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Tec2, a Second Transposon-Like Element Demonstrating Developmentally Programmed Excision in *Euplotes crassus*

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The analysis of a repetitive DNA interruption of the micronuclear precursor to a 0.85-kb macronuclear gene in the hypotrich *Euplotes crassus* has led to the identification of a second transposon-like element named Tec2. Two copies of this element, one inserted into the other, compose the interruption. The Tec2 element resembles the previously characterized Tec1 element in overall size, copy number, length, and extreme terminal sequence of its inverted repeats and in the apparent use of a 5′-TA-3′ target site. In addition, extrachromosomal circular forms of Tec2 appear in DNA isolated from cells undergoing macronuclear development at the same time and with the same conformation as extrachromosomal circular forms of Tec1. These similarities suggest that the Tec1 and Tec2 elements may be under the same type of regulation during macronuclear development.

Repetitive sequence elements constitute a significant portion of the eukaryotic genome. These repetitive sequences have frequently been found to resemble transposable elements in structure and in ability to move throughout the genome. Detailed molecular and genetic characterizations of a wide variety of eukaryotic genomes have revealed that multiple mobile genetic elements, differing in both general structure and transposition mechanism, can exist within one species (reviewed in reference 3). The Tec1 element in the hypotrichous ciliated protozoan *Euplotes crassus* was originally identified as a highly prevalent, repetitive sequence family (20). DNA sequence analysis demonstrated that this repetitive sequence structurally resembles transposons in that it possesses 698-bp terminal inverted repeats flanked by an apparent 0- to 4-bp target site duplication (1, 19). Additional studies, however, revealed that the Tec1 element has several properties that set it apart from previously described transposons, including an unusually high copy number (20,000 to 40,000 copies per genome) and an excision that appears to be developmentally coordinated as the majority of the copies become extrachromosomal circles at a discrete time period during the sexual phase of the *E. crassus* life cycle (19).

The hypotrichs, like all ciliated protozoa, are unique in that they contain two distinct nuclei within the same cytoplasm (reviewed in references 22 and 39). The micronucleus contains typical eukaryotic chromosomes, but it is transcriptionally inactive. This nucleus appears to serve as a germ line nucleus because its main function is reserved for the sexual phase of the organism's life cycle. The exchange and fusion of meiotic products of the micronucleus between conjugating cells result in a new zygotic nucleus from which both nuclei of the cell are subsequently derived. The macronucleus contains linear gene-size DNA molecules that are responsible for all of the transcriptional activity that occurs during the vegetative growth of the organism. For this reason it is often referred to as the cell's somatic nucleus. Unlike the micronucleus, the macronucleus is not carried over during the sexual phase of the life cycle: it is destroyed and regenerated from a mitotic copy of the above-mentioned zygotic nucleus via a dramatic, genome-wide rearrangement of DNA sequences.

In hypotrichous ciliates like *E. crassus*, the development of a new macronucleus occurs over a 3- to 4-day time span (22, 34). Initially, the chromosomes within the developing macronucleus (also referred to as an anlagen) undergo multiple rounds of DNA replication to form polytene chromosomes. A proteinaceous material then encases these polytene chromosomes in structures called vesicles. Once formed, there is a dramatic reduction in the sequence complexity of the chromosomes within the vesicles as up to 95% of the DNA is eliminated. The vesicles then break down, and the remaining DNA molecules are replicated to the high ploidy found in the mature macronucleus. The resulting macronuclear genome is composed of approximately 24,000 different linear molecules between 0.4 and 20 kb in size, each carrying a single gene (12, 14, 21, 38).

Recent studies examining the micronuclear DNA sequence organization in *E. crassus* have demonstrated that repetitive DNA homologous to Tec1 elements preferentially associates with, and in several examples interrupts, the micronuclear precursors to macronuclear genes (1, 20). The developmentally programmed excision of Tec1 elements during macronuclear development therefore plays an important role in the formation of macronuclear genes. Similar examples of repetitive DNA interrupting the micronuclear precursors to macronuclear genes have been observed in other ciliates. Two members of the TBE transposon-like element family in the related hypotrich *Oxytricha fallax* have been found to interrupt the micronuclear precursor to a macronuclear gene (15). These elements are completely absent from all copies of the mature macronuclear gene, suggesting that TBE elements, like Tec1 elements, are precisely excised during macronuclear development (17). A family of transposon-like elements that are eliminated during macronuclear development has also been identified in the holotrichous ciliate *Tetrahymena thermophila* (6). The existence of transposon-like repetitive element families that preferentially associate with and/or interrupt the micronuclear precursors to macronuclear genes thus appears to be a common feature of ciliate micronuclear genome organization.

