The Neurospora Transposon *Tad* Is Sensitive to Repeat-Induced Point Mutation (RIP)

John A. Kinsey,* Philip W. Garrett-Engele,† Edward B. Cambareri* and Eric U. Selker†

**Department of Microbiology, Molecular Genetics and Immunology, The University of Kansas Medical Center, Kansas City, Kansas 66160, and †Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403*

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ABSTRACT

RIP (repeat-induced point mutation) efficiently mutates repeated sequences in the sexual phase of the *Neurospora crassa* life cycle. Nevertheless, an active LINE-like retrotransposon, *Tad*, was found in a *N. crassa* strain from Adiopodoumé. The possibility was tested that *Tad* might be resistant to RIP, or that the Adiopodoumé strain might be incompetent for RIP. *Tad* elements derived from the Adiopodoumé strain were found to be susceptible to RIP. In addition, strains lacking active *Tad* elements, including common laboratory strains and strains representing seven species of Neurospora, were found to have sequences closely related to *Tad* but with numerous mutations of the type resulting from RIP (G:C to A:T). Even the Adiopodoumé strain showed *Tad*-like elements with mutations characteristic of RIP. Results of crossing of an Adiopodoumé transformant with progeny of Adiopodoumé suggest that the Adiopodoumé strain is proficient at RIP. We conclude that *Tad* is an old transposable element that has been inactivated by RIP in most strains. Finding relics of RIP in both heterothallic and homothallic species of Neurospora implicates RIP across the genus.

UNLIKE most eukaryotes, *Neurospora crassa* has little redundant DNA (KRUMLAUF and MARZLUF 1980). With the exception of rRNA and tRNA genes, all Neurospora genes that have been described appear to be unique in the genome. This may reflect the operation of repeat-induced point mutation (RIP), a mutagenic process that occurs in the sexual stage of the *N. crassa* life cycle (SELKER *et al.* 1987; SELKER 1990). RIP efficiently detects duplications of gene-sized DNA segments, whether linked or unlinked, and generates G:C to A:T mutations in both copies of the duplicated DNA (SELKER and GARRETT 1988; CAMBARERI *et al.* 1989). Sequences altered by RIP are typically, although not invariably, methylated. RIP occurs only in the ascogenous cells formed by fertilization that contain haploid nuclei from both parents (SELKER *et al.* 1987). Thus repeated sequences are safe from RIP so long as they do not go through a cross. In a cross, unlinked duplications typically have a 50:50 chance of being missed by RIP; linked duplications escape less frequently (SELKER *et al.* 1987); J. IRELAN, A. HAGEMANN and E. SELKER, in preparation; E. CAMBARERI and E. SELKER, unpublished).

The characteristics of RIP, and the fact that *N. crassa* is normally an out-crossing species, should make *N. crassa* an inhospitable host for transposons. A transposon that entered would be safe from RIP provided it did not transpose by a duplicative process, but it would be subjected to RIP mutation during the next sexual cycle if more than one copy were present. Consistent with this interpretation, no active transposons were detected in standard laboratory strains using the *am* gene as a transposon trap (J. KINSEY, unpublished); however, KINSEY and HELBER (1989) discovered an active LINE-like element in a strain of *N. crassa* collected from Adiopodoumé, Ivory Coast, using the same system. The Adiopodoumé strain harbors about 40 copies of this transposon, named *Tad*. The existence of this functional multicopy transposon suggested two possibilities: (1) *Tad* is inherently resistant to RIP or (2) some strains of *N. crassa*, such as the Adiopodoumé strain, are deficient in RIP. We report here the results of testing these hypotheses.

MATERIALS AND METHODS

DNA manipulations: Neurospora DNA was isolated by the method of METZENBERG and co-workers (METZENBERG and BAISCH 1981; STEVENS and METZENBERG 1982). Restriction digests were carried out using buffers and conditions specified by the manufacturer. Restriction digests were fractionated on 0.7 or 1.0% agarose gels and transferred to nylon membranes. Two protocols were used for transfer and hybridization. In protocol A, Zetabind (Cuno) membranes were used and hybridizations were performed overnight at 60° in 5% dextran sulfate/0.4% sodium dodecyl sulfate/0.45 M NaCl/0.045 M sodium citrate/60 mM sodium phosphate, pH 6.6/10 mM EDTA/0.06% bovine serum albumin/0.06% Ficoll/0.06% polyvinylpyrrolidone/50 μg/ml denatured salmon sperm DNA. After hybridization, membranes were washed at 50° in 50 mM NaCl/20 mM sodium phosphate, pH 6.8/1.0 mM EDTA/0.1% sodium dodecyl sulfate. In protocol B, transfer was to Genescreen Plus and hybridization was performed overnight at 65° in 10% dextran sulfate/1.0% sodium dodecyl sulfate/1.0 M NaCl/100 μg/ml denatured salmon sperm DNA. After hybridization, membranes were washed at 65° in 300 mM NaCl/30 mM sodium citrate/1.0% sodium dodecyl sulfate. Hybridization probes were prepared by the random oligomer-primer method of FEINBERG and VOGELSTEIN (1984).

