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Genetics

Transnuclear retrotransposition of the Tad element of *Neurospora*

(LINE-like element/retrotransposon/heterokaryon)

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ABSTRACT **Tad is a LINE-like DNA element found in *Neurospora crassa*. A *Neurospora* artificial intron based on the first intron of the *am* (glutamate dehydrogenase) gene was constructed and introduced, in the correct orientation, into a unique *Nru* I site in open reading frame 1 of an active Tad element, Tad1-1. Transformants containing the Tad element with the artificial intron were placed in forced heterokaryons with strains lacking Tad elements. Tad was shown to transpose between nuclei in these heterokaryons. Examination of the transposed Tad elements showed that the intron had been precisely removed in all cases. This confirms that Tad is a retrotransposon and that there is a cytoplasmic phase in these retrotransposition events.**

LINEs (long interspersed repetitive DNA elements) and LINE-like elements constitute one of the most abundant classes of transposable elements. They have been identified in a wide variety of eukaryotic organisms and may constitute as much as 5% of the genome (1–4). Despite their wide distribution and presumed importance in genome dynamics, relatively little is known about the mechanism of transposition or how transposition is regulated.

Tad is a transposable element found originally in Adiopodoumé, a strain of *Neurospora crassa* collected from the Ivory Coast (5). It was initially identified by using the *am* (NADP-specific glutamate dehydrogenase) gene as a transposon trap (5). DNA sequence analysis of two active Tad elements (E. Cambareri, J. Helber, and J.A.K., unpublished results) revealed that Tad is a LINE-like transposon with two major open reading frames (ORFs) on the plus strand. As is typical of LINE-like elements, ORF1 of Tad shows significant homology with ORF1 from other elements in this group only near the carboxyl terminus of the putative 486-amino acid protein. ORF2 contains several blocks of conserved sequence characteristic of reverse transcriptase (RT) (6). Analysis using several different algorithms suggests that the RT domains are most closely related to the RT domains of other LINE-like elements. Like other LINE-like elements, Tad has no terminal repeats. Since LINE-like elements were thought to be retrotransposons (and therefore might have a cytoplasmic intermediate), the ability of Tad to transpose between nuclei within heterokaryons was tested (7). Transnuclear transposition was observed, indicating that there is a cytoplasmic intermediate in Tad transposition, consistent with the idea that Tad is a retrotransposon.

To test for retrotransposition of a yeast Ty element, Boeke *et al.* (8) placed a block of sequence containing a yeast intron into a marked Ty element and demonstrated that the intron was precisely spliced during transposition. This was the first direct demonstration that a presumed retrotransposon actually transposed through an RNA intermediate. Recently, similar experiments have been used to demonstrate that members of the LINE-like family of transposons are retrotransposons as had been predicted (9–12). In this study I have constructed an artificial intron (AI) for *Neurospora* and placed it directly within the coding sequences of ORF1 of the active Tad1-1 element. If this intron were not spliced from the Tad RNA it would result in premature termination of translation of ORF1. I have found that the AI is efficiently and precisely spliced from Tad during transposition, confirming that Tad is a retrotransposon.

MATERIALS AND METHODS

Plasmid Construction. The plasmid pNCi was constructed by ligation of the PCR product shown in Fig. 1 into pBS(−) (Stratagene) which had been cut with *Sma* I. The AI was removed from pNCi by digestion with *Sna*BI and *Pvu* II and ligated into various plasmids as described in the text. Restriction enzymes were obtained from BRL, New England Biolabs, Promega, or Stratagene. DNA ligase was obtained from BRL. All enzymes were used with buffers supplied by the manufacturers and according to their instructions.

***Neurospora* Transformation.** Cells were made competent as described (13). Spheroplasts were transformed with plasmid DNAs with selection for either am^+ (growth on minimal medium containing glycine at 1.5 mg/ml) or resistance to hygromycin (300 μg/ml). Primary transformants were made homokaryotic by repeated streaking and single conidial colony isolation on selective medium.

Heterokaryon Transfer Experiments. Forced heterokaryons between transformants bearing introduced Tad elements and naive strains were constructed, passaged, and broken down as described (7).