In this report, we describe the identification and initial

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characterization of a second transposon-like element, named Tec2, in the micronuclear genome of *E. crassus*. Two copies, one inserted into the other, of Tec2 have been found interrupting the micronuclear precursor to a macronuclear gene, indicating that the excision of these elements may also be essential to the formation of the macronuclear genome. Although Tec2 fails to cross-hybridize with the previously characterized Tec1 element, it resembles Tec1 in copy number, size of inverted repeats, and overall length. Extrachromosomal circular forms of Tec2 appear in DNA isolated from cells undergoing macronuclear development at the same time extrachromosomal circular forms of Tec1 are present. Thus, it appears that the elimination of each element during macronuclear development is mechanistically related.

MATERIALS AND METHODS

Cells strains and culturing. *E. crassus* strains 8 and 24 were grown and maintained in supplemented artificial seawater (Tropic Marin) with the marine alga *Dunaliella salina* as the initial food source as described by Roth et al. (34). When the supply of algae was exhausted, cells were fed *Escherichia coli* at a concentration of 3 liters of bacteria per 28 liters of *E. crassus* cells. After 5 to 7 days, cells were harvested for DNA isolation or used in mating experiments.

DNA isolation. For the isolation of total *E. crassus* DNA, up to 28 liters of cells were filtered through 45-μm-pore-size Nitex filters (Tetko, Inc., Lancaster, N.Y.) and concentrated on 15-μm-pore-size Nitex filters to a final volume of 200 ml. Cells were pelleted by centrifugation (100 × *g*, 3 min) and subjected to proteinase K digestion and CsCl-ethidium bromide banding as described by Jahn (18). For isolation of total DNA from cells undergoing macronuclear development, the proteinase K lysate was treated with hexadecyltrimethylammonium bromide (CTAB; Sigma, St. Louis, Mo.) as described by Murray and Thompson (30) and were then phenol (twice) and chloroform (twice) extracted and ethanol precipitated.

Recombinant phage and plasmid DNA were isolated and purified as described by Maniatis et al. (27).

Restriction endonuclease digestions and gel electrophoresis. Restriction endonuclease digestion of DNAs was carried out according to the manufacturer (Bethesda Research Laboratories, Bethesda, Md., and Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The sizes of the digested DNAs were determined by electrophoresis through agarose or low-melting-point-agarose gels made with 1× TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) or 1× TAE (40 mM Tris-acetate, 1 mM EDTA).

Labeling reactions and Southern hybridizations. Nick translations were carried out as described by Maniatis et al. (27). Individual restriction fragments were isolated in low-melting-point agarose and labeled as described by Feinberg and Vogelstein (11). Labeled DNAs were passed through G-50 spin columns to remove unincorporated triphosphates.

DNA was blotted to Zeta Probe nylon membranes (Bio-Rad, Richmond, Calif.) as described by Southern (37) except for the substitution of 0.4 N NaOH as the transfer solution. After 16 to 24 h, the membrane was rinsed in 0.2 M Tris-HCl (pH 7.5) and allowed to air dry. Prehybridization was carried out by soaking membranes in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–0.25% nonfat dry milk at 65°C for 2 to 4 h. Hybridizations were performed at 65°C in 6× SSC–0.5% sodium dodecyl sulfate (SDS)–0.25% nonfat dry milk for 12 to 18 h. Membranes were washed three times in 2× SSC–0.5% SDS and three times in 1× SSC–0.5% SDS at 65°C (approximately 20 min per wash). Membranes that were to be reused were kept moist during exposure to X-ray film and then washed twice (30 min each time) in 0.1× SSC–0.5% SDS at 95°C.

Quantitation of autoradiograms was performed by using the AMBIS Radioisotope Scanning System II (San Diego, Calif.).

DNA sequencing and PCR. Restriction fragments were subcloned into the Bluescribe plasmid vector (Stratagene, La Jolla, Calif.) and sequenced by the dideoxy chain termination method (35), using Sequenase version 1 or 2 (United States Biochemical Corp., Cleveland, Ohio). M13 universal and reverse primers (United States Biochemical Corp.) were used as well as synthetic oligonucleotide primers. Polymerase chain reactions (PCR) were carried out as described by Mullis et al. (29), using Amplitaq polymerase and a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.).

Nucleotide sequence accession numbers. Sequence data were submitted to GenBank as the following files (accession numbers in parentheses): (i) micronuclear 0.85-kb precursor sequences (M73022, M73023), (ii) macronuclear 0.85-kb molecule sequence (M73025), (iii) inverted repeat sequence of Tec2-2 (M73024), (iv) sequences adjacent to Tec2-1 in Tec2-2 (M73026, M73027), and (v) partial sequence of fragment A of Tec2-1 (M73201).