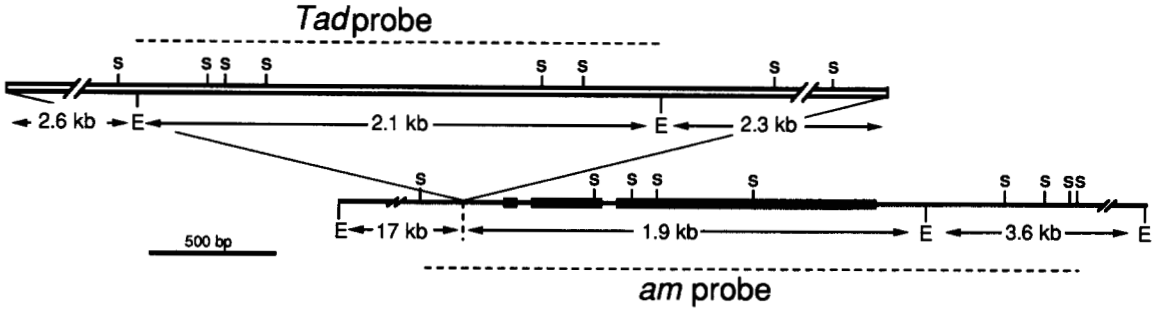

FIGURE 1.—Map of *Tad*3-2/*am* region showing relevant *Eco*RI (E) and *Sau*3AI (S) sites, and segments used for probing blots such as that shown in Figure 2. The positions of the exons of *am* are indicated by heavy lines.

DNA amplification by polymerase chain reaction (PCR): Genomic DNA from a variety of Neurospora stains was subjected to PCR amplification (SAIKI *et al.* 1985; MULLIS and FALOONA 1987). Primers used to amplify the 3′ junction between *Tad*3-2 and the *am* gene were, primer 1, 5′-CGGAATTCAGCTGTGGTGAAAAATCAAGCT, and primer 2, 5′-GGGATCTACGGACGACACTA. Degenerate primers used to amplify a fragment of *Tad* elements from a variety of strains were: primer 1, 5′AARAARARRRARRATATARYT and primer 2, 5′RAYYTYTAYYTTYRRRAAAA. PCR fragments were either sequenced directly using Cycle sequencing with the BRL kit, as per manufacturer's specifications, or the PCR fragments were cloned into plasmid vectors (pBS, Stratagene) and sequenced by the dideoxy method of SANGER and COULSON (1975).

Strains and culture conditions: The Adiopodoumé strain, FGSC 430, and standard Oak Ridge strains, 74-OR23-1VA (FGSC 2489) and ORSa (FGSC 2490), were obtained from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas. Other strains were derived in these experiments, were from the stock collection of JAK, or were obtained from DAVID PERKINS. Standard Neurospora culture and crossing conditions and media were used (DAVIS and DESERRES 1970). Adiopodoumé was transformed using standard procedures for Neurospora (VOLMER and YANOFSKY 1986).

RESULTS

Early work with *Tad* demonstrated that this element is sexually transmissible (KINSEY and HELBER 1989). Indeed, the transposon was initially identified in progeny from a cross of Adiopodoumé with a laboratory strain, J852 (*lys-1*). *Tad* was discovered in two strains within which independent transposition events had occurred that affected expression of the *am* (glutamate dehydrogenase) gene. One of the mutations was due to insertion of *Tad* within the coding sequences which completely inactivates the gene; however, the other mutation, *am::Tad*3-2 (referred to hereafter as 3-2), was due to the insertion of the *Tad*3-2 element into the *am* gene 5′ non-coding sequences (see Figure 1). This resulted in an unstable $Am^{+/-}$ phenotype, apparently due to intermittent interference of *am* transcription by *Tad* (E. CAMBARERI and J. KINSEY, unpublished). Although most sexual progeny harboring 3-2 continue to show the unstable $Am^{+/-}$ phenotype, some are stably Am^{+} and others are stably Am^{-}. It seemed possible that loss of the unstable Am phenotype in sexual progeny resulted from RIP.

Southern blot analysis reveals mutation and methylation in progeny of 3-2: As a first step to determine whether *Tad* is sensitive to RIP, we examined the *Tad/am* region of several $Am^{+/-}$, Am^{+}, and Am^{-} progeny by Southern hybridization. We used the isoschizomer pair *Sau*3AI and *Mbo*I, to look for hallmarks of RIP–numerous mutations and *de novo* DNA methylation. *Sau*3AI, but not *Mbo*I, is sensitive to methylation of the C in GATC sites (NELSON and MCCLELLAND 1991). Figure 2 shows representative results. The predominant fragments detected in the Adiopodoumé strain (lane pair 1), using an internal *Tad* probe, showed no indication of DNA methylation; the *Sau*3AI and *Mbo*I bands were of equal intensity. As expected, these bands were not evident in DNA from the standard laboratory strain (Oak Ridge; lane pair 8). The significance of the higher molecular weight bands seen in all lanes will be discussed below. The stable Am^{-} and unstable $Am^{+/-}$ strains examined showed heavy hybridization to the expected *Sau*3AI and *Mbo*I fragments of *Tad*, indicating that these strains harbor multiple unmethylated *Tad* elements (lane pairs 2, 3, 4, 7). In contrast, the two stable Am^{+} strains examined (lane pairs 5 and 6) showed a marked reduction of these *Mbo*I bands and an apparent absence of the corresponding *Sau*3AI bands. These results indicate that these stable Am^{+} strains do not have multiple normal copies of *Tad*. Southern hybridizations using other enzymes demonstrated that *Tad* was still present in the *am* upstream region (data not shown). A probing of the *Sau*3AI/*Mbo*I blot with an *am* probe indicated that the *Tad* element at *am* (3-2) had become methylated and showed evidence of mutation (Figure 2, right panel). The ≈700-bp *Mbo*I fragment spanning the *Tad-am* junction was replaced by a ≈1.3-kb *Mbo*I fragment in strain J1702 (lane pair 6). Both stable Am^{+} strains showed an approximately *Tad*-sized *Sau*3AI fragment (arrow in Figure 2) indicative of extensive methylation. Reduced intensity of the 0.4-kb *Sau*3AI *am* band, but not of the 1.0-kb *Sau*3AI *am* band indicated that the methylation extended at least to the *Sau*3AI site 160 bp into the third exon of *am* but not as far as the site 390

