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restless, an Active *Ac*-like Transposon from the Fungus *Tolypocladium inflatum*: Structure, Expression, and Alternative RNA Splicing

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Elements of the *hAT* transposon family, such as the maize *activator* (*Ac*), have been discovered in a large number of eukaryotic species. This type of class II transposon, present in both plants and animals, has not been previously detected in any fungal organism. However, using a differential screening method to detect repetitive DNA, we have identified a *hAT* transposon in the hyphomycete *Tolypocladium inflatum*. The transposon, which we named *restless*, is 4,097 bp long, carries 20-bp inverted repeats and an 8-bp target site duplication, and encodes a long open reading frame which is interrupted by a single intronic sequence. The derived mRNA exhibits alternative splicing, resulting in the formation of two transcripts that may be translated into polypeptides of 157 or 803 amino acids. The predicted amino acid sequence of the larger polypeptide demonstrates significant homology with transposases from the *hAT* transposon family. A chromosomal analysis using pulsed-field gel electrophoresis showed that all seven chromosomal bands carry copies of the 4.1-kb transposon. This was confirmed in hybridization experiments with rare-cutting restriction endonucleases which indicate that about 15 copies are present in *T. inflatum*. The genomic distribution of *restless* and its transcriptional expression, alternative mRNA splicing, and genomic mobility all imply a potential role for this element in developing a transposon tagging system for use in filamentous fungi.

Eukaryotic transposons can be divided into two major groups (18, 42). Class I transposable elements are able to transpose via an RNA intermediate, a process which employs a reverse transcriptase. Well-known members of this type include retrotransposons such as the *copia* element from *Drosophila melanogaster* (17) and the Ty elements from *Saccharomyces cerevisiae* (61).

Class II elements, which transpose via DNA intermediates, demonstrate direct excision prior to their integration into target sequences. The best-known examples are the *Ac-Ds* elements of maize, discovered about half a century ago by Barbara McClintock (40, 41).

Both classes of transposons have previously been identified in filamentous fungi. In a few cases, such as the *Neurospora crassa Tad* element (37) and the *Fusarium oxysporum Fot1* and *impala* elements (10, 39), this was achieved by the use of a transposon trap. Others were discovered by characterizing repeated DNA sequences, leading to the isolation of the *MGR* (24) and *grasshopper* (13) elements from *Magnaporthe grisea*, the *Foret* element from *F. oxysporum* (31), and the *CfT-1* element from *Cladosporium fulvum* (43). The majority of these fungal transposons are class I transposable elements, while only a few belong to class II (*Fot1* and *impala*). Those elements, whose prototypes were found in *Caenorhabditis elegans* or in *D. melanogaster* (7, 28, 46), were characterized as *Tc1-mariner*-like transposons (9).

Here, we describe the isolation and characterization of a class II transposon from *Tolypocladium inflatum* ATCC 34921 (synonym *Beauveria nivea* [20, 60]). This filamentous fungus has gained major biotechnological importance because of its ability to produce cyclosporin, a neutral cyclic peptide of 11 amino acids (11 aa) which is used as an effective immunosuppressant in organ transplantations (6). Genetic manipulation of this fungus is particularly difficult because of the lack of a sexual cycle. Therefore, the discovery of a transposable element may widen the genetic analysis of this fungus by a transposon-based tagging system.

The transposon carries short inverted repeats and 8-bp target site duplications and encodes a large open reading frame interrupted by a single intronic sequence which exhibits alternative splicing. The predicted amino acid sequence deduced from this frame shows significant homology with transposases from the *hAT* transposon family (15, 62). Members of this family include the maize *activator* (*Ac*) element (45) and the *D. melanogaster hobo* transposon (8), which have not yet been discovered in any fungal organism. The genomic distribution of the fungal transposon, its transcriptional and splicing characteristics, and its mode of transposition activity all suggest its potential use in development of a transposon tagging system for filamentous fungi.

MATERIALS AND METHODS

Strains and culture conditions. The following fungal strains were used in this study: *T. inflatum* ATCC 34921, CBS 824.70, and DSM63544; *B. nivea* ATCC 42437 and ATCC 38656; *Tolypocladium cylindrosporum* CBS 719.70; and *Tolypocladium geodes* CBS 723.70. Culture conditions were as described previously (54). *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.) was used for in vitro recombination under standard culture conditions.

Oligonucleotides. Oligonucleotides as given in Table 1 were synthesized for use as sequencing primers and/or for specific PCR and reverse transcription-PCR (RT-PCR) amplifications.

Gene libraries. A library of partially *Sau*3AI-digested genomic DNA of *T. inflatum* ATCC 34921 in vector Lambda-GEM11 was kindly provided by G. Weber (Biochemie GmbH, Kündl, Austria). Another *Sau*3A gene library was constructed with a DASHII lambda vector using XL-packaging extracts as recommended by the manufacturer (Stratagene) in order to obtain larger genomic inserts.

DNA and RNA isolation. Fungal DNA was isolated according to the methods described by Shure et al. (53) and Zhu et al. (65). Total RNA was isolated from fungal strains as follows. A 10-g (fresh weight) sample of mycelia, grown for 7 days, was ground under liquid nitrogen in a mortar, and the powder was mixed with an equal amount of boiling extraction buffer (0.2 M boric acid, 30 mM

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TABLE 1. Numbers and DNA sequences of oligonucleotides used in this investigation

No.	Sequence (5′→3′)	Position (nucleotides) in *restless* sequence or procedure used
FK47	TCAGTGGCCATCCGACAG	3754–3737
FK57	ACGAGAATCCCACGCCTA	1619–1636
FK61	X-TCCGAATTCAGATCTGGC-X[a]	5′ RACE
FK62	ATTTAAATCTATAAAATTAGG	1080–1100
FK63	TGCTACGCGACCCGACGC	1261–1278
FK66	CCTCTCCCAAGCCATCAAGG	3255–3236
FK67	AAGATCGGAGATATCCGTTGG	3945–3965
FK68	CCTTGACCCCTGGAATTAGAAC	176–155
FK71	GATGCGCTGGTCGGCAGG	2033–2016
FK74	GGCGCGCCAGATCTGAATTC	5′ RACE
FK87	TACAGCTGGAAGCGCGTG	2399–2382
FK88	CTCGCGCTTCCAGCTGTA	2382–2399
FK89	TGTCTGCGGGTAAAGAGTC	2703–2685
FK90	GACTCTTTACCCGCAGACA	2685–2703
FK91	AATCCGATTGAGGTATCTGCGGA	2966–2944
FK93	CCTGCCGACCAGCGCATC	2016–2033
FK96	CAGAGTGCGTAATCAACCAA	1–20, 4099–4080
FK97	CTACTTCCAACCGTTGAAGCC	21–41
FK101	TCGGTAGGCGTGGGATTCTCG	1640–1620
FK104	CTCGCGCCATTCTGGCGTC	326–344
FK105	CCGTCTACTCTCACCATCCC	645–664
FK106	TATCAGCGGTCTTCGTCAATA	701–681
FK107	GCTTCCCAGCCGCCACTGC	1442–1460

[a] 5′ X, PO_4; 3′ X, DMT-C6-3′ amine.

