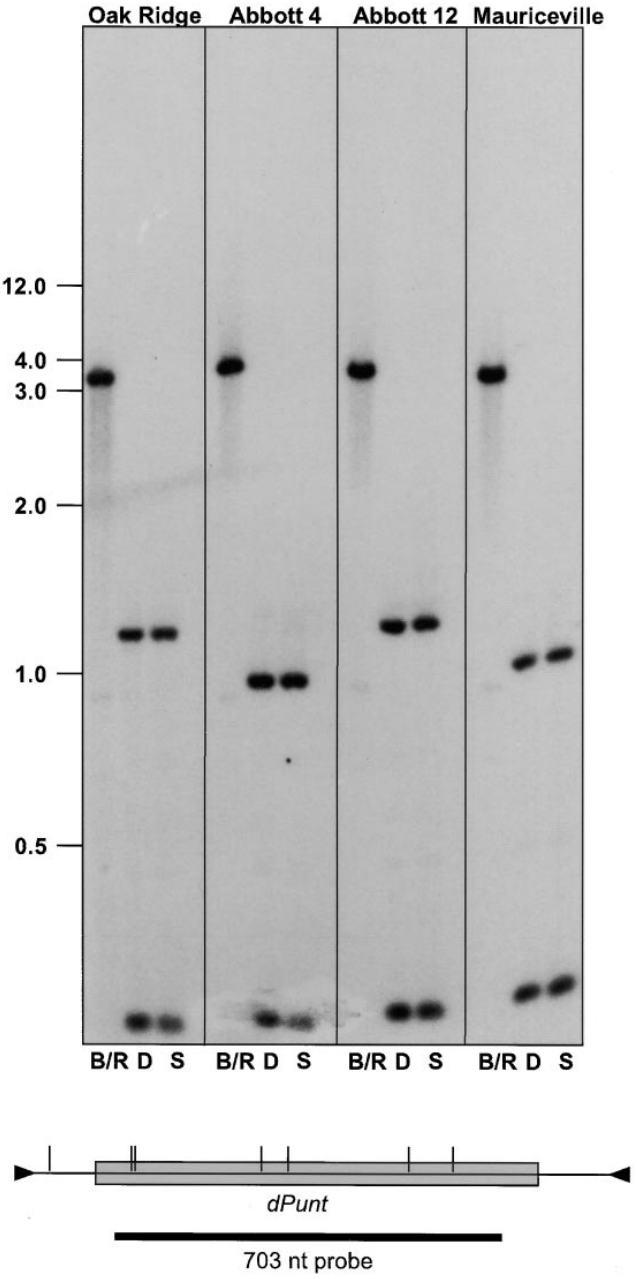

Figure 6.—Southern hybridizations of *dPunt* to genomic DNA of wt *N. crassa* strains. Genomic DNA from the indicated strains was digested with *Bam*HI and *Eco*RI (B/R), *Dpn*II (D), or *Sau*3AI (S) and probed with the *Ava*II fragment of *dPunt*. A DNA ladder is indicated. The positions of size markers (kb) are indicated. A map of *dPunt* with the probe indicated is shown below the Southern hybridization. The inverted terminal repeats are indicated by arrowheads and the region of *dPunt* that is homologous to the Fot1 open reading frame is indicated by a gray box.

revealed only the bands expected from the *dPunt* sequence. This suggested that the other sequences that hybridize to a $Punt^{RIP1}$ probe had been modified by RIP. To further investigate the nature of the sequences detected with the $Punt^{RIP1}$ probe, we designed PCR primers for segments of the *dPunt* sequence that have relatively few of the sites preferred by RIP. Both C and T or G and A were incorporated into the primers at the few CpA and TpG sites, respectively, to increase our chance of amplifying sequences that had been mutated by RIP.