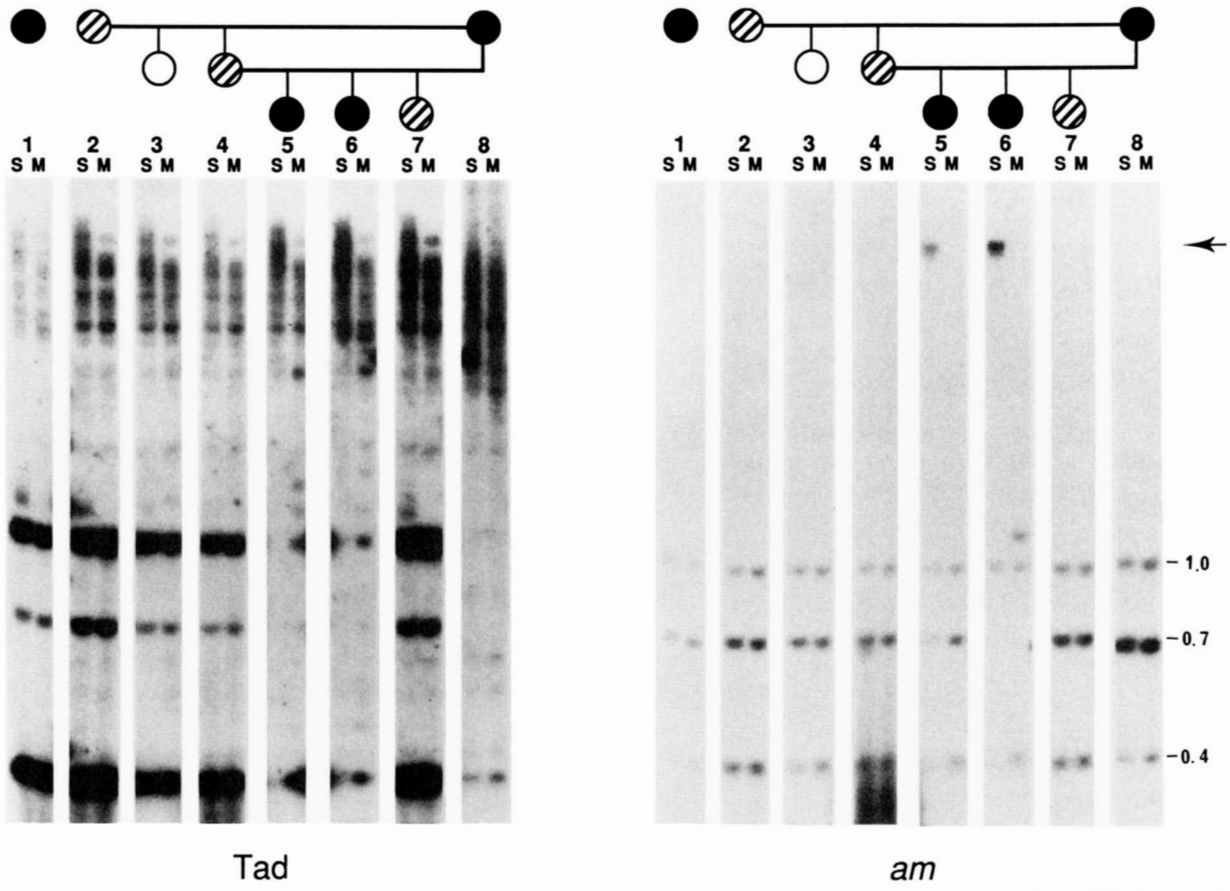

FIGURE 2.—Examination of sexual progeny of *Tad*3-2 strains for evidence of RIP. Genomic DNA from Am^+ (filled circles), Am^- (open circles) or unstable $Am^{+/-}$ (hatched circles) strains were digested with an excess of *Sau*3AI (S) or *Mbo*I (M), fractionated by agarose gel electrophoresis and probed with the 2.1-kb *Eco*RI fragment of *Tad* (see Figure 1). The Southern blot was then stripped and probed with the 2.6-kb *am* fragment. Relationships of the selected strains are shown above the lanes, and the size (in kilobases) of the native *am* fragments are indicated to the right. The arrow points to bands resulting from methylation near the *Tad/am* junction. Strain designations are Adiopodoumé (1), 86.2.9.23 (2), J1557 (3), J1577 (4), J1602 (5), J1607 (6), J1603 (7) and 74-OR8-1a (8).

bp further downstream. These results suggested that *Tad* is sensitive to RIP.