EDTA, 1% sodium dodecyl sulfate; pH 9.0, adjusted with NaOH). The supernatant was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1). After an additional extraction with chloroform-isoamyl alcohol (24:1), nucleic acids were ethanol precipitated, and the pellet was dissolved in distilled water. RNA was selectively precipitated by adding LiCl to a final concentration of 2 M. Poly(A) mRNA was isolated with a Dynabead mRNA Direct kit (Dynal, Hamburg, Germany). Bacterial plasmid DNA was isolated by using Quickprep spin columns (Qiagen, Hilden, Germany).

Gel electrophoresis, blotting, and hybridization conditions. Agarose gels, Southern blotting, and DNA-DNA hybridization were performed according to standard procedures (48). A Decaprime labeling kit (Ambion, Austin, Tex.) was used to label 20 to 30 ng of template with [α-^{32}P]dATP. RNA was separated on denaturing formaldehyde gels (48) and transferred to a positively charged nylon membrane (Boehringer, Mannheim, Germany) using a Posiblot apparatus (Stratagene).

Subcloning. Specific fragments from lambda clones and RT-PCR amplifications were cloned into the corresponding restriction sites of the pBluescript/SK$^+$ vector (Stratagene). The corresponding designations and characteristics are given in Table 2.

TABLE 2. Subcloning of DNA fragments from lambda clones 6-39 and 1805-3 into vector pBluescript/SK$^+$

Plasmid	Insert or description
pCE96	PCR amplification product with primers FK57 and FK87 using RNA as a template
pDH54	3.1-kb *Sac*I fragment of λ clone 6-39
pDH55	1.5-kb *Sac*I fragment of λ clone 6-39
pDH56	1.4-kb *Sac*I fragment of λ clone 6-39
pDH57	0.8-kb *Sac*I fragment of λ clone 6-39
pDH82	6.5-kb *Eco*RI fragment of λ clone 6-39
pDH83	2.8-kb *Eco*RI fragment of λ clone 6-39
pDH86	4.4-kb *Hin*dIII fragment of λ clone 6-39
pDH91	1.5-kb *Hin*dIII fragment of λ clone 1805-3
pDH93	2.0-kb *Eco*RI fragment of λ clone 1805-3
pDH94	7.8-kb *Eco*RI fragment of λ clone 1805-3
pFK62	0.8-kb *Hin*dIII fragment of λ clone 6-39
pFK63	1.5-kb *Hin*dIII fragment of λ clone 6-39
pFK64	1.9-kb *Hin*dIII fragment of λ clone 6-39
pML44	2.4-kb *Sac*I fragment of λ clone 6-39
pML45	3.3-kb *Hin*dIII fragment of λ clone 6-39

Sequencing. All sequence analysis was done with double-stranded DNA used as a template. Sequencing was performed either by employing universal and reverse sequencing primers or by using the oligonucleotide primers indicated below. In addition, nested DNA fragments were obtained with a nested deletion kit (Pharmacia, Freiburg, Germany). Sequence reactions were performed by the method of Sanger et al. (49) with a Sequenase kit (Amersham/USB, Cleveland, Ohio) under conditions recommended by the manufacturer. Sequence analysis was performed using the HUSAR-GENIUS program package of the Deutsche Krebsforschungszentrum (Heidelberg, Germany).

Nitrate reductase (*niaD*) mutants. Spore suspensions (10^7/ml) of *T. inflatum* were grown on agar plates containing sodium chlorate (24 g of sodium chlorate, 20 g of sucrose, 3 g of sodium nitrate, 1 g of L-arginine, 0.5 g of $MgSO_4$, 0.5 g of KCl, 0.1 g of $FeSO_4$, and 2% agar-agar in 75 mM potassium phosphate buffer, pH 5.8). Mutant colonies able to grow on chlorate medium were isolated after 10 to 14 days and tested for stability by being replated on chlorate-containing medium. Stable strains were further analyzed in order to distinguish nitrate reductase mutants from mutants with a modified molybdenum cofactor. This was done by making use of the ability of nitrate reductase mutants to utilize hypoxanthine as a nitrogen source (hypoxanthine medium: 20 g of sucrose, 3 g of sodium nitrate, 1 g of hypoxanthine, 0.5 g of $MgSO_4$, 0.5 g of KCl, 0.1 g of $FeSO_4$, and 2% agar-agar in 75 mM potassium phosphate buffer, pH 5.8). Putative nitrogenase mutants were checked for their inability to grow on media with sodium nitrate as the sole nitrogen source. For this purpose, the medium described above was used but hypoxanthine was omitted. Uptake mutants are unable to utilize hypoxanthine. Strains unable to grow on sodium nitrate but showing strong growth on hypoxanthine were tentatively considered physiological nitrate reductase mutants and further analyzed (50).

PCR and RT-PCR amplification. PCR and RT-PCR were performed in accordance with the methods of Saiki et al. (47) and Kawasaki and Wang (33) with some modifications: 0.5 to 1.0 μg of poly(A) mRNA was treated for 1 h at 37°C with 10 U of RNase-free DNase (Boehringer). RT was conducted in a 20-μl volume employing a random hexamer mix (Boehringer) and 50 U of superscript reverse transcriptase (Stratagene); following an incubation at 37°C for 1 h, the enzyme was inactivated at 100°C for 5 min. Subsequent PCR amplification was performed by adding specific primers, 3.0 U of *Taq* polymerase (Boehringer), buffer, and water to a final volume of 50 μl. In parallel, PCR amplification using fungal DNA as a template was also carried out. PCR amplification was performed in a Perkin-Elmer PE9600 thermocycler using the following program: 2 min at 94°C; 40 cycles of 60 s at 94°C, 60 s at 55°C, and 90 s at 72°C; and 15 min at 25°C. Aliquots taken from PCR or RT-PCR amplifications were analyzed by gel electrophoresis. PCR products were separated from unincorporated nucleotides and primer by using a PCR purification kit (Qiagen). Amplification of DNA fragments larger than 3 kb was performed with a PCR-Expand kit (Boehringer).

5′ RACE and asymmetric PCR. The rapid amplification of cDNA 5′ ends (5′ RACE) and asymmetric PCR method used was based on the original protocols of Frohmann et al. (19) and Belyavski et al. (5) as modified by Apte and Siebert