RESULTS

The micronuclear precursor to a 0.85-kb macronuclear gene is interrupted by repetitive DNA. Our laboratory has previously shown that micronuclear clone EC2 contains homology to several macronuclear genes, including one that is 0.85 kb in size (20). In this study, it was demonstrated that the 0.85-kb macronuclear-homologous sequence is interrupted by repetitive DNA (20). One portion of the macronuclear-homologous sequence was found to lie just to the right of the single *Kpn*I site in EC2 (Fig. 1); the other was found to lie over 4 kb away in a 5.4-kb *Eco*RI fragment. To more accurately determine the location of the macronuclear-homologous sequence within the *Eco*RI fragment, we used a clone of the 0.85-kb macronuclear gene to probe Southern blots of restriction digests of the 5.4-kb *Eco*RI fragment. The results, summarized in Fig. 1, demonstrate that one portion of the 0.85-kb macronuclear-homologous sequence is contained within a 1.9-kb *Xba*I-*Hin*dIII fragment. The repetitive DNA interrupting the 0.85-kb macronuclear-homologous sequence is therefore approximately 10 kb in size. The interruption of micronuclear precursors to macronuclear genes by long stretches of repetitive DNA has previously been reported (1). Although hybridization and partial sequence analysis demonstrated that these repetitive sequences bore partial homology to the Tec1 element, they were not characterized in any detail. We therefore undertook the characterization of this interruption, reasoning that its necessity for elimination during macronuclear development may provide insight as to how such repetitive sequences are eliminated to generate mature macronuclear molecules.

To examine the DNA sequence of the interruption's termini, the 0.85-kb macronuclear gene was initially sequenced in both directions from the *Kpn*I site, and the resulting sequence data were used to construct two oligonucleotide primers (Fig. 1). Because this *Kpn*I site represents the approximate location of the interruption, these primers, when annealed to restriction fragments from the micronu-

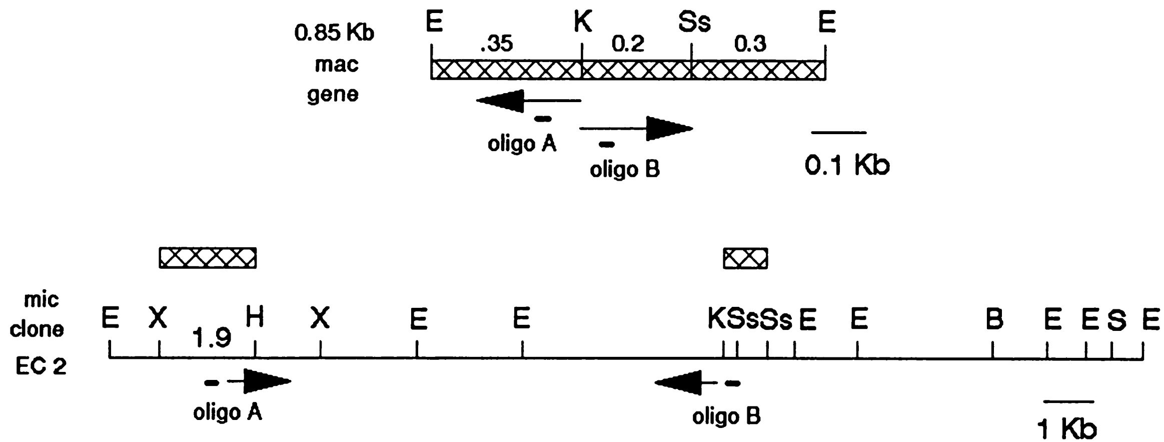

FIG. 1. Restriction maps of the 0.85-kb macronuclear gene and micronuclear clone EC2, showing that micronuclear clone EC2 contains a 0.85-kb macronuclear-homologous sequence that is interrupted by a repetitive DNA element that structurally resembles a transposon. Restriction fragments in EC2 possessing homology to the macronuclear gene are denoted by the cross-hatched boxes. The regions of the macronuclear gene that were sequenced are indicated by the arrows, and the approximate locations of synthetic oligonucleotides A and B are shown. Restriction sites: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Ss, *Sst*I; X, *Xba*I.

clear clone, generated the DNA sequence across each end of the interruption. The sequence data (Fig. 2A) show that the interruption of the 0.85-kb macronuclear-homologous sequence within micronuclear clone EC2 begins 25 bp to the left of the *Kpn*I site. A comparison of the DNA sequence of the entire 0.85-kb macronuclear gene with the DNA sequences adjacent to each side of the interruption revealed not only that the entire 0.85-kb macronuclear gene was represented in the micronuclear clone but that the two sequences share 100% identity with each other. Thus, we expect that micronuclear clone EC2 may contain the micronuclear allele that gives rise to the mature 0.85-kb macronuclear gene that we have cloned.