The new *Sau*3AI fragment detected in the Am^+ strains was in a region of the gel where we detected many weak bands using the *Tad* probe in both *Sau*3AI and *Mbo*I digests (Figure 2, left panel). Probings with other parts of *Tad* produced equivalent results (data not shown). These bands were present not only in the $Am^{+/-}$, Am^-, Am^+ and Adiopodoumé strains, but also in Oak Ridge and other strains thought to be devoid of *Tad*. Furthermore, differences between the bands detected in *Sau*3AI and *Mbo*I digests were suggestive of DNA methylation. Washing the blots at higher temperatures preferentially reduced the intensity of these higher bands relative to the expected *Tad* bands, implying that the ubiquitous sequences were similar but not identical to *Tad* (data not shown). These large, partially methylated *Mbo*I fragments, related to *Tad*, were reminiscent of products of RIP observed in previous studies (SELKER *et al.* 1987; CAMBARERI *et al.* 1991). These results suggested that *Tad* is sensitive to RIP and that a variety of *N. crassa* strains, ranging from normal laboratory strains to the Adiopodoumé strain from which active *Tad* was isolated, contain *Tad* or *Tad*-like elements that had been modified by RIP.

The results from our analyses of the small set of $Am^{+/-}$, Am^+, and Am^- progeny, all carrying *Tad*3-2 upstream of *am*, suggested that Am^+ progeny result from alterations (probably by RIP) in the *Tad/am* region. We explored this further by analyzing 45 additional progeny from a cross of the original unstable $Am^{+/-}$ strain (J1518) with an *am* deletion strain J1264 (am^{132}) and 42 additional progeny from a cross of the unstable $Am^{+/-}$ strain J1577 (derived from J1518 by two back-crosses to Oak Ridge strains) with am^{132}. Six of the progeny from each cross were selected for am^+ function. Forty-nine of the 87 strains from the combined progeny sets had the 3-2 rather than the am^{132} allele. Four of the 37 unselected strains with the 3-2 allele were Am^+. About half of the others were clearly unstable $Am^{+/-}$ strains, producing Am^+ conidia at frequencies ranging from $\approx 10^{-3}$ to ≈ 0.5. The others were classified as Am^-, but may have included

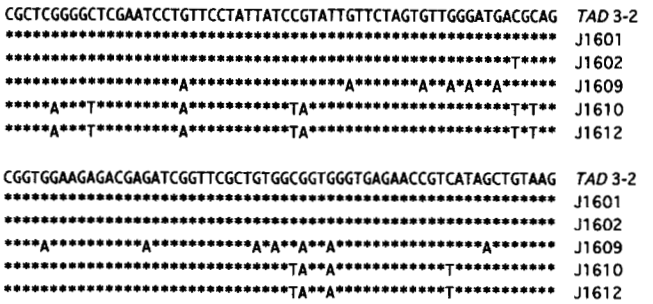

FIGURE 3.—DNA sequence of a 120 bp segment of *Tad*3-2 from progeny of a cross of J1577 (*am::Tad*3-2) × J1264 (am^{132}). DNA from progeny was used as template for PCR amplification (see MATERIALS AND METHODS). The region of the PCR products between nucleotides 6637 and 6756 in 3-2 was sequenced by Cycle sequencing. The sequences obtained are compared with that from the cloned original *Tad*3-2 sequence, which is shown on the top line (CAMBARERI *et al.* 1994). Only differences are indicated.

strains more revertible than standard *am* mutants. Southern hybridizations using *Eco*RI, *Sau*3AI and *Mbo*I digests probed with *am* fragments revealed sequence alterations and DNA methylation in the *Tad/am* junction region of most Am^+ strains (data not shown). Am^- and $Am^{+/-}$ strains occasionally, but less frequently, showed alterations and methylation. Results from probing Southern blots with *Tad* suggested that a subset of the progeny of each class (Am^+, Am^- and $Am^{+/-}$) no longer carried an intact *Tad* element (data not shown).

DNA sequence analysis of *Tad*3-2 elements from Am^+ progeny: To test directly the suggestion that *Tad* is sensitive to RIP, we isolated and sequenced segments of *Tad*3-2 from Am^+ progeny isolated from a cross of J1577 (*am::Tad*3-2) × J1264 (am^{132}). DNA isolated from the progeny was used as template to amplify a ≈1-kb fragment spanning the *am-Tad*3-2 junction. Segments of the PCR products were directly sequenced. Figure 3 shows the sequence of 120 bp of this region from four stable Am^+ progeny (J1602, J1609, J1610 and J1612) and one $Am^{+/-}$ progeny (J1601) compared with that of the original *Tad*3-2. The region shown spans nucleotides 6637–6756 of the *Tad* sequence. All of the stable Am^+ progeny differ from *Tad*3-2 by changes of the type expected for RIP (CAMBARERI *et al.* 1989). More extensive sequencing of these DNAs revealed additional mutations of only the type expected from RIP (G:C to A:T mutations). The sequence shown reveal no differences between two of the Am^+ progeny, J1610 and J1612; however additional sequence analysis (not shown) indicated that they are independent strains with differing patterns of RIP.