DNA Isolation and Analysis. DNA was isolated by the methods of Metzenberg and coworkers (14, 15). Colony blot hybridization was performed as described (16). Southern blots were prepared (5) with charged nylon membranes (NEN or Cuno). PCR was carried out according to the protocol from the manufacturer of the *Taq* polymerase used (Perkin–Elmer). Primers are described in Fig. 1 and the text. Sequencing of constructs and cloned PCR products was by the dideoxynucleotide procedure (17) using Sequenase (United States Biochemical) and protocols provided by the manufacturer.

RESULTS AND DISCUSSION

Construction of the *N. crassa* AI. It has been shown (17, 23) in yeast that an AI integrated into a variety of genes is efficiently and precisely spliced. The AI was constructed so that it could be introduced into any site created by restriction enzymes that leave blunt ends. These results were consistent with a model that assumes that all of the specific sequences required for efficient splicing in yeast are contained within the intron.

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Abbreviations: ORF, open reading frame; AI, artificial intron; ex-het, ex-heterokaryotic homokaryon.

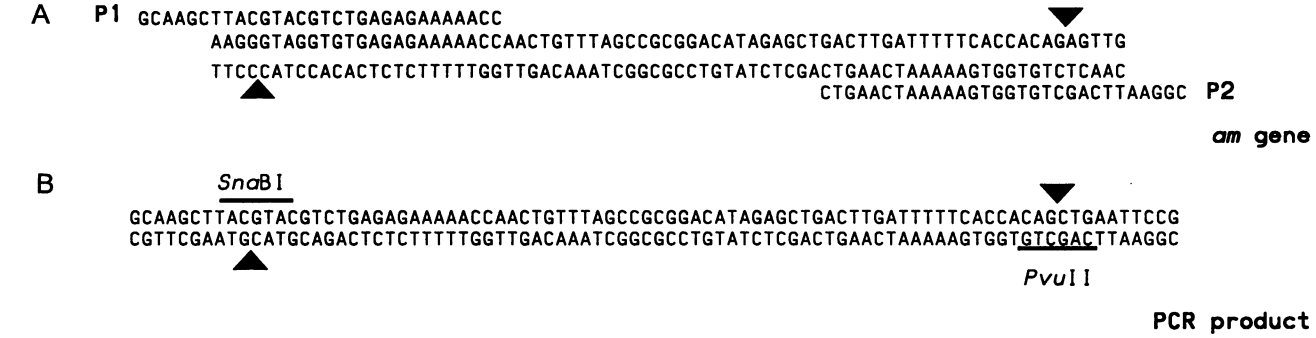

FIG. 1. Sequence of intron 1 of the *am* gene and primers used to make an artificial intron. (*A*) Sequence of the *am* gene surrounding intron 1 is shown as double-stranded DNA. Primer P1 can anneal to the bottom strand, and primer P2 to the upper strand. Arrowheads indicate the intron–exon junctions. (*B*) Sequence of the PCR product obtained when primers P1 and P2 were used for amplification. Note that digestion with *Sna*BI and *Pvu* II would precisely release the intron.

Examination of sequences surrounding introns that are found within a variety of *N. crassa* genes (ref. 19 and unpublished work) suggested that *Neurospora*, like yeast, might require no specific sequences from outside of the introns for precise splicing. The first intron of the *am* gene is a typical *Neurospora* intron. It is 66 nucleotides long (20), beginning with the sequence 5′-GTA-3′ (half of an *Sna*BI site) and ending with the sequence 5′-CAG-3′ (half of a *Pvu* II site). Consequently I used a PCR technique to construct a cloned intron that could be precisely removed from surrounding sequences by *Sna*BI/*Pvu* II double digestion (see Fig. 1 for sequence of the intron and the primers used for amplification). The PCR product was cloned into the *Sma* I site of pBS to give a plasmid designated pNCi.

The *Neurospora* AI Is Efficiently Spliced from a Heterologous Site in the *am* Gene. To determine whether the AI could be accurately spliced when introduced at a heterologous site in *Neurospora*, the AI was inserted into a unique *Eco*RV (blunt) site within the coding sequences of the *am* gene (20) contained in plasmid pJR2 (21). Two AI-bearing plasmids were isolated (Fig. 2), pJR2i (with the AI in the correct orientation with respect to *am* transcription) and pJR2ir (with the AI in the inverted orientation). All junctions and orientations were confirmed by DNA sequencing. These plasmids were used in transformation experiments with strain J1264, which contains the am^{132} deletion of the *am* locus.