The structure of the interruption resembles that of a transposable element in that its endpoints are nearly perfect inverted repeats flanked by direct repeats of the dinucleotide 5′-TA-3′. Only one copy of this direct repeat is found in the mature macronuclear gene, suggesting that the flanking repeats may be the result of a target site duplication. The DNA sequence from each end of the interruption toward the middle shows near 100% identity for 705 bp. The inverted repeats of this interruption are therefore approximately the same size as the inverted repeats of the Tec1 element (19). A DNA sequence comparison between the inverted repeats of Tec1 and those of the interruption shows greater than 80% identity for the first 60 bp. This identity decreases to approximately 70% for the next 60 bp and then decreases to less than 55% for the remainder of the inverted repeats (partial data shown in Fig. 2B). The lack of identity between the inverted repeats of the interruption and the inverted repeats of Tec1 suggests that the interruption may be composed of a different transposon-like element within *E. crassus*. This was further supported by the failure of internal restriction fragments of Tec1 to hybridize to Southern-blotted restriction fragments of the interruption. The analysis of the interruption was therefore continued under the presumption that it was composed of a second repetitive element in the micronuclear genome of *E. crassus*.

The interruption is composed of two members of a novel element family named Tec2, one interrupting the other. When a restriction fragment containing a portion of one inverted repeat from the interruption was used as a probe to restriction digests of the interruption, four restriction fragments showed homology; the two that were expected and two internal restriction fragments. DNA sequence analysis of the fragments showing homology to the inverted repeat probe confirmed that they all contained inverted repeat sequences in the orientations depicted in Fig. 3. These results indicated that the interruption could be composed of two transposon-like elements, one inserted into the other. To determine whether both elements were members of the same repetitive family, two restriction fragments (fragments A and B; Fig. 3) that make up the majority of the internal, uninterrupted element were used as radioactive probes to Southern-blotted restriction digests of the entire interruption. Fragment A hybridized to restriction fragments on both sides of the internal, uninterrupted element, whereas fragment B hybridized to restriction fragments only on the right side (results summarized in Fig. 3). The observed cross-hybridization between the two elements composing the interruption indicates that both elements belong to the same repetitive element family. Because this repetitive element structurally resembles a transposon but shows no homology to the Tec1 element, it has been given the name Tec2 (transposon-like *E. crassus* 2).

For easier description, the internal, uninterrupted Tec2 element will be referred to as Tec2-1 and the external, interrupted Tec2 element will be referred to as Tec2-2 (Fig. 3). The hybridization results indicated where within Tec2-2 the Tec2-1 insertion occurred. Because restriction fragment A from Tec2-1 hybridizes to sequences on both sides of the Tec2-1 element, Tec2-2 appears to have been interrupted in a region corresponding to fragment A of Tec2-1. To confirm this, fragment A was sequenced from its *Eco*RI site along with the regions of Tec2-2 immediately adjacent to the inverted repeats of Tec2-1. The sequence data (Fig. 2C) confirm that the sequence of Tec2-2 on both sides of Tec2-1's inverted repeats shows greater than 85% identity to the sequence of fragment A from Tec2-1. The sequence data also show that Tec2-1 inserted into Tec2-2 at a 5′-TA-3′ corresponding to a 5′-TA-3′ located 80 bp to the left of the *Eco*RI site of fragment A in Tec2-1. A copy of this TA is found flanking both inverted repeats of Tec2-1, again implicating the use of this sequence as a target site for Tec2 elements.

Tec2-1 possesses the most prevalent restriction map compared with other elements in the genome. Comparison of the

A

```
0.85 Kb mac gene     ...ATCAACAACGTTAGAAAATGAAATCGGTGTGTGCCACTGATGGACTACTT
                        **************************************************
mic clone EC2        ...ATCAACAACGTTAGAAAATGAAATCGGTGTGTGCCACTGATGGACTACTT


CAGAAGGCCTA                                                      TCCGTTACAACC
***********                                                      ************
CAGAAGGCCTAtagagggataaaaat....interruption....attattatccctctataTCCGTTACAACC


ATTACCTCGCCAGGGTACCCTGAAGCTAGTGCTTACAACATGATTGGGCGATCATCATTGACTTCATGGCCG...
************************************************************************
ATTACCTCGCCAGGGTACCCTGAAGCTAGTGCTTACAACATGATTGGGCAATCATCATTGACTTCATGGCCG...
             KpnI
```