***Tad* elements showing evidence of RIP are present in Adiopodoumé and laboratory strains of *N. crassa*:** As indicated above, Southern blot experiments with Adiopodoumé and laboratory (Oak Ridge) strains of *N. crassa* using *Tad* sequences as probes showed many weak bands suggestive of relics of RIP operating on *Tad* (see Figure 2, lane 8, *Tad* probe). We had not observed

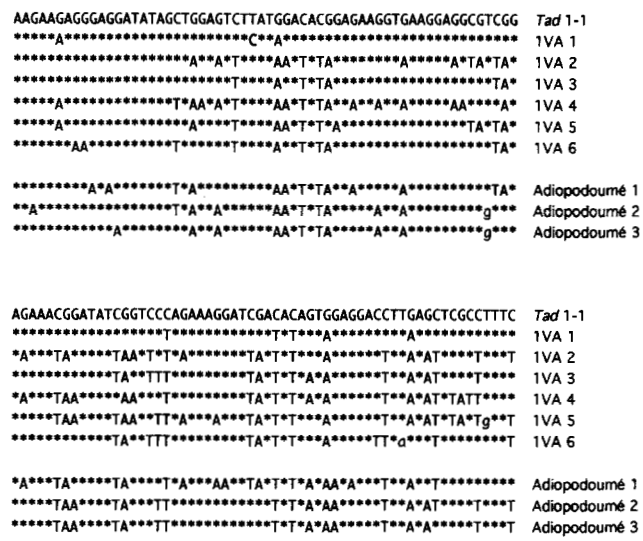

FIGURE 4.—DNA sequence of a segment of *Tad*-related sequences from Adiopodoumé and a laboratory strain of *N. crassa*. Genomic DNA from either the laboratory wild-type 74-OR23-1VA (FGSC 2489; IVA) or Adiopodoumé (FGSC 430) was used with degenerate primers (see MATERIALS AND METHODS) to amplify segments of *Tad* elements, whether or not they had been mutated by RIP. Fragments of the expected size (550 bp) were obtained with both amplifications, and representatives from each strain were cloned and sequenced. Sequence differences relative to an active *Tad* element, *Tad*1-1 (CAMBARERI *et al.* 1994) in a segment between nucleotides 2461 and 2581 are indicated. Polarized transition mutations of the type generated by RIP are indicated in upper case letters and transversions are shown in lower case letters. Transitions in the opposite direction from that expected by RIP operating on *Tad*1-1 are indicated in bold.

these bands in previous studies, when more stringent hybridization and washing conditions were employed (KINSEY 1989). We confirmed that the putative *Tad*-related elements are detectable using the less stringent protocol (A; MATERIALS AND METHODS), but not with the more stringent protocol (B; MATERIALS AND METHODS). To investigate the possibility that the weakly hybridizing sequences of Adiopodoumé and laboratory strains of *N. crassa* are relics of RIP, we designed primers with degeneracies to amplify segments of *Tad* elements, whether or not they had been mutated by RIP. PCR products of the expected size (550 bp) were obtained with DNA either from the standard Oak Ridge strain 1VA or from the Adiopodoumé strain as template (data not shown). We cloned and sequenced several products from each reaction to determine whether the amplified segments were indeed closely related to *Tad*. Figure 4 shows sequence data for six clones obtained using 1VA DNA and three clones obtained using Adiopodoumé DNA, compared to the sequence found in both active elements, *Tad*3-2 and *Tad*1-1. The region shown corresponds to nucleotides 2461–2581 of the *Tad* sequence (CAMBARERI *et al.* 1994). These data demonstrate that both strains contain elements unmistakably related to *Tad* but with numerous mutations. Three additional

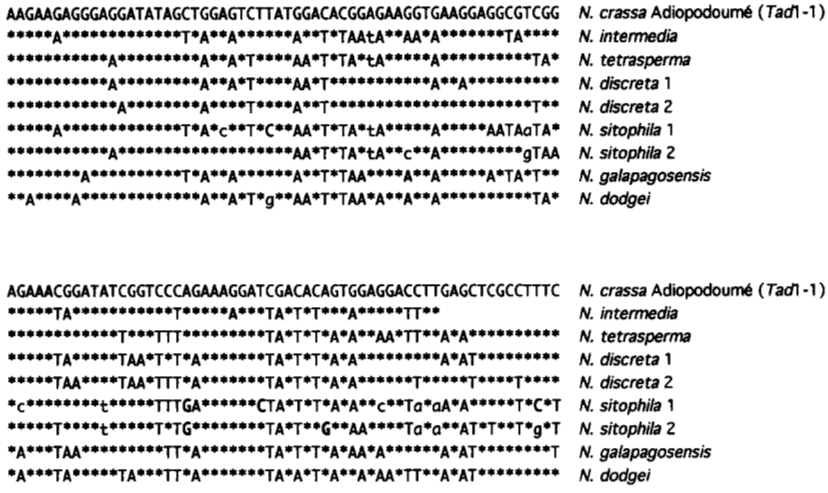

FIGURE 5.—DNA sequence of a segment of *Tad*-related sequences from strains representing a variety of *Neurospora* species. The region examined was the same as for the *N. crassa* strains (Figure 4). Two examples from both *N. discreta* and *N. sitophila* are shown. Transition mutations are indicated in upper case letters and transversions are shown in lower case letters. Transitions in the opposite direction from that expected by RIP operating on *Tad*1-1 are indicated in bold.