PCR fragments from *N. crassa* and *N. sitophila* were cloned and sequenced. Of five clones obtained from Oak Ridge, one was identical to *dPunt*, two were identical to $Punt^{RIP1}$, and two represented new sequences related to *dPunt*. This confirmed that the primers were able to amplify both unmutated and mutated sequences. The two new sequences related to *dPunt*, $Punt^{RIP2}$ and $Punt^{RIP3}$, were 87 and 89% identical to *dPunt*, respectively, and 98% identical to each other. The TpA/ApT and (CpA+TpG)/(ApC+GpT) ratios for the *Punt* sequence of $Punt^{RIP2}$ are 1.11 and 0.89, respectively, and 1.11 and 0.84, respectively, for that of $Punt^{RIP3}$, suggesting that both sequences had been modified by RIP. Inspection of the nature of the differences between *dPunt* and the elements in $Punt^{RIP2}$ and $Punt^{RIP3}$ reinforced the conclusion that the sequences in $Punt^{RIP2}$ and $Punt^{RIP3}$ are relatives of *dPunt* that had been modified by RIP. The differences are primarily transition mutations in which there was a C to T or G to A change of the sequences in $Punt^{RIP2}$ or $Punt^{RIP3}$ (Table 2). Furthermore, most of these changes are at the sites preferred by RIP (CpA and TpG).

The only active transposon found in *N. crassa* to date, *Tad*, was found in the wild-type strain Adiopodoumé. We were therefore encouraged to look for active copies of *Punt* in Adiopodoumé. One *Punt*-like sequence was obtained from Adiopodoumé. This sequence was so similar to *dPunt* (97% identical) that it is not possible to say whether the differences between *dPunt* and the *Punt* homologue, *Punt*-Al, are characteristic of RIP (Table 2). The paucity of differences suggests, however, that *Punt*-Al was not mutated by RIP.

One *Punt*-like sequence was also obtained from *N. sitophila*. This sequence, $Punt^{RIP4}$, is 84% identical to *dPunt* and the TpA/ApT and (CpA+TpG)/(ApC+GpT) ratios are 0.95 and 0.79, respectively, suggesting that this sequence too may have been modified by RIP. It should be noted, however, that it is not known whether RIP occurs in *N. sitophila*; nor is it known whether the RIP-index values calculated for *N. crassa* are applicable for *N. sitophila*. The spectrum of differences between the *N. sitophila* sequence and *dPunt* are suggestive of two processes operating on these sequences, RIP and spontaneous mutation, leading to gradual sequence divergence (Figure 8). Twenty-five of 43 of the differences are transition mutations oriented such that there is a G:C to A:T change of the *N. sitophila* sequence. These differences are primarily located at the favored sites of RIP. The *N. sitophila* sequences also show nine transition mutations in the opposite direction. These differences are primarily at dinucleotides not favored by RIP, consistent with the idea that they were not caused by RIP. Finally, the *N. crassa* and *N. sitophila* sequences differ by