B

```
                                           25                       50
Tec1 IVR              TAGAGGGAGTTTTA-TATTGAAACAATATAAATTAATAATTATAATTA-A
                      ******** *   * *********** ** ****************** *
Interruption IVR      TAGAGGGA-TAAAAATATTGAAACAA-ATTAATTAATAATTATAATTATA
                                 (T)

                  75                      100                     125
TA-ATTGAACAATTTAATGTT--AAA-TAATACTCTTTCTATCT-ACC-T-TTC-TCACCT---TATTAAGT-TT
 * ****** ** *** * **   *** ** * * ******* ** *** * *** ** ***    ****   * **
AATATTGAAAAAATTA-T-TTCCAAACTA-T-C-CTTTCTAACTTACCCTCTTCCTCTCCTAACTATTCTCTATT
                                                         (A)

                 150                     175                      200
-CCACAT---A--A-AC-AAGCT-T--TCATTTTGCATCCT--TA--TGATCATCACTT-AAТТ-TGTCTTT---
 *** **    *  *  *  ** ** *  ****    *  **    **  **** *  **** ** * *** **
ACCAAATGTGATGATATTAA-CTATAATCAT---G--TC-TAATAGCTGATTA--ACTTCAACTATGT-TTCAAA
```

C

```
Tec2-1  Fragment A
...ACTTACTTCATGATCAAATATATAAATGATTAAATTAGGATTAATTTCATAATATTTAATATGGAAACACGT
   ******* ***** ********************** * ***************************** **
...ACTTACTCCATGACCAAATATATAAATGATTAAATTTGAATTAATTTCATAATATTTAATATGGAAACCCGC
Tec2-2 L

AATTCAGCGAAGAAGAAAGA                                       TATCAAGACCAAAGACT
********************                                       *****************
AATTCAGCGAAGAAGAAAGAtatagagggataataat......attattatccctctaTATCAAGACCAAAGACT
                    Tec2-1 IVRL              Tec2-1 IVRR

                                                                EcoRI
TCTTTGAATATGAGATGATACCTTTCAAAACACATAGAGATTCAGCAATGGAGAAGAAGAGTAAGAATTC...
*** ****************** *** ******* ****   **** ********** ***** **    **
TCTATGAATATGAGATGATACCATTCCAAACACAGAGAGCATCAGTAATGGAGAAGCAGAGTTAGCCCTC...
                                                                Tec2-2 R
```

FIG. 2. Sequence analysis of the interruption. (A) Partial sequence data generated by annealing oligonucleotides A and B to the appropriate restriction fragments from EC2. The 0.85-kb macronuclear-homologous sequence contains an interruption (lowercase letters) that begins 25 bp to the left of the *Kpn*I site. Approximately 1.2 kb of the micronuclear clone in the regions surrounding the interruption has been sequenced. The entire 0.85-kb molecule is accounted for in the clone, and the micronuclear sequence is 100% identical to the 0.85-kb macronuclear molecule. Shown are the sequences immediately adjacent to both sides of the interruption (uppercase letters). The TA target site duplication, of which only one copy is present in the macronuclear gene, is highlighted. (B) Comparison of the first 200 bp of the interruption's left end with the first 200 bp of an inverted repeat from Tec1. Nucleotide differences between IVRL and IVRR of the interruption are denoted by nucleotides in parentheses. (IVR indicates inverted repeat; L and R denote left and right, corresponding to the map drawn in Fig. 1.) The identity to Tec1 is approximately 85% for the first 60 bp but decreases to less than 60% overall for the remainder of the inverted repeat sequence. Gaps were introduced to facilitate the best alignment. (C) DNA sequence generated from the sites shown