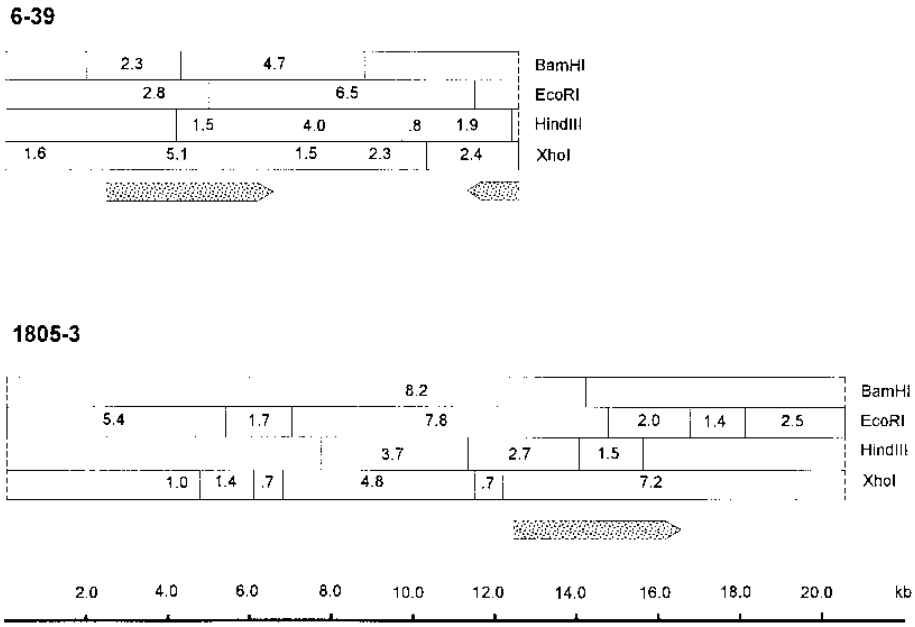

FIG. 1. Physical maps of two λ clones carrying copies of the *restless* transposon. Clone 6-39 is a GEM11 derivative, while 1805-3 has been derived from vector DASHII. Four restriction enzyme patterns are shown, with fragment sizes given in kilobases. The ends of inserts (dashed lines), generated by using enzymes which have no restriction site in the vectors' multiple cloning sites, and the positions of complete copies of the repeated DNA sequence (open rectangles) are indicated. (The bar at the bottom is a size scale.) The orientation of the insert is shown. In λ clone 6-39, a second, partial copy of the sequence is present.

(2). To facilitate direct sequencing, a 0.2- to 3-μl aliquot taken from a successful PCR amplification was used in a further amplification. Reaction conditions were as previously described, with the exceptions that 10 pmol of oligonucleotide FK74 was added and only 0.04 pmol of the specific downstream oligonucleotide FK71 was used. A large excess of single-stranded RNA was obtained, suitable for sequencing with oligonucleotide FK101.

Inverse PCR. Genomic DNA (60 ng) was digested with restriction enzyme *Xho*I, which does not cut transposon *restless*. As a result, DNA fragments which carry copies of transposon *restless* surrounded by its adjacent genomic sequences are generated. The DNA was then ligated with 1 U of T4 DNA ligase (Boehringer). This DNA was then used for PCR amplification employing oligonucleotides FK67 and FK68. Thus, DNA fragments which consist of genomic sequences surrounded by the transposon are amplified selectively.

Pulsed-field gel electrophoresis and chromosomal analysis. Chromosomal DNA was isolated and run on pulsed-field gels as described elsewhere (54). A restriction analysis of high-molecular-weight chromosomal DNA was undertaken, using rare-cutting endonucleases in a previously described method (34, 35).

Nucleotide sequence accession number. The nucleotide sequence in Fig. 3 is available in the EMBL data library under accession number Z69893.

RESULTS

Cloning of repeated sequences, a potential source of transposons. Genetic information in fungi is encoded mainly by single-copy genes; only a few occur in larger copy numbers, e.g., the cluster of genes coding for rRNA (rDNA) (25, 56). Eukaryotic transposons are also present in higher copy numbers, generally known as middle repetitive sequences (17). Consequently, the isolation of repeated DNA sequences may act as a first step in the identification of transposable elements. Recombinant lambda clones which contained repeated DNA sequences from a GEM11 library were identified by using ^{32}P-labeled total genomic DNA as a probe for plaque hybridization (for details, see reference 34). This method allowed the differentiation of clones carrying single- or multicopy sequences (24). ^{32}P-labeled yeast rDNA (pMY60 [59]) was used to identify clones containing *T. inflatum* rDNA sequences (34). About 1% of 4,800 plaques tested contained repeated DNA sequences exhibiting no rDNA sequence homology. Of 50 lambda clones, 9 were shown to have homology with each other, while 2 other clones each contained a 4.1-kb repeated element (RE) flanked by unique sequences. The other seven phage clones contained only fragments of the 4.1-kb element. Restriction fragments of the two lambda clones 6-39 and 1805-3 (Fig. 1) were ligated into vector pBluescript/SK$^+$ as indicated in Table 2.

Genomic distribution of the repeated DNA element. The identification of two copies of an RE, with unique flanking sequences, suggested that this RE might be a transposable element. If this was the case, then the RE should be present in several copies, distributed throughout the entire genome. To confirm this assumption, intact chromosomes were isolated, subjected to pulsed-field gel electrophoresis, and Southern blotted. The chromosomes were then probed with lambda clone 6-39, which contains two copies of the RE (Fig. 2A). All seven chromosomes of *T. inflatum* (54) hybridized with the probe, indicating that the RE is indeed present on all chromosomes. A more detailed view of the RE's genomic distribution was obtained via an analysis of chromosomal DNA restriction fragments (34, 35). *T. inflatum* chromosomes were digested with rare-cutting enzymes, and the fragments were then separated by pulsed-field gel electrophoresis, Southern blotted, and probed with the RE (Fig. 2B). Depending on the restriction enzyme used, up to 11 bands were visible. The different intensities of these bands led us to conclude that more than 11 copies of the RE are present in the genome of *T. inflatum* ATCC 34921.

In order to prove that each RE copy is flanked by unique genomic sequences and to determine the exact copy number, total DNA from *T. inflatum* ATCC 34921 was digested with *Eco*RI, *Hin*dIII, and *Sac*I, subjected to gel electrophoresis, Southern blotted, and probed with fragments from the RE of lambda clone 6-39 (Fig. 2C). A single intensive hybridization band could be seen on the autoradiogram when *Hin*dIII-digested DNA was probed with a ^{32}P-labeled 1.5-kb