clones were obtained and sequenced from the Adiopodoumé strain, but they showed no differences in sequence from that of *Tad*3-2. The sequence divergence of the *Tad*-related elements relative to *Tad* ranged from 7 to 32% (23.4% average) in the segment examined. Almost all (98%) of the sequence differences were of the type expected from RIP (G:C to A:T) operating on *Tad*. We conclude that both Adiopodoumé and laboratory strains of *N. crassa* have *Tad* elements that had been inactivated by RIP. Interestingly, the sequences of the *Tad*-related elements obtained are all distinct, consistent with the Southern data that suggested that the copy number of inactivated *Tad* elements in Adiopodoumé and laboratory strains of *N. crassa* is rather high.

***Tad* elements inactivated by RIP are present in most or all species of Neurospora:** Southern blots at reduced stringency had revealed the presence of *Tad*-related elements in a variety of strains representing different species of Neurospora (data not shown). We therefore used the degenerate primers described above to test whether *Tad* elements inactivated by RIP are widespread in the genus Neurospora. Fragments of the expected size were amplified from a variety of strains, representing most of the species of Neurospora, including both heterothallic species like *N. crassa* and the homothallic species *Neurospora africana*, *Neurospora galapagosensis* and *Neurospora tericola* (Table 1). We cloned and sequenced PCR products from representatives of most of the species. In each case, we found evidence of *Tad* elements that had been extensively mutated by RIP. Sample data are shown in Figure 5. The vast majority (98%) of the differences observed relative to an active *Tad* element are transitions and most (93%) of these are in the direction expected. The sequences of the various *Tad*-related sequences determined are all different. These results suggest that all species of Neurospora, or a common ancestor, have hosted *Tad* and that RIP has inactivated almost all copies of this transposon.

Is Adiopodoumé defective in RIP?: Since an active *Tad* element survived in the Adiopodoumé strain, we wished to determine whether this strain is defective in RIP. The observation of *Tad*-related relics of RIP in Adiopodoumé suggested that this strain was, at least at one time, competent for RIP. Similarly, we identified another repeated sequence (AdRS-2) present in the Adiopodoumé strain and found evidence that it too had been altered by RIP (J. KINSEY, unpublished). In order to test whether Adiopodoumé is still proficient for RIP we needed to look for RIP in crosses in which both parents were of the Adiopodoumé genetic background. This could not be done directly since *N. crassa* is a heterothallic species and the only strain known with active *Tad* elements is Adiopodoumé (KINSEY 1989). Consequently, we used crosses involving progeny of Adiopodoumé to test for RIP.

First, we transformed the Adiopodoumé strain with the plasmid pES201, which contains the bacterial *hph* (hygromycin phosphotransferase) gene driven by the *Aspergillus nidulans trpC* promoter and the *N. crassa am* gene driven by it own promoter. Transformants were selected for resistance to hygromycin and then screened for copy number by Southern blot hybridization. One transformant, T430-ES201-2 (referred to hereafter as T-2), was identified as having a single copy of the transforming DNA integrated at a site unlinked to the *am* gene. This transformant, which contains two copies of the am^+ gene and is Am^+, was crossed to a series of other am^+ strains to test genetically for RIP. In the first set of crosses (Table 2 crosses 14 and 15) T-2 was crossed to strains with the standard Oak Ridge background, ORSa and *inl* (37401)*a* (FGSC 4075). New *am* mutants were detected in these crosses at frequencies of 0.01 and 0.04, respectively. This indicated that RIP can occur in crosses in which the Adiopodoumé strain is one of the parents. Thus if Adiopodoumé were defective for RIP, this trait must be recessive.

In a second set of crosses, we used F_1 progeny from a cross of ORSa × Adiopodoumé to test with the Adiopodoumé transformant. A total of 10 progeny of the *a* mating type were crossed to T-2 and the progeny

TABLE 1

Strains used to amplify putative *Tad*-related sequences using degenerate primers

Species	Designation	FGSC no.
N. africana	africana N200	1740
N. crassa	74-OR23–1VA	2489
N. crassa	Adiopodoumé	430
N. discreta	Bandipur	6789
N. discreta	Gouana	6794
N. discreta 2[a]	Homestead-1k	3268
N. discreta 1[a]	Kirbyville-6	3228
N. dodgei[a]	PR300	1692
N. galapagosensis	G349	1739
N. galapagosensis var. *dominicana*[a]	D301	4628
N. intermedia[a]	Bambaroo	3996
N. intermedia	Mareeba	5105
N. intermedia	Big Emma	5908
N. intermedia	Harbin	3983
N. sitophila 1[a]	Arlington	1843
N. sitophila 2[a]	Kinkala-1	6559
N. sitophila	Pobina	6802
N. terricola	WFS5000	1889
N. tetrasperma[a]	Liberia	965
N. tetrasperma	Bernard	6576

[a] PCR products from these strains were cloned and sequenced (Figure 5).