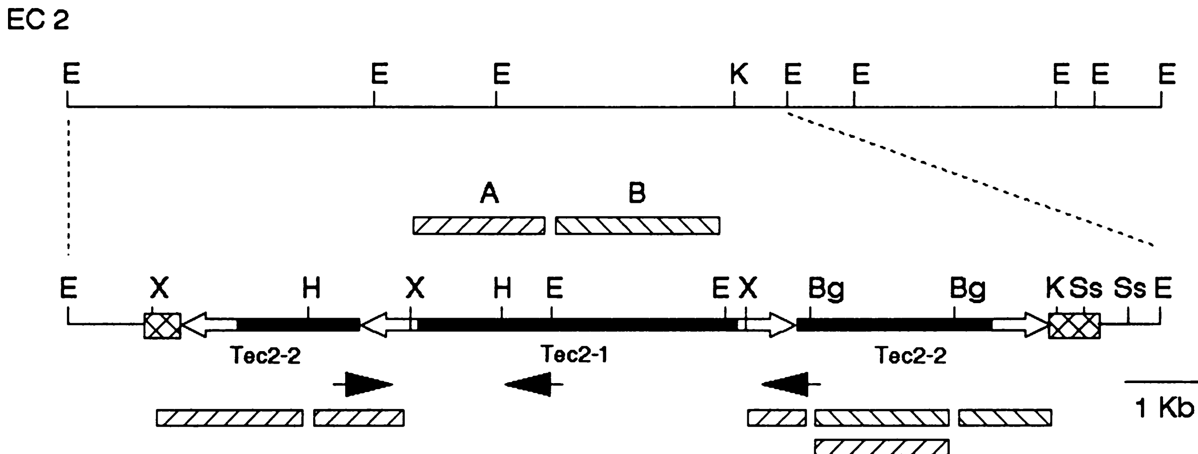

FIG. 3. Evidence that the interruption is composed of two transposon-like elements of the same family, one interrupting the other. The region of EC2 containing the interruption is expanded, and a detailed restriction map is displayed. Cross-hatched boxes denote locations of the precursors to the 0.85-kb macronuclear gene. Open arrows denote the locations and orientations (confirmed by DNA sequence analysis) of both elements' inverted repeats. Two restriction fragments denoted by boxes A and B were used to probe a Southern blot of restriction digests of the interruption. Fragments external to the internal element showing homology to probe A or B are denoted with similar hatched patterns. Closed arrows denote regions of the interruption that were sequenced. The internal, uninterrupted element is named Tec2-1; the external element is named Tec2-2. Restriction sites: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Ss, *Sst*I; X, *Xba*I.

restriction sites present in Tec2-1 and Tec2-2 shows that the two elements possess different restriction maps. To determine the most prevalent restriction map of Tec2 compared with other elements in the genome, restriction fragments A and B from Tec2-1 were used as radioactive probes to Southern blots of total *E. crassus* DNA digested with various restriction enzymes. The total DNA used in these Southern blots is composed of both micronuclear and macronuclear DNA sequences. In undigested DNA, both fragments hybridize solely to high-molecular-weight micronuclear DNA sequences. In genomic digests, however, the probes hybridize to lower-molecular-weight restriction fragments of the various Tec2 elements present in the genome. The results in Fig. 4A and B show that the most prevalent restriction fragment sizes detected in each genomic digest are also present in the region of Tec2-1 from which each probe was derived (data summarized in Fig. 4C). Both probes, however, do show weaker hybridization to restriction fragments that are presumably derived from copies of Tec2 that contain restriction site polymorphisms. These results demonstrate that the Tec2-1 element in micronuclear clone EC2 contains a restriction map that is shared by the majority of the Tec2 elements in the genome. Summation of the Tec2-1 fragment sizes indicates that the Tec2-1 element, and thus the majority of Tec2 elements in the genome, is approximately 5.3 kb in size. Although Tec2 is similar in size to the previously reported 5.5-kb Tec1 elements, the restriction maps of the most prevalent version of each element in the genome are markedly different (19).

Having determined that the majority of the Tec2 elements in the genome contain a 2.2-kb *Eco*RI fragment, we isolated this restriction fragment from Tec2-1 and used it to estimate the number of Tec2 elements within the micronuclear genome of *E. crassus*. A known quantity of *Eco*RI-digested total DNA, along with a dilution series of the 2.2-kb *Eco*RI fragment, was electrophoretically separated on an agarose gel, Southern blotted, and probed with the radioactively labeled 2.2-kb *Eco*RI fragment. The resulting autoradiograph was analyzed on an AMBIS beta scanner, and the amount of 2.2-kb *Eco*RI fragment in total DNA was determined. The results demonstrated that the Tec2 element family represents approximately 2% of the micronuclear genome, which corresponds to about 20,000 copies of the Tec2 element. The Tec1 and Tec2 elements are therefore present in approximately the same number of copies in the micronuclear genome of *E. crassus* (19).

Extrachromosomal forms of Tec2 exist in developing macronuclear DNA. The majority of the Tec1 elements in *E. crassus* have been shown to excise as extrachromosomal circles at a specific time during macronuclear development (19). The similarities between Tec2 and Tec1 in overall size, copy number, and DNA sequence of the first 60 bp of their inverted repeats suggest that Tec2 elements may be excised by the same machinery that excises Tec1 elements during macronuclear development. If this were true, one would predict that extrachromosomal circular forms of Tec2 elements also exist within developing macronuclear DNA. To investigate this hypothesis, total DNA was isolated from *E. crassus* cells 30 h after cells of opposite mating type were mixed together. This total DNA, which includes micronuclear, macronuclear, and anlagen sequences, was electrophoresed undigested on a 1% agarose gel, Southern blotted, and probed with a PCR-amplified fragment containing approximately the last 250 bp of Tec2-2's inverted repeats.

as arrows in Fig. 3. The DNA sequence from the *Eco*RI site of fragment A from Tec2-1 is compared with DNA sequences of Tec2-2 immediately adjacent to the inverted repeats (IVRL and IVRR) of Tec2-1. Tec2-1 inserted into Tec2-2 at a TA (highlighted) located 86 bp from a polymorphic *Eco*RI site that is present in Tec2-1 but not present in Tec2-2. Sequences immediately adjacent (uppercase letters) to the inverted repeats of Tec2-1 (IVRL and IVRR; lowercase letters) show greater than 75% identity to the corresponding sequence from Tec2-2. For the sequences shown as Tec2-1, fragment A, and Tec2-2 R, the opposite strand was sequenced.