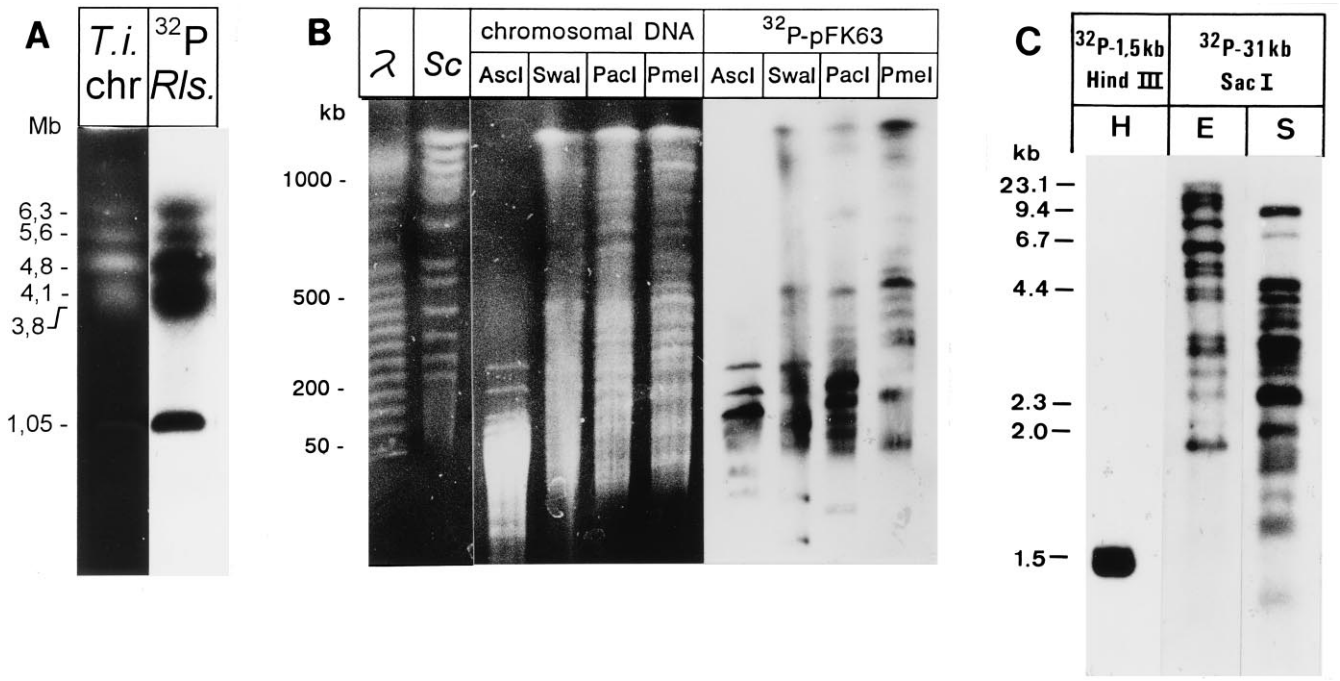

FIG. 2. (A) Chromosomal location of *restless* analyzed by subjecting intact *T. inflatum* chromosomal DNA (*T.i.* chr) to pulsed-field electrophoresis. The gels were blotted and probed with ^{32}P-labeled repeated DNA (^{32}P *Rls.*) (1.5-kb *Hin*dIII fragment of λ clone 6-39, pFK63). All bands hybridize with the probe. (B) Prior to pulsed-field gel electrophoresis, *T. inflatum* chromosomal DNA was digested with rare-cutting restriction enzymes *Asc*I, *Pac*I, *Pme*I, and *Swa*I. A λ ladder (λ) and *S. cerevisiae* (Sc) chromosomal DNA were both used as contour-clamped homogeneous electric field gel electrophoresis size markers. The gel was probed as outlined for panel A. The autoradiogram of the Southern blot shows numerous bands, each indicating a chromosomal fragment hybridizing with the repeated DNA element. (C) Hybridization of *T. inflatum* genomic DNA with different restriction fragments from lambda clone 6-39.

*Hin*dIII fragment from clone 6-39. This single band corresponds to an internal fragment, of equal size, derived from all genomic copies of the RE, suggesting that each RE carries a copy of the 1.5-kb clone 6-39 *Hin*dIII fragment, while *Eco*RI- and *Sac*I-digested DNA was probed with a ^{32}P-labeled 3.1-kb *Sac*I fragment from clone 6-39, in order to detect sequences from the right-hand end of the RE and the adjacent genomic DNA. Since the locations of *Eco*RI or *Sac*I sites outside the RE depend on the adjacent genomic sequences, digestion with an appropriate enzyme should yield each copy of the RE in a unique restriction fragment. This result is shown in Fig. 2C. Taking the different intensities of the bands on the autoradiogram into account, the RE is present in about 15 genomic copies.

Finally, we screened different strains of related fungi (see Materials and Methods) for the presence of the RE. Under stringent hybridization conditions, no other strain showed homology with the *restless* element.

Sequence characteristics of the RE. The two complete RE sequences from lambda clones 6-39 and 1803-5 were established. A third RE sequence, which is only partially present in lambda clone 6-39, was determined (positions 3211 to 4097 in Fig. 3). The two complete 4,097-bp RE sequences seem to be almost identical, except for the few nucleotide substitutions indicated in Fig. 3. Genomic PCR employing an oligonucleotide that hybridizes with the very end of the element also led to the amplification of a single 4.1-kb DNA fragment. No larger or smaller fragments were detected (data not shown). We therefore conclude that all genomic copies have a uniform size. The 20-bp sequence 5′-CAGAGTGCGTAATCAACCAA-3′ is located at the left-hand terminus of the element, and the right-hand end of the RE terminates with a perfect inverted repeat of the same sequence, as expected for a transposon. Clustered within 150 bp of the left- and right-hand termini are six repeats of the short sequence 5′-CCAAC-3′ (Fig. 3). In addition, a larger repeat, 5′-CAAYCCAACTG-3′, present subterminal to the left-hand end is repeated three times at the right-hand terminus.

A large open reading frame interrupted by a small, 47- or 54-bp intron (see Fig. 5) has the capacity to code for a polypeptide of 803 amino acids (aa). Among the transposon copies sequenced, four nucleotide differences were observed. Two, at positions 2826 and 3645, both affect the third base of a codon but do not change the encoded amino acid residue. At position 2657, a GC-to-CG exchange alters a serine codon into a threonine codon, while another substitution at position 1473 (T versus C) also leads to a change in the deduced amino acid sequence (leucine to serine); this position is localized four codons downstream of the proposed ATG start codon. The sequence immediately 5′ and 3′ of the ATG start codon (5′-CTGCAATGG-3′) is apparently similar to a proposed fungal consensus 5′-YCA(C/A)(A/C)ATGG-3′ (58).

Figure 4 shows genomic sequences adjacent to five different copies of the element. It is obvious that 8-bp direct repeats are present immediately upstream and downstream of the termini. The sequences of these direct repeats are not conserved themselves.

Eukaryotic transposons that transpose without an RNA intermediate create short target site duplications upon insertion at a new genomic site; they possess short terminal inverted repeats (TIRs), and they are also frequently characterized by subterminal repeats which serve as transposase binding sites (38, 42, 44). Because the RE described here has all these structural properties, we propose that it is a transposon and have named it *restless.*