Plasmid pJR2i gave transformation frequencies that were indistinguishable from those obtained with the parent plasmid, pJR2 ($\approx 10^3$ transformants per μg of DNA), whereas no *am* prototrophic transformants were obtained with pJR2ir in repeated attempts. This suggested that the AI placed in the correct orientation in the *Eco*RV site was efficiently and accurately spliced.

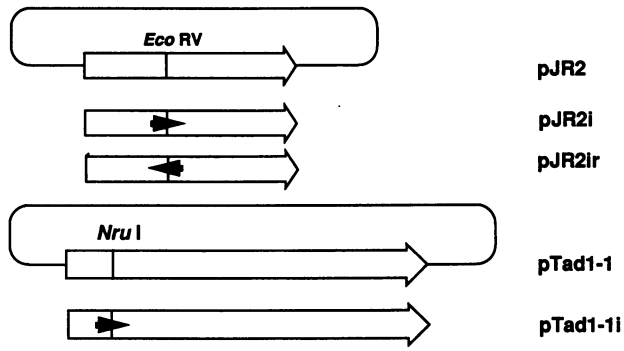

FIG. 2. Schematic representation of plasmids used in this study. In each case the open arrow indicates either the *am* gene (pJR2 and derivatives) or the Tad transposon. The arrow is pointing in the direction of transcription. The black arrow indicates the intron integrated into these genes. When the open and black arrowheads are pointed in the same orientation, the intron is inserted in the correct orientation.

Introduction of the AI into Tad1-1. E. Cambareri, J. Helber, and I (unpublished data) have isolated and sequenced an active Tad element which has been designated Tad1-1. Within the sequence that codes for ORF1 there is a unique *Nru* I site. Cleavage at this site interrupts codon 92 of the putative ORF1 protein. To introduce the AI into this site, the plasmid pTad1-1 (which contains the Tad1-1 element with 2 kb of surrounding chromosomal DNA within the *Bam*HI site of pBS) was cut with *Nru* I and ligated with the AI fragment. A plasmid with the intron in the correct orientation (confirmed by sequence analysis) was designated pTad1-1i.

The pTad1-1i plasmid was introduced into the am^{132} strain J1264 (which has no sequences that hybridize to Tad under stringent hybridization conditions) by cotransformation with the hygromycin-resistance plasmid pDH25 (22). Hygromycin-resistant transformants were selected and made homokaryotic by repeated streaking and single conidial colony isolation. Strains that contained at least one integrated copy of full-length Tad1-1i element were identified by hybridization of colony blots and genomic Southern blots with a variety of Tad probes (data not shown).

The Tad1-1i Element Retains Activity. Active Tad elements can transpose between nuclei in heterokaryons (7). The AI in the *Nru* I site of ORF1 introduces an in-frame UGA termination codon. Since a functional ORF1 is presumed to be essential for Tad activity, the Tad element in pTad1-1i should only be able to transpose between nuclei if the intron has been removed by splicing. To test this, forced heterokaryons were constructed, as described (7), between Tad 1-1i-containing transformants (*am* was used as the forcing marker) and a Tad-naive strain, J326, bearing the *lys-1* marker. After five or six serial transfers on medium selective for the heterokaryon, lysine-requiring ex-heterokaryotic homokaryons (ex-hets) were recovered as described (7). Ex-hets were screened for the presence of Tad DNA sequences by colony blots. Several ex-het strains with positive signals indicated that introduction of the AI at the *Nru* I site did not inactivate Tad.