TABLE 2

Frequency of RIP at the *am* locus in crosses of transformant T430-ES201-2 with various strains

Cross no.	Strain	Total spores	Am⁻ spores	Frequency of Am⁻ spores
1	F_1 (92-69-04)[a]	400	6	0.02
2	F_1 (92-69-09)[a]	350	12	0.03
3	F_1 (92-69-10)[a]	400	6	0.03
4	F_1 (92-69-11)[a]	331	14	0.04
5	F_1 (92-69-12)[a]	400	11	0.03
6	F_1 (92-69-14)[a]	400	21	0.05
7	F_1 (92-69-18)[a]	300	8	0.03
8	F_1 (92-69-19)[a]	300	11	0.04
9	F_1 (92-69-21)[a]	268	8	0.03
10	F_1 (92-69-22)[a]	155	21	0.13
11	6XBC (14)[b]	100	31	0.31
12	6XBC (1)[b]	100	10	0.10
13	6XBC (8)[b]	100	21	0.21
14	FGSC 4075 (*inl*)	101	4	0.04
15	FGSC2490 (ORSa)	470	3	0.01

[a] Strains that were F_1 progeny of a cross of Adiopodoumé to ORSa.
[b] Strains from D. D. PERKINS that resulted from six successive backcrosses of progeny from a cross of *eas* (UCLA191) *a* × Adiopodoumé to the Adiopodoumé parent strain.

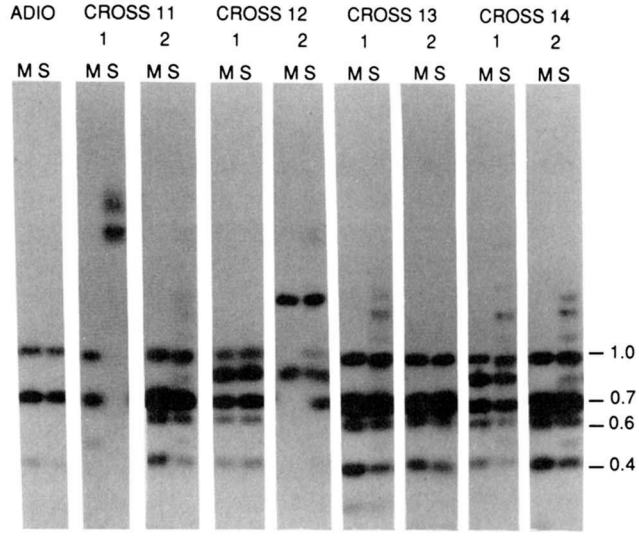

FIGURE 6.—Evidence of RIP in progeny of Adiopodoumé. Genomic DNA of *am* progeny from crosses 11, 12, 13 and 14 (Table 2) were digested with either *Mbo*I (M) or *Sau*3AI (S), fractionated by agarose gel electrophoresis and probed with the *Bam*HI-*Eco*RI fragment of the *am* gene (Figure 1). Two progeny from each cross are shown. The T-2 parent (not shown) gives the same restriction pattern as that shown by progeny 2 from cross 11. Progeny may or may not have the 0.65-kb band depending upon segregation and RIP of the ectopic copy of *am*. The prominent bands seen in *Mbo*I and *Sau*3AI digests of Adiopodoumé are approximately 1.0, 0.7 and 0.4 kb (Figure 1).

were screened for *am* mutants (Table 1). All crosses produced numerous *am* mutants; Am⁻ strains were produced at frequencies ranging from 0.02 to 0.13. Assuming the sought RIP defect were due to a single gene not linked to mating type, these crosses rule out the possibility that Adiopodoumé is defective in RIP, with greater than 99% confidence levels. As a further test, we made use of strains that resulted from six successive backcrosses to Adiopodoumé (Table 2). Using formulae provided by LESLIE (1981), these progeny were calculated to have an allogenic tract centered around the mating type locus of average length 24 cM. Because a recombination block extends for approximately 30 cM around the *eas* mutation that was present in these crosses (D. PERKINS, personal communication), a similar allogenic block of approximately 42 cM was present at the right end of LGII. Aside from these two blocks, the average allogenicity of these progeny will consist of a single block of about 15 cM located at random. The likelihood that three such progeny would share the same 15-cM block is approximately 3×10^{-6}.

Three such am^+ strains, which resulted from six backcrosses to Adiopodoumé, were crossed to T-2 and the progeny screened for inactivation of *am* by RIP. Numerous *am* mutants were generated in all three crosses (Table 2, crosses 11–13). To be certain that the mutants were the result of RIP, random *am* progeny from these crosses and from a control cross (Table 2, cross 14) were analyzed in the *am* region by Southern hybridization using *Sau*3AI and *Mbo*I digests as described above (Figure 6). Progeny with the ectopic copy of *am* will have an additional band at 0.65 kb in addition to the normal *am* bands of approximately 1.0, 0.7 and 0.4 kb. Bands of other sizes are due to methylation (*Sau*3AI digests only) or mutation (both digests). Most of the mutants showed higher molecular weight *Sau*3AI fragments indicative of methylation and several (cross 11 progeny 1; cross 12, progeny 1 and 2; cross 14, progeny 1) showed alterations of *Mbo*I fragments indicative of nucleotide sequence changes. Thus the hallmarks of RIP are seen in crosses

of the Adiopodoumé transformant with each of the three progeny from the serial backcrosses to Adiopodoumé. Since these progeny should be almost equivalent to the original Adiopodoumé strain (but of opposite mating type), it is extremely unlikely that the occurrence of active *Tad* elements in Adiopodoumé is due to a defect in RIP.