Transcript analysis. We were unable to detect transcripts

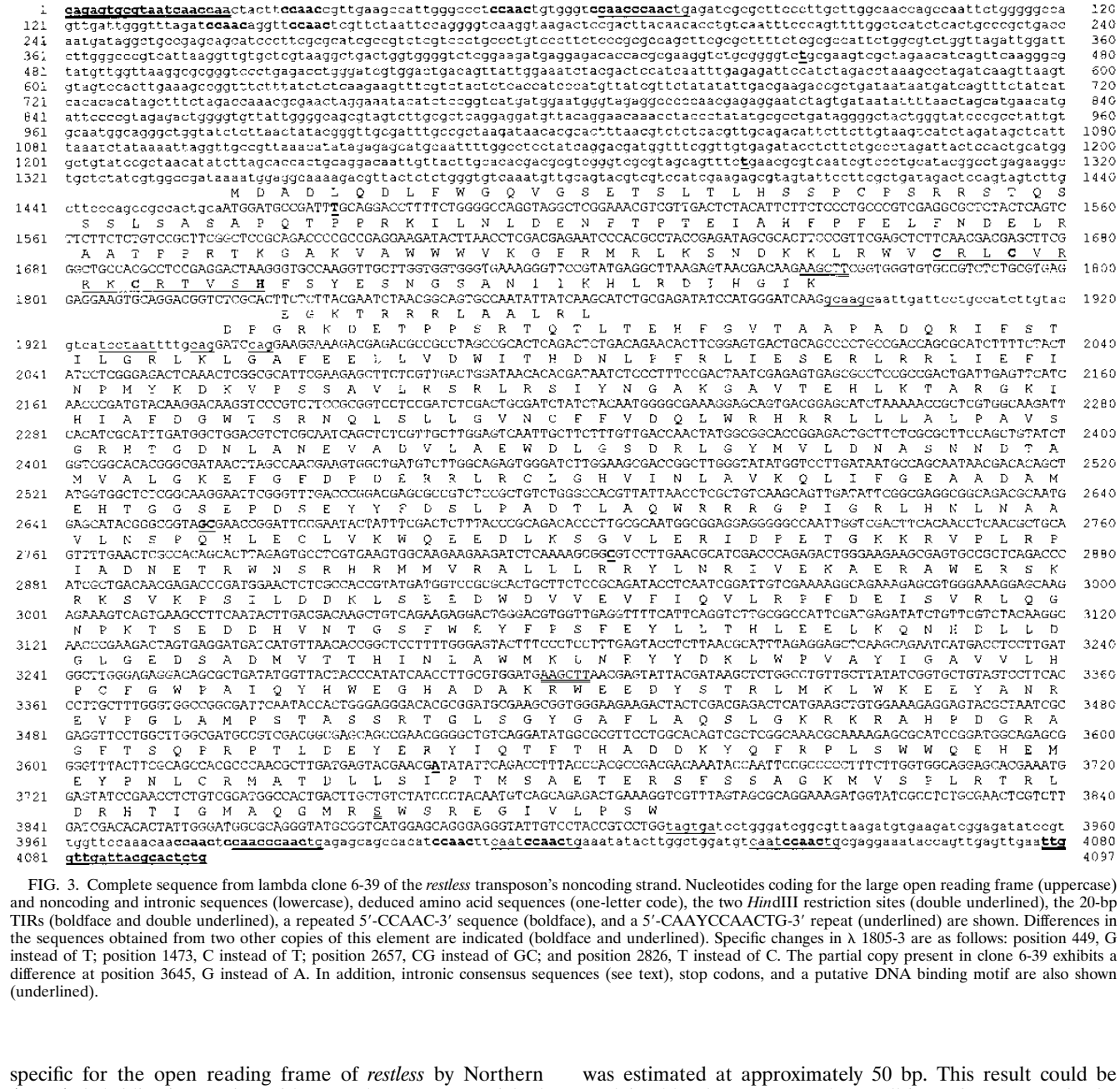

FIG. 3. Complete sequence from lambda clone 6-39 of the *restless* transposon's noncoding strand. Nucleotides coding for the large open reading frame (uppercase) and noncoding and intronic sequences (lowercase), deduced amino acid sequences (one-letter code), the two *Hin*dIII restriction sites (double underlined), the 20-bp TIRs (boldface and double underlined), a repeated 5′-CCAAC-3′ sequence (boldface), and a 5′-CAAYCCAACTG-3′ repeat (underlined) are shown. Differences in the sequences obtained from two other copies of this element are indicated (boldface and underlined). Specific changes in λ 1805-3 are as follows: position 449, G instead of T; position 1473, C instead of T; position 2657, CG instead of GC; and position 2826, T instead of C. The partial copy present in clone 6-39 exhibits a difference at position 3645, G instead of A. In addition, intronic consensus sequences (see text), stop codons, and a putative DNA binding motif are also shown (underlined).

specific for the open reading frame of *restless* by Northern (RNA) hybridizations using either total RNA or enriched poly(A) mRNA. This result may indicate either a low level of transcription or no transcription at all. We also employed the more sensitive reverse-PCR technique as described in Materials and Methods. Enriched poly(A) mRNA was treated with RNase-free DNase to remove any DNA contamination; PCRs were performed with specific primers from cDNA, generated with nonspecific hexamer primers; and corresponding DNA fragments were amplified as a control. Two primer combinations (FK93-FK91 and FK90-FK47) gave rise to identical-size PCR products irrespective of whether a DNA or an RNA template was used (Fig. 5A). In contrast, the reverse-PCR product obtained with primers FK107 and FK71 and an RNA template had a slightly increased electrophoretic mobility compared with that of the PCR product derived from a DNA control. By comparing DNA and RNA template derivatives with a 123-bp size marker, the difference in PCR product size was estimated at approximately 50 bp. This result could be explained by the presence of a small intronic sequence in the DNA copy. To test this assumption, cDNAs from RT-PCR were cloned, sequenced, and compared with the genomic DNA sequence. Surprisingly, two different cDNAs were detected. The two have the same 5′ splice site, 5′-GCAAGC-3′, which is unusual, as almost all other fungal introns carry a thymidine residue rather than a cytosine residue at the second position of the 5′ splice site (58). The location of the 3′ splice site (5′-CAG-3′) in each cDNA varies by 4 nucleotides so that the introns have sizes of 47 and 54 bp (Fig. 5B). A total of 17 cDNAs were analyzed, 5 of which carried the 47-bp intron and 12 of which carried the 54-bp intron. The latter creates a reading frame shift, and consequently a truncated polypeptide may be translated from this differentially processed mRNA. Alternative splicing may therefore lead to the expression of two polypeptides, one of 803 aa and the other of 157 aa.