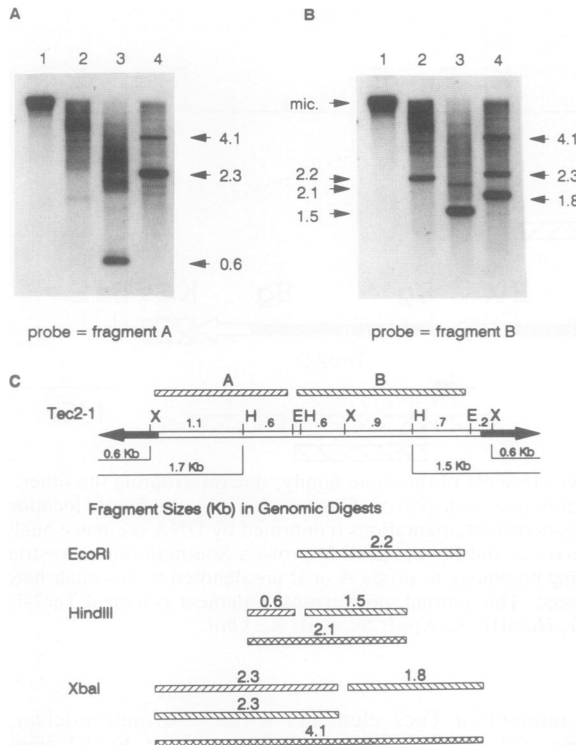

FIG. 4. Evidence that Tec2-1 contains the most prevalent restriction map compared with other Tec2 elements in the genome. (A) Undigested (lane 1) total *E. crassus* DNA was electrophoresed side by side with *Eco*RI-digested (lane 2), *Hin*dIII-digested (lane 3), or *Xba*I-digested (lane 4) DNA on a 1% agarose gel, which was Southern blotted and probed with fragment A (Fig. 3). (B) A Southern blot similar to the one shown in panel A was probed with fragment B (Fig. 2A). Sizes (in kilobases) of the most prevalent restriction fragments are shown. mic. denotes hybridization to high-molecular-weight micronuclear DNA sequences. (C) The results shown in panels A and B are summarized. The restriction map of Tec2-1 is shown along with the locations of probes A and B. The most prevalent restriction fragments observed in genomic digests for each probe are shown as boxes with similar hatched patterns. Less prevalent restriction fragments observed by both probes (representing Tec2 elements polymorphic for a particular restriction site) are shown as rectangles with cross-hatched patterns.

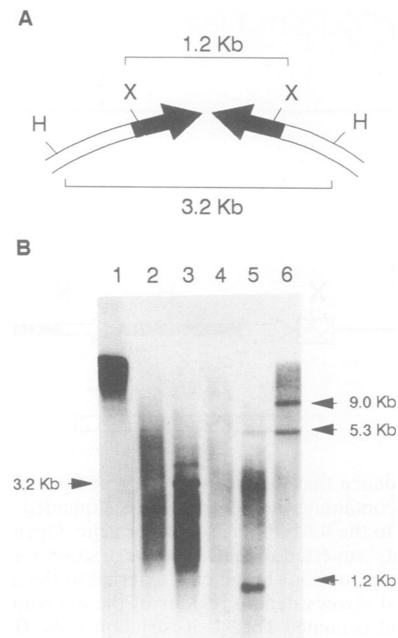

FIG. 5. Presence of extrachromosomal circular forms of Tec2 in DNA isolated from cells undergoing macronuclear development. (A) Diagram showing the predicted restriction fragment sizes that would be generated if developing macronuclear DNA-containing circular forms of Tec2 with their inverted repeats in a head-to-head orientation were digested with *Hin*dIII (H) or *Xba*I (X). These sizes are based on the known distances of these restriction sites from the ends of the Tec2-1 element (shown in Fig. 4C). (B) Comparison of total vegetative *E. crassus* DNA (lanes 1, 2, and 4) with total DNA isolated from *E. crassus* cells 30 h after mixing of cells of opposite mating type (lanes 3, 5, and 6). Five micrograms of each sample, either undigested (lanes 1 and 6) or digested with *Hin*dIII (lanes 2 and 3) or *Xba*I (lanes 4 and 5), was electrophoresed on a 1% agarose gel, Southern blotted, and hybridized with a probe specific for the inverted repeats of Tec2. The sizes of the observed prevalent bands are indicated. mic. denotes hybridization to high-molecular-weight micronuclear DNA sequences.