The Intron Is Spliced from Tad1-1i During Transposition. Genomic Southern blot analysis was used to show that Tad sequences were present in ex-hets and to give preliminary evidence that the intron is precisely removed. Fig. 3 shows an example of the kinds of data obtained. DNA from the transformant T5.4 (T) was digested with *Eco*RI, *Nru* I, or both, and the fragments were separated by gel electrophoresis, blotted to nylon membrane, and probed with an internal *Eco*RI–*Nru* I fragment from Tad1-1 (see Fig. 3*A*). Transformant T5.4 has at least four independently integrated copies of Tad1-1i as indicated by the bands in the DNA digested with only *Eco*RI. Double digestion with *Eco*RI and *Nru* I would

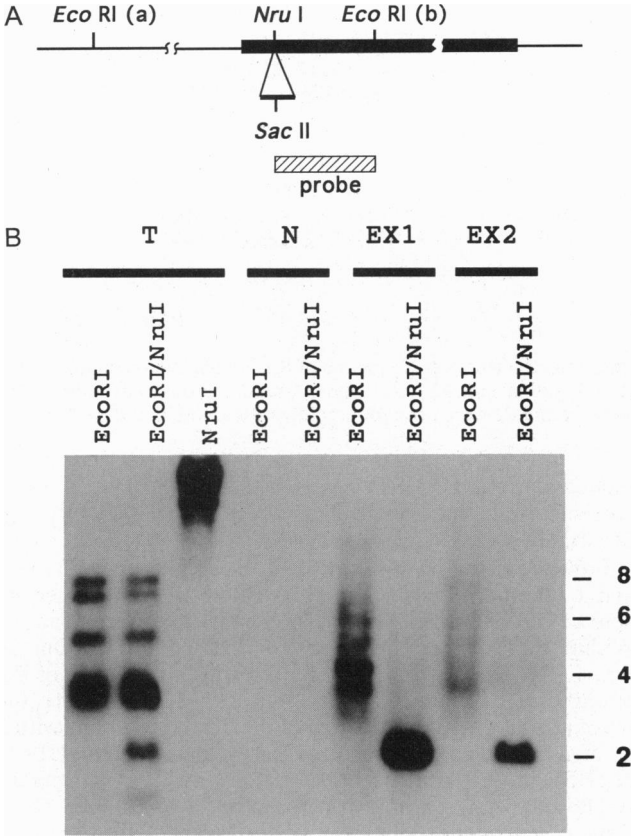

FIG. 3. Genomic Southern digest of a transformant, the naive nuclear type, and two ex-het strains. (*A*) A schematic representation of pertinent restriction sites. The thin bar represents chromosomal DNA flanking a Tad element. Tad is shown as a thick line. *Eco*RI (a) represents a flanking site at a variable distance from the 5′ end of Tad elements. *Eco*RI (b) is within Tad. The *Nru* I site is within Tad elements that lack an AI. The intron, shown below Tad, disrupts the *Nru* I site and introduces a *Sac* II site. The probe used in *B* is shown as a hatched bar. (*B*) One microgram of DNA was digested in each case with the enzyme(s) indicated. T, transformant T5.4; N, strain J326 (*lys-1*), which was used as the naive nuclear type in heterokaryons; EX1 and EX2, two independently isolated lysine-requiring ex-hets (see text for details). Numbers at right refer to positions of size standards in kilobases.

not be expected to change the size of bands which still contain the intron, unless there were an *Nru* I site in nearby flanking DNA. As expected, the bands in the transformant were unaffected by *Nru* I digestion. The appearance of a new band of the size expected if the intron had been removed, without the loss of the major bands seen in the digest with *Eco*RI alone, suggests the presence of numerous transposition events, each of which is characteristic of only a few nuclei in the population. If this were the case, such events would be expected to contribute to the general background in *Eco*RI-digested DNA since the flanking *Eco*RI (a) sites would be more or less at random distances from the internal *Eco*RI (b) site (see Fig. 3*A* for location of sites). In the double digest where both sites are internal, all of the dispersed copies of Tad (from which the intron has been removed) would give a single-sized band. The lanes labeled N show that the naive strain was devoid of Tad sequences prior to being placed in the heterokaryon with T5.4. The lanes labeled EX1 and EX2 contain DNA from two independent lysine-requiring ex-hets. The two strains have different patterns of *Eco*RI bands. When DNA from either strain was digested with *Eco*RI and *Nru* I, all of the bands were reduced to the size expected if the intron had been precisely removed, regenerating the *Nru* I site. This suggests that the intron is efficiently removed in the process of transposition.