The low steady-state abundance of *restless*-specific tran-

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c1 ---cgagCTTGCCGGcagag---//---ctctgCTTGCCGGcgcc---
c2 ---gttgAGAACGACcagag---//---ctctgAGAACGACcttg---
c3 ---tgaaGATGGAAAcagag---//---ctctgGATGGAAAcgcc---
c4 ---aggaAGTTCTGGcagag---//---ctctgAGTTCTGGcgat---
c5 ---cttcGTCCTGCAcagag---//---ctctgGTCCTGCAtatc---
   genomic--TSD-->&lt;====restless====&gt;--TSD-->genomic
```

FIG. 4. *restless* transposon target site duplications. Fragments overlapping the termini of five transposon copies were cloned and sequenced by using primers FK67 and FK68, specific for the right- and left-hand ends of the fragment, respectively. The five copies were named c1 to c5 (from λ clones 6-39, 1805-3, 2037-2, 2037-3, and 2037-4, respectively). Terminal nucleotides from each of the five *restless* transposon copies are given in lowercase italics. The remaining sequences (– – –//– – –) are not shown. The 8-bp target site duplication (TSD), a short direct repeat generated by the transposon, and adjacent genomic sequences are shown. For more details, see the text.

scripts made it impossible to map the transcription start site by standard techniques, such as primer extension. Instead, we used a modified version of the 5′-RACE technique (5, 19). As shown in detail in Fig. 5C, transcription is initiated at position 1282, 167 bp in front of the ATG start codon. Potential promoter elements 5′-CAAT-3′ and 5′-TAGATTA-3′ are located 157 and 101 bp upstream, respectively. A map of the *restless* element showing the positions of exons and introns is given in Fig. 5D.

Despite its rather low abundance, the existence of a spliced mRNA from the *restless* transposon, initiated 167 bp in front of the ATG codon, strongly indicates that the element is active.

***restless* is an active transposon.** In order to test the ability of *restless* to excise and to reintegrate elsewhere in the host genome, we analyzed *T. inflatum* lines obtained from a program to generate strains with a mutated nitrate reductase gene (*nia*$^-$) (unpublished data). Nitrate reductase genes have been cloned from a number of fungi (e.g., see references 11, 23, and 63) and are useful as transposon traps (e.g., see references 10 and 39). Putative *nia* mutants can be distinguished from uptake or cofactor mutants by their ability to utilize hypoxanthine as a nitrogen source (see Materials and Methods). DNA was isolated from *nia* mutants, digested with *Eco*RI, subjected to gel electrophoresis, Southern blotted, and probed with an *Eco*RI fragment (pDH83) from lambda clone 6-39 carrying 2.5 kb of *restless* and flanking genomic sequences. The hybridization patterns in Fig. 6A were produced by using both wild-type DNA and DNAs isolated from the five individual isolates generated as mentioned above. On the autoradiogram, each band represents a specific copy of *restless* at a different genomic location. As may be deduced from the figure, one of the several bands present in the *T. inflatum* wild-type strain is absent from all the other isolates, while these isolates all carry additional bands not present in the wild-type DNA. The data suggest that single copies of *restless* are able to excise and reintegrate at different locations in the *T. inflatum* genome. In order to distinguish recombination events from true transposition, inverse PCRs were performed with genomic DNA from *nia*$^-$ mutants, followed by separation of the amplification products on agarose gels. As shown in Fig. 6B, some PCR fragments occur exclusively in specific *nia*$^-$ mutants, indicating that they are the result of transposition events. This was further tested by cloning and sequencing of the two bands indicated in Fig. 6B. In both cases, target site duplications of 8 bp were present (Fig.

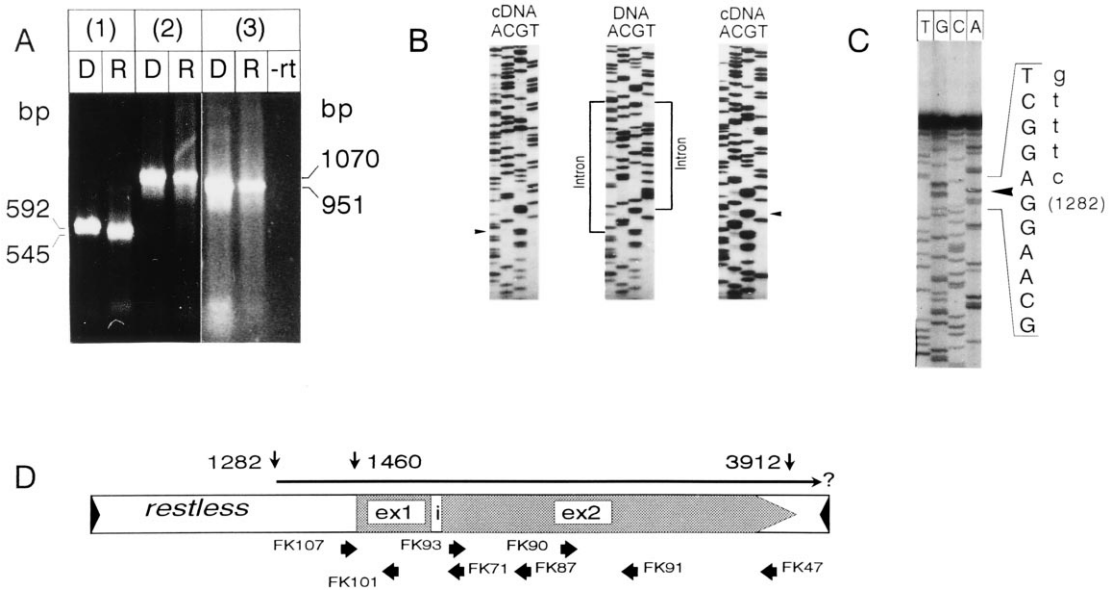

FIG. 5. (A) Transcript analysis by RT-PCR. *restless*-specific oligonucleotide pairs were employed in a PCR using random synthesized cDNAs. Abbreviations: D, DNA template; R, RNA template (RT-PCR); −rt, no RT prior to PCR. Lanes 1, oligonucleotides FK107 plus FK71; lanes 2, FK90 plus FK47; lanes 3, FK93 plus FK91. (B) Presence of an intron, which exhibits alternative splicing, in the *restless* transposon's coding region. Both cDNAs from RT-PCR and genomic PCR amplification products obtained with FK107-FK71 were cloned and sequenced with oligonucleotide FK71. The cDNAs lack a 47- or 54-bp fragment, respectively, depending upon the splice site consensus sequences (see text). (C) Identification of the transcription initiation site by 5′ RACE. By using oligonucleotide FK87, located at positions 2380 to 2394, a cDNA was synthesized. A specially synthesized oligonucleotide (FK61) with blocked 3′ ends and phosphorylated 5′ ends was ligated to the 3′ end of the cDNA. A first PCR amplification employed oligonucleotides FK74 (which binds to FK61) and FK87. Seminested PCR was performed using oligonucleotides FK74 and FK71. The final amplification product was approximately 770 bp (data not shown). To obtain the transcript's exact initiation point, the amplified DNA was directly sequenced. The point at which the *restless* sequence fuses into that of oligonucleotide FK61 (uppercase letters) is indicated (arrowhead). The expected genomic DNA sequence of *restless* at this position (lowercase letters) is also shown. (D) Schematic representation of the *restless* transposon and the locations of oligonucleotides used in the different experiments. The exons (ex1 and ex2) (shaded boxes), separated by an intron (i), are shown. The orientation is given by the arrow at the 3′ terminus of exon 2. TIRs (black triangles), transcript initiation sites or translational start and stop codons (vertical arrows), and the orientations and locations of oligonucleotides used in the various experiments (horizontal arrows) are indicated.

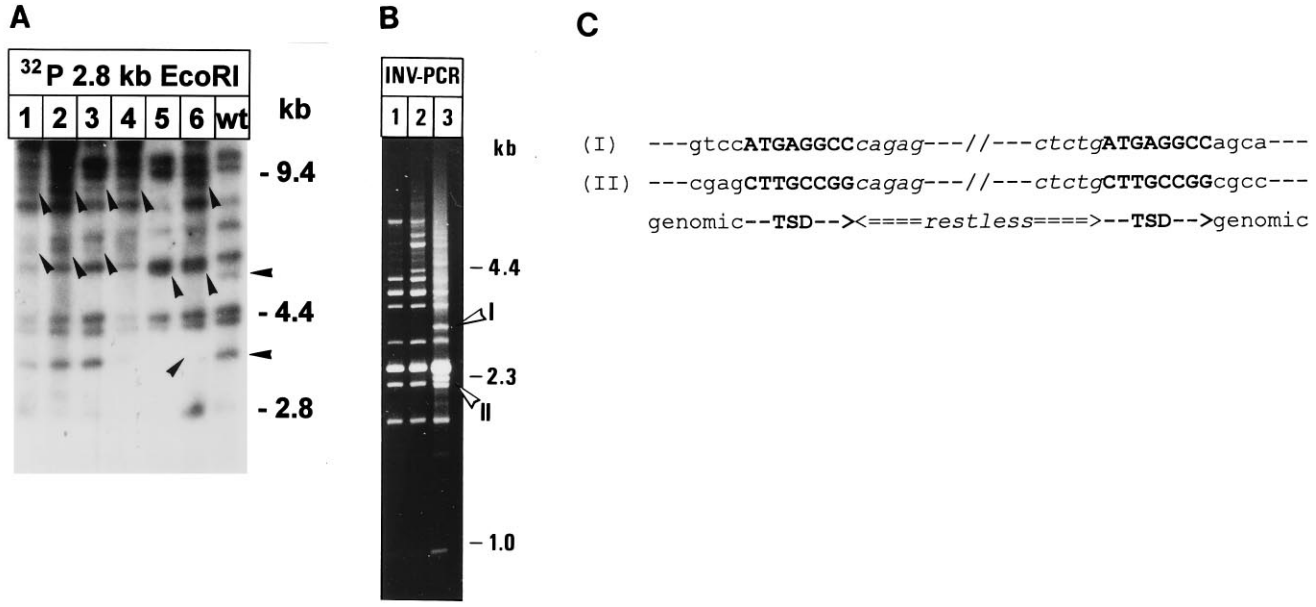