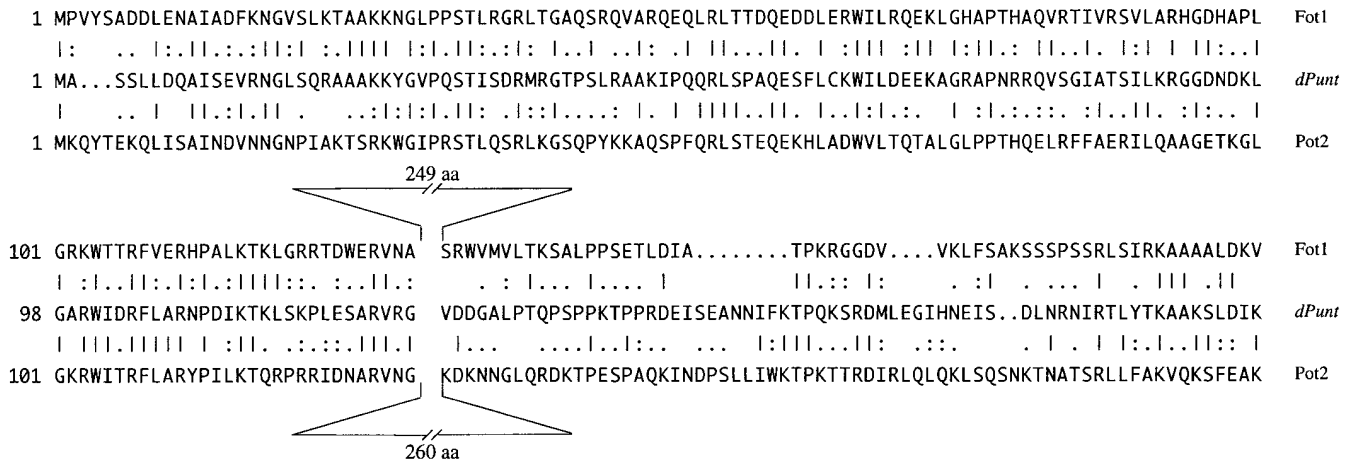

Figure 7.—Sequence comparison of the *dPunt* open reading frame with the Fot1 and Pot2 transposases. Positions of identity are indicated with a vertical slash. Positions of similarity are indicated with one or two dots depending upon the level of similarity. Regions of Fot1 and Pot2 that are not shown are indicated above and below the alignments, respectively.

a handful of transversions (nine in the 279-nt sequence analyzed).

DISCUSSION

Our interest in the control and function of DNA methylation in *N. crassa* led us to a previously unknown transposable element. Only a few methylated regions of the *N. crassa* genome have been characterized: the tandemly repeated 9-kb rRNA repeats (Russell *et al.* 1985; Perkins *et al.* 1986), which are also methylated in a number of other eukaryotes (Goyon *et al.* 1996; Kochanek *et al.* 1996; Amado *et al.* 1997; Buckler *et al.* 1997); the ζ-η region, which resulted from RIP operating on an ancestral tandem duplication encompassing a 5S rRNA gene (Grayburn and Selker 1989); sequences in the vicinity of a copy of the LINE-like transposable element *Tad* integrated at the *am* gene (Kinsey *et al.* 1994; Cambareri *et al.* 1996); sequences related to *Tad* elsewhere in the genome, which apparently have been mutated by RIP (Kinsey *et al.* 1994); and the Ψ_{63} region, characterized in this study. We found a transposon, *Punt*^{RIP1}, integrated in the 5S rRNA pseudogene named Ψ_{63}. *Punt*^{RIP1} shows evidence of mutagenesis by RIP and is methylated at all of the sites examined. Further analysis revealed that there are repeated sequences related to *Punt*^{RIP1} in Neurospora wild-type strains. In addition to these relics of *Punt* acted upon by RIP, we discovered one copy of a *Punt*-like sequence that appears to have not been modified by RIP. This element, called *dPunt*, is defective due to a large deletion of its central region. Presumably, it was this deletion that protected *dPunt* from RIP. The remaining segments would not be expected to trigger RIP because of their short lengths. Available data suggest that unlinked duplications shorter than about 1 kb are rarely mutated by RIP (Selker *et al.* 1989; Selker 1990). Furthermore, each strain appears to have only one copy of a *Punt*-like sequence not mutated by RIP. The differences between *dPunt* and *Punt*^{RIP1} that are not consistent with the action of RIP could have occurred either before or after the transposon entered *N. crassa*.

The fact that *dPunt* appears to be present at the same locus in all of several strains tested, including exotic strains that show extensive polymorphisms relative to laboratory strains, suggests that the deletion event that protected *dPunt* from RIP did not occur recently. Neurospora is very efficient at inactivating duplicated sequences including transposons such as *Tad* (Kinsey *et al.* 1994). It is therefore curious that many copies of *Punt* are present in the genomes of Neurospora strains. It is possible that *Punt* was already present in multiple copies at the time that RIP developed. However, various Neurospora strains show evidence of different distributions of *Punt*, suggesting that *Punt* spread through the *N. crassa* genome relatively recently, presumably despite the existence of RIP.

Because RIP is limited to the sexual cycle of Neurospora (Selker 1990), it is possible that *Punt* was initially spared because its host(s) rarely entered the sexual cycle. Proliferation of *Punt* could have occurred even if the host did not completely avoid the sexual cycle because RIP is not 100% efficient and therefore active copies of *Punt* could have escaped any given round of