The AI Has Been Precisely Removed from Transposed Copies of Tad1-1i. PCR primers that surround the *Nru* I site of Tad1-1 (5′-GTCCCTCACGATGTCCTGCG-3′ and 5′-AGGGTCGTCTTCGTGTCTTC-3′) were used to amplify fragments from transformant and ex-het strains. The primers were expected to amplify a 611-bp fragment from Tad lacking the AI and a 677-bp fragment from Tad with the AI at the *Nru* I site. Fragments with the AI should lack the *Nru* I site but have an *Sac* II site within the AI. Fig. 4 shows examples of PCR products obtained. When the control plasmid pTad1-1 was used as substrate, a 611-bp fragment was obtained (lane K) which could be cut into two smaller bands by *Nru* I (lane D). With pTad1-1i, a 677-bp band was obtained (lane J) which could be cut into two bands by *Sac* II (lane C). When DNA from the transformed strain T5.4 was used, two bands were observed (lane N), one the size expected for fragment with intron and one of the size expected for fragment without intron. When these PCR products were digested with *Sac* II (lane G) the upper band became fainter, and two bands of the appropriate size for intron-containing fragments were seen (compare lane G with lane C). When the T5.4 products were digested with *Nru* I (lane H), very faint bands of the size expected for fragment without intron were seen (not visible in Fig. 4). This result suggested that the majority of the lower band seen in uncut product does not represent the Tad fragment without intron. When DNA from the naive strain J326 was tested, a PCR product of the same size as fragment without intron was seen (lane O); as expected, this band was cut with neither *Sac* II (lane E) nor *Nru* II (lane F). I have cloned and sequenced copies of this DNA and found that it represents what appears to be a highly degenerate version of Tad sequence preexisting in the naive strain. As such it presents an inconvenient band but does not effect the results

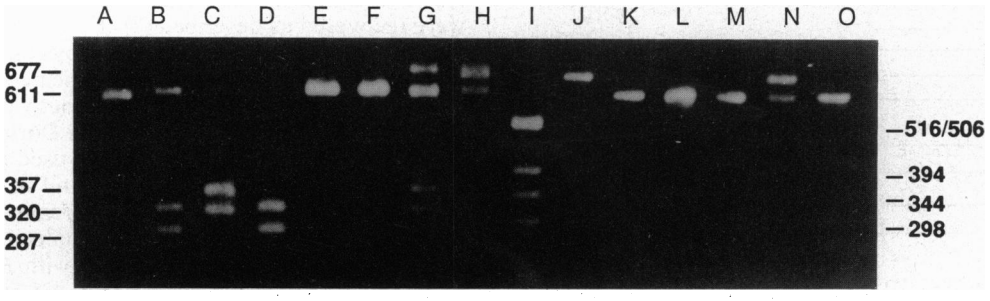

FIG. 4. Gel electrophoresis of products of PCR amplification with primers surrounding the *Nru* I site of Tad1-1. Plasmid or genomic DNA was used for PCR amplification. DNAs used were from ex-het 5.4-1 (lanes A, B, and M), pTad1-1i (lanes C and J), pTad1-1 (lanes D and K), naive strain J326 (lanes E, F, and O), transformant T5.4 (lanes G, H, and N), and ex-het 5.4-3 (lane L). Lane I contained size standards. PCR products in lanes A, C, E, and G were cut with *Sac* II. Products in lanes B, D, F, and H were cut with *Nru* I. Numbers at left refer to sizes (lengths in base pairs) of PCR fragments or their digestion products. Numbers at right refer to sizes of known fragments in lane I.

presented here. A detailed analysis of this and other related sequences will be presented elsewhere. When DNA from two ex-het strains (5.4-1, lane M, and 5.4-3, lane L) was used, a single band at the size expected for intronless fragment was seen. The product from neither 5.4-1 (lane A) nor 5.4-3 (data not shown) was cut by *Sac* II. In contrast, much of the DNA in this band (which consists of both Tad and the degenerate Tad) was cut by *Nru* I (results for 5.4-1 shown in lane B). These results confirm that the ex-het strains have Tad with a regenerated *Nru* I site, as expected if the AI had been precisely removed.

PCR fragments from ex-hets 5.4-1 and 5.4-3 were cloned into pBS and the inserts that contained an *Nru* I site were sequenced. In each case the sequence was identical to that of Tad1-1 in this region. This result confirms that the intron has been precisely removed from the Tad elements that transpose to naive nuclei. Taken together, these data clearly indicate that Tad transposes between nuclei via an RNA intermediate.

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