FIG. 6. Transposition of the *restless* element. (A) Single spore lines (*nia*$^-$ mutants) are indicated by numbers and were obtained as described in Materials and Methods. Total DNAs from the different lines were isolated, digested with *Eco*RI, subjected to gel electrophoresis, and Southern blotted. The blot was hybridized with the 2.8-kb *Eco*RI fragment from lambda clone 6-39 (pDH83). Differences between the hybridization patterns obtained from the individual *nia*$^-$ mutants and the original wild-type *T. inflatum* strain (wt), used for selection of mutants, are indicated (arrowheads). For more details, see the text. (B) Inverse-PCR amplification of DNAs from *nia*$^-$ mutants 2 (lane 1), 4 (lane 2), and 5 (lane 3). Genomic DNA was prepared for inverse PCR as described in Materials and Methods. PCR amplification was carried out with oligonucleotides FK67 and FK68. Two amplification products (I and II) that are present only in *nia*$^-$ mutant 5 are indicated. (C) Sequence analysis of the two amplification products (I and II) indicated in panel B. Sequences were obtained by using oligonucleotides FK67 and FK68. In both cases, target site duplications of 8 bp are present. For symbols and abbreviations, see the legend to Fig. 4.

6C). This result shows clearly that the two copies analyzed by DNA sequencing are the result of transposition events that occurred in the *nia*$^-$ mutants.

DISCUSSION

The *restless* transposon belongs to the eukaryotic *hAT* transposon family. Quite a number of transposable elements in filamentous fungi have been isolated and characterized. Besides the multiple class I transposons (24, 31, 37, 43), some class II transposons, belonging to the so-called *Tc1-mariner*-like transposon family, have also been identified (9, 10, 22, 32, 39). This transposon family was previously identified in the worm *Caenorhabditis elegans* and the fruit fly *D. melanogaster* (7, 28, 46). In addition, a very small transposon was identified in *N. crassa* (64). However, the transposon described in this paper differs from all these class II elements, both structurally and by its capacity to code for a large polypeptide. While the fungal counterparts of the *Tc1-mariner* family have larger TIRs and very short target site duplications of 2 bp (e.g., see reference 10), *restless* has 20-bp TIRs with 8-bp target site duplications. The *restless* TIR sequence exhibits similarity with TIR sequences from the *hAT* transposable element family (Fig. 7A), in which 8-bp target site duplications are a characteristic feature. The best known representative of this group is the maize *Ac* transposon (45). The affiliation between *restless* and the *hAT* family is further demonstrated by the presence of three conserved amino acid sequence motifs, located within the putative polypeptide encoded by exon 2 of the *restless* open reading frame (Fig. 7B). These motifs are typically found among *hAT*-encoded polypeptides (15). Therefore, *restless* apparently represents a novel type of fungal transposable element.

***restless* is a dispersed genetic element.** The *restless* transposon copy number was estimated to be about 15 per haploid genome. Other transposons from the *hAT* family usually occur at a frequency of 10 to 50 copies (*hobo* [55]), while fungal class II transposons generally have copy numbers between 2 and 100 (*Fot1* [10]). The *restless* copy number lies within this range. In contrast, class I transposons often occur in much higher copy numbers, which in the case of some plants even exceed 10^6 (18).

hAT transposons may exist in two forms: autonomous copies, capable of independent transposition (e.g., *Ac* [45] [see above]), and nonautonomous elements (e.g., *Ds* [14]), which must be *trans* activated by the gene product of an autonomous element in order to transpose. Nonautonomous copies often arise as the result of internal deletions (14). However, *restless* exists as a homogeneous size class in the *T. inflatum* genome. PCR and Southern hybridization experiments gave no evidence for copies smaller than 4.1 kb (data not shown). It is still possible that there exist nonautonomous copies which carry a small number of point mutations.

Finally, our data suggest that *restless* is present exclusively in *T. inflatum* ATCC 34921. Strain specificity is not an uncommon feature of transposable elements. For example, the class II transposon *Tag1* from *Arabidopsis thaliana* has been found only in a single ecotype (57).

Evolution of *hAT* transposons. *hAT* transposons have been found in plants, fungi, insects, and worms (for a review, see reference 15). Their presence in such diverse organisms suggests that they were once present in ancestors common to them all. The alternative is that horizontal gene transfer must have occurred frequently among completely unrelated organisms. The latter possibility is unlikely, although horizontal gene transfer might have contributed to the spread of *hAT* transposons among related strains and species, e.g., the *hobo* element of *D. melanogaster* (36). The fact that strains and species

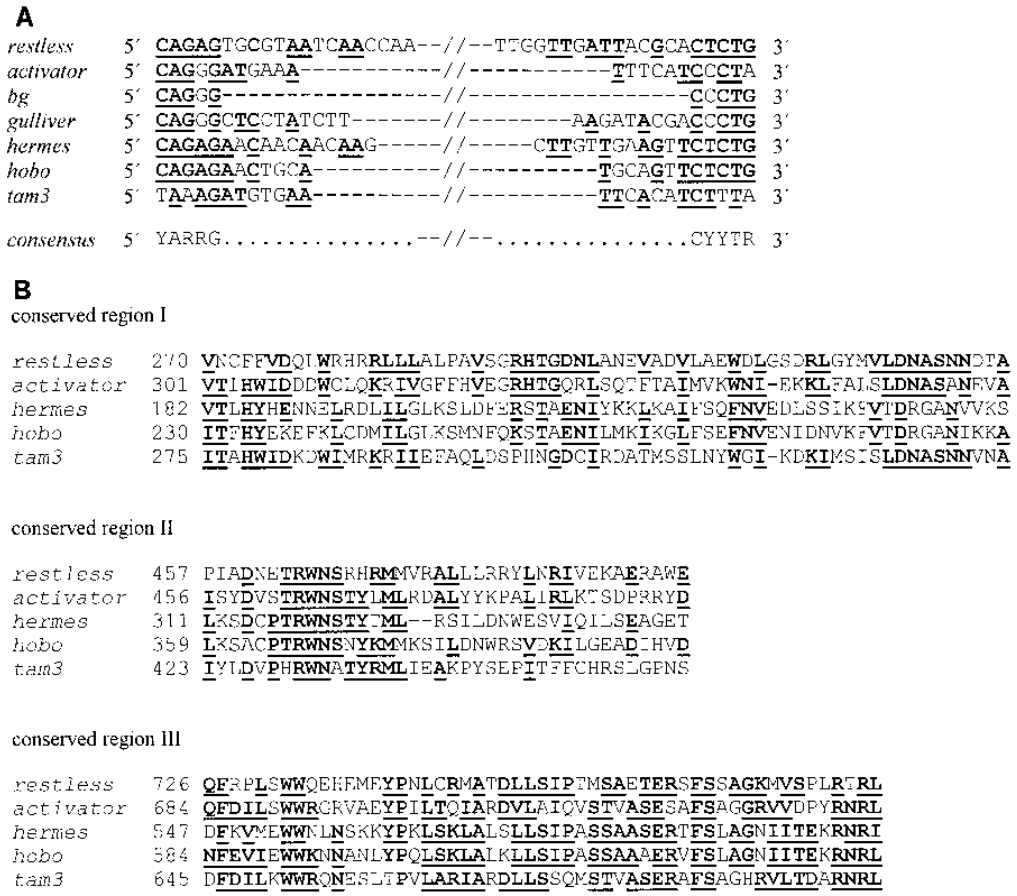

FIG. 7. (A) Comparison of TIR sequences from *restless* with those of other transposons from plant and insect sources: *activator* (*Zea mays*), *hermes* (*M. domestica*), *hobo* (*D. melanogaster*), and *Tam3* (*Antirrhinum majus*). Only the inverted repeat sequences at the ends of the transposons are shown; internal nucleotides are indicated by interrupted dashes (– – –//– – –). Residues common to *restless* and any of the other transposable elements (underlined boldface letters) and two nucleotides which are conserved among all transposons (boldface) are indicated. (B) Comparison of putative transposase amino acid sequences from the *hAT* element family. Three main conserved regions are shown. Regions II and III are as previously described (15). Amino acid sequences from the *Ac* element from *Z. mays* (45), *hermes* from *M. domestica* (62), *hobo* from *D. melanogaster* (8), and the *Tam3* element from *A. majus* (27) were compared. Identical or similar amino acid residues with respect to *restless* are indicated (underlined boldface lettering).