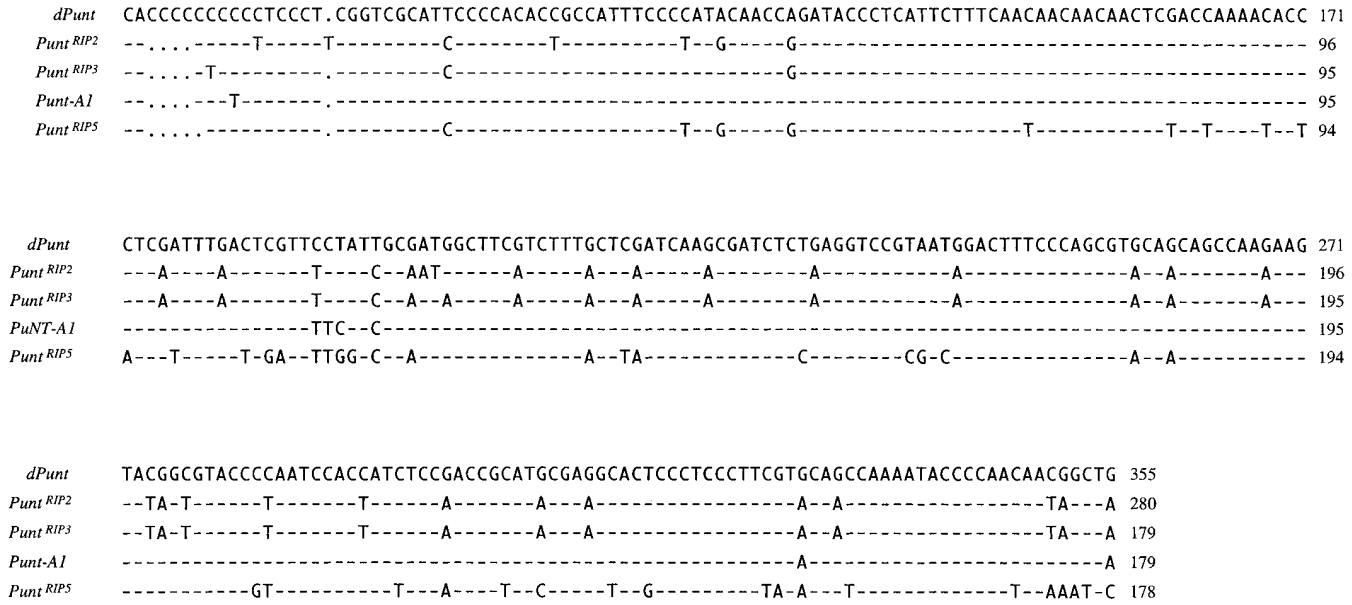

Figure 8.—DNA sequences of *dPunt* and relatives of *Punt* from *N. crassa* and *N. sitophila*. Positions of identity are indicated by dashes in the sequences of the *Punt* relatives below the *dPunt* sequence. The *dPunt* sequence is numbered starting from the left boundary of the transposon. The other four sequences are numbered from the first nucleotide of sequence.

RIP. Thus there may have been two competing processes occurring in the genome: propagation of *Punt* through transposition and inactivation of *Punt* by RIP. Considering the apparent absence of active copies of *Punt* in the Neurospora genomes examined, it appears that RIP has at least temporarily prevailed in its kinetic battle with *Punt*.

The only known active transposable element in any strain of Neurospora, *Tad*, was found in a strain collected from the wild, Adiopodoumé (Kinsey and Helber 1989). Interestingly, a section of a *Punt* element isolated from Adiopodoumé appears not to have been modified by RIP. It is possible that Adiopodoumé tolerates repeated sequences better than do other *N. crassa* strains. Even Adiopodoumé harbors transposons that were inactivated by RIP, however (Kinsey *et al.* 1994). Perhaps the ancestors of Adiopodoumé went through the sexual cycle relatively infrequently. In a battle between two opposing processes, RIP and transposition, a change in the rate of either process could shift the outcome.

Although *N. crassa* efficiently inactivates transposable elements and other repeated sequences, many other fungi do not. Undoubtedly *N. crassa* pays a price in fitness for its genome defense system beyond the mere cost of making the machinery of the RIP process. The organism is virtually unable to maintain more than one copy of conventional genes as well as transposons, and this may be the largest part of the cost of RIP. However, it seems possible that the absence of transposon-mediated shuffling of the genome could also be detrimental to the long-term adaptability of the species facing a changing environment. The continued existence of an organism carrying the RIP system suggests that, at least in the short and medium term, the cost in fitness has been less than the potential cost of genomic destabilization by transposable elements. Whether this has been true over a very long term would require evidence about the antiquity of the RIP system.

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