DISCUSSION

A priori, one might assume that active transposons like *Tad* should not exist in *N. crassa*. Repeated sequences are heavily mutated by RIP in the period between fertilization and karyogamy and are typically also left methylated (for a review see SELKER 1990) Thus, any transposon that enters the sexual cycle with more than one copy in the genome risks destruction. The presence of *Tad*, an active LINE-like element, in the Adiopodoumé strain of *N. crassa* (KINSEY 1989) suggested either that this strain was defective in RIP, that *Tad* was a new transposon, or that *Tad* was immune to RIP. One of the original *am* mutations caused by insertion of *Tad* in the 5′ noncoding sequences (*am::Tad*3-2) resulted in an unstable $Am^{+/-}$ phenotype; however, this strain could give rise to progeny that were either stably Am^+ or stably Am^-. We considered that this might be due to RIP. The results indicate that in those progeny that are stably Am^+, the *Tad*3-2 element had indeed been extensively mutated by RIP. We interpret these results to suggest that mutations in *Tad* can, either directly or indirectly, prevent it from interfering with *am* expression. Likewise, the stable Am^- strains might have resulted from the "spillage" of RIP mutations into the *am* coding sequences (FOSS *et al.* 1991). Evidence of mutations by RIP and associated cytosine methylation in *Tad* elements was also found in many of the other progeny, regardless of the phenotype expressed by the *am* allele. Thus it is clear that the *Tad* elements that came from the Adiopodoumé strain are not inherently resistant to RIP. In fact, in some of the progeny, all of the *Tad* elements were apparently so extensively mutated that little or no hybridization was observed when their DNA was probed with *Tad* under stringent conditions (data not shown).

The presence of bands that weakly hybridized to a *Tad* probe in a variety of strains not believed to harbor *Tad*, suggested that these strains might contain *Tad* elements that had been subjected to RIP. Using PCR with degenerate probes, we were able to amplify and sequence fragments of *Tad*-related elements from Adiopodoumé, as well as from the standard *N. crassa* laboratory strain 74-OR-231VA and representatives of almost all species of Neurospora. In each case we found *Tad* elements that had been extensively mutated by RIP; approximately 50% of the G-C pairs had been changed to A-T pairs and very few other kinds of mutations were evident. The *Tad*-related elements from *N. sitophila* showed several transition differences polarized in the opposite direction of that expected (*i.e.*, G:C pairs at positions with A:T pairs in *Tad*1-1 and *Tad*3-2) suggesting that an element ancestral to *Tad*1-1 and *Tad*3-2 may have survived limited mutagenesis by RIP. Altogether, our findings suggest that *Tad* is an old transposable element that spread throughout the genus Neurospora but then became completely inactivated in almost all strains. These results also imply that RIP is, or was, not limited to *N. crassa*. Even homothallic species showed evidence of RIP. This suggests that either a species that was ancesteral to the homothallic species was competent for RIP or that RIP was not limited to the heterothallic species.

The only known strain in which *Tad* has escaped complete inactivation by RIP is Adiopodoumé. In the small sample of elements from Adiopodoumé represented by our PCR clones, half of the elements (3/6) showed clear signs of RIP. The direct tests for RIP proficiency indicated that if Adiopodoumé is deficient in RIP, the gene responsible must be recessive and located within approximately 12 cM to the right or left of mating type. Taken together these data suggest that Adiopodoumé is proficient in RIP. This poses the question of how *Tad* survived in the Adiopodoumé strain.

One can imagine a number of possible explanations for the occurrence of active *Tad* elements in Adiopodoumé. Duplications escape RIP at appreciable frequencies (typically 50% per sexual cycle), so it is conceivable that the active elements in Adiopodoumé survived simply by chance. It is possible that something about the behavior of *Tad* in this particular strain gives it an advantage over similar elements in other strains. For example, the mechanism of transposition in this strain may be timed to insure that copies of *Tad* would always be in the cDNA or other cytoplasmic stage during the time when RIP occurs (KINSEY 1990, 1993). This would allow the elements to repopulate both nuclei after RIP had completed its action. Conceivably, *Tad* might stay ahead of RIP by virtue of an exceptionally high transposition activity in this strain.

Another possible explanation for active *Tad* elements in Adiopodoumé is that one or more copies of *Tad* in this strain are immune to RIP because they are in a hypothetical protected chromosomal location. There is some evidence that the tandemly repeated rDNA genes of Neurospora are protected because of their location in the nucleolar organizing region (K. HAACK and E. SELKER, unpublished). It is also possible the presence of active *Tad* elements in Adiopodoumé reflects a new horizontal transfer of *Tad* from some other organism. Each of these scenarios make specific and different predictions for the behavior of *Tad* in crosses. Experiments are currently under way to determine if any of these predictions are fulfilled.

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