related to *T. inflatum* do not possess the *restless* element may be explained in two ways. (i) Even among closely related species, such as *D. melanogaster* and *Musca domestica*, the *hAT* transposons differ significantly, making it difficult to identify elements by Southern hybridization (62). (ii) Some fungi might have lost or actively eliminated transposons. For instance, in *N. crassa*, a mechanism that is able to inactivate repeated DNA sequences by introducing point mutations has been detected (50a, 51).

Expression and activity of the *restless* element. The most important question concerning a newly identified transposon in any genome is its biological activity. We have undertaken several approaches to elucidate this problem. First, we compared the sequences from two different copies of *restless*, which were virtually identical with the exception of a few point mutations. The data are consistent with those expected for an active transposon because without selective pressure, a sequence might not be conserved. Alternatively, either a recent introduction of the transposon into *T. inflatum* or a case of concerted evolution may be assumed. Second, transcription of the *restless* element was analyzed. Our results indicate that it is transcribed at a very low level. The identification of intronic sequences spliced from a precursor RNA proves that *restless* is transcriptionally active. Interestingly, the 5′ splice site differs significantly from most fungal sequences of this type (58), since a cytidine, rather than a thymidine residue, is located at the second position. However, the significance of this finding is not known. Most surprisingly, our data indicate that alternative splicing occurs in the pre-mRNA of *restless*. As far as we are aware, this is the first case of its kind, not only for a *hAT* transposon but also for fungi. Alternative splicing itself has been repeatedly reported for several organisms (e.g., references 12 and 29). For example, in *D. melanogaster*, alternative splicing of transposon *P* restricts its activity to generative cells (52). Surprisingly, the difference in *restless* intron length is only 7 bp, although recently a similar case of alternative splicing was reported for the green alga *Euglena gracilis*. In this case, alternative splicing of the chloroplast *roaA* transcript results in two polypeptides 514 and 516 aa in length (30). However, while alternative splicing of the *restless* transcript is caused by choice of a different 3′ splice site, it is the use of alternative 5′ splice sites that generates different *roaA* mRNAs.

Studies of the related transposase from the *Ac* element revealed that a DNA-binding region located towards the N terminus of the polypeptide binds to subterminal repeated *Ac* sequences (16, 38). As shown in this paper, similar subterminal repeats are present in the *restless* transposon and are potential binding targets for the *restless*-encoded polypeptide (trans-

posase). About two-thirds of the cDNAs might be translated into 157-aa polypeptides, while only a minor part of all cDNAs encode the full-length 803-aa polypeptide. Although the existence of these proteins still has to be proven, they may provide the basis of a regulatory mechanism. Within the coding region of the 157-aa polypeptide, a potential DNA binding motif is present (Fig. 3). Further studies will focus on whether this 157-aa polypeptide binds to *restless* subterminal repeats, thereby blocking binding between these sites and the full-length polypeptide.

The 5′ end of the mRNA was mapped 180 bp in front of the putative methionine initiation codon by employing the 5′-RACE technique (2, 5, 19). The function of sequences further upstream is unknown but may be associated with *cis*-acting elements involved in transcriptional regulation.

Evidence presented in Fig. 6 let us conclude that at least some of the *restless* transposons are capable of excision and transposition into a new genomic site. We can exclude recombination as the source of changes observed, since the transposed copies possess target site duplications, which are generated through transposition events only. The question remains whether only a few transposon copies are able to excise. This may be influenced by factors such as chromatin structure. Alternatively, one may assume that only a few transposon copies may be functional within the genome. Although all copies of the transposon are identical in size, point mutations, as shown in Fig. 3, may render most copies inactive.

***restless*, a valuable tool for use in gene tagging.** The insertion of a transposable element both disrupts and tags a structural gene. These elements can therefore provide an invaluable role in isolation and identification of genes (4, 21). For instance, the maize *Ac-Ds* transposable system was successfully employed in a large number of plants, including *Petunia* (26), tobacco (3), and *Arabidopsis* (1) plants. We therefore suppose that a fungal representative of this type of transposon would be a promising candidate for use in a fungal transposon tagging system.

Strain improvement in *T. inflatum* is hampered by the inability of this fungus to undergo a sexual cycle. In an attempt to circumvent this problem, electrophoretic karyotypes of both *T. inflatum* and its related strains were established (54). In addition, a strain-specific unique repeated DNA element (*CPA*) was also characterized (34). To date, isolation of genes involved in regulating cyclosporine synthase gene expression has been unsuccessful, largely because of limitations in standard molecular genetic approaches. However, *restless* might be the appropriate tool with which to circumvent some of these disadvantages. This assumption is based on *restless*'s transcriptional activity as well as its ability to be excised and reintegrate into the genome. As a first step towards developing a fungal gene-tagging system, the transposon will be cloned into a suitable vector, which will allow us to select for excision from its donor site.

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