This probe did not cross-hybridize to a restriction fragment containing the entire inverted repeat of Tec1 under the stringency conditions used; thus, this probe is specific for Tec2 elements. The results in Fig. 5B show that two bands, corresponding to 9.0 and 5.3 kb in size, are observed in undigested total DNA from cells undergoing macronuclear development (lane 6) that are not present in undigested total DNA from vegetative cells (lane 1). Similar-size bands were previously observed when anlagen DNA isolated from *E. crassus* cells was probed with a Tec1 element-specific probe. These two bands were shown to represent circular and linear extrachromosomal forms of Tec1 elements (19).

The similarity in size of the 5.3-kb band to the size of an intact Tec2 element (e.g., Tec2-1) suggests that this band represents linear forms of Tec2 elements. The results of two different experiments indicated that the 9.0-kb band may represent extrachromosomal circular forms of Tec2. First, when the same 30-h total DNA sample was run undigested on a lower-percentage agarose gel (0.7%), this putative circular form migrated with increased mobility corresponding to 6.2 kb. Second, unique-size restriction fragments were present in digests of developing macronuclear DNA that were not present in similar digests of vegetative DNA when probed with the Tec2-specific inverted repeat probe. These unique-size restriction fragments are of the size predicted if the circular forms of the elements have their inverted repeats joined in an end-to-end orientation. Figure 4C indicates the distances between internal *Xba*I and *Hin*dIII sites within Tec2-1 (the most prevalent version of Tec2 in the genome) and the termini of the element. Figure 5A illustrates that 1.2- and 3.2-kb restriction fragments would be generated if circular forms possessing this head-to-head configuration were digested with *Xba*I and *Hin*dIII, respectively. Figure 5B shows that the expected 1.2-kb *Xba*I and 3.2-kb *Hin*dIII restriction fragments are present in developing macronuclear DNA but completely absent from *Xba*I- and *Hin*dIII-digested vegetative DNA (lanes 2 and 4, respectively). Faint hybridization can be seen to the 5.3- and 9.0-kb bands in the lane containing *Xba*I-digested developing macronuclear DNA. The 9.0-kb band in this lane could represent circular forms that are completely lacking in *Xba*I sites. A significant number of Tec2 elements in the genome are polymorphic for at least one *Xba*I site (Fig. 4A and B). The 5.3-kb band in this

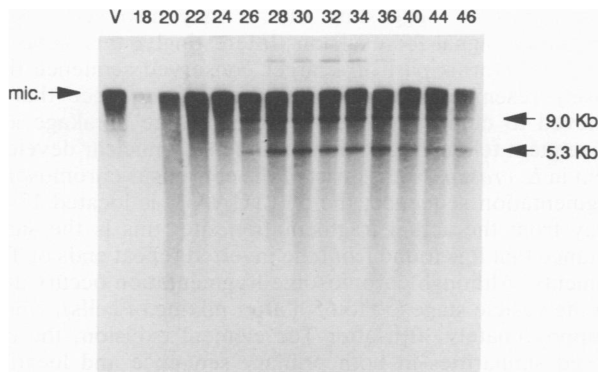

FIG. 6. Appearance of extrachromosomal forms of Tec2 in developing macronuclear DNA beginning at 26 h after mixing. Total DNA was isolated from *E. crassus* cells at 2- to 4-h intervals beginning at 18 h after mixing of cells of opposite mating type. Five micrograms of each sample was electrophoresed undigested on a 1% agarose gel, Southern blotted, and hybridized with a probe specific for the inverted repeats of Tec2. The number above each lane denotes the time after mixing that the sample was isolated. V, vegetative DNA. The sizes, based on relative mobilities of the extrachromosomal forms, are indicated.

lane could represent versions of Tec2 that contain only one *Xba*I site. Digestion of developing macronuclear DNA containing circular forms with a single *Xba*I site would generate such linear forms. The observance of the expected, unique restriction fragment sizes in digests of developing macronuclear DNA combined with the band's increased mobility in lower-percentage agarose indicates that the 9.0-kb band represents circular forms of Tec2.

Extrachromosomal forms of Tec2 appear at a specific time point during macronuclear development. The extrachromosomal circular forms of the Tec1 element have previously been shown to appear in developing macronuclear DNA beginning at 26 h after mixing of cells of opposite mating type (19). To determine the timing of Tec2 excision, total DNA was isolated from cells at 2- to 4-h intervals throughout macronuclear development beginning at 18 h after mixing of cells of opposite mating type. These DNA samples were electrophoresed undigested on an agarose gel, Southern blotted, and probed with the PCR-amplified, Tec2-specific inverted repeat fragment. As shown in Fig. 6, the 9.0- and 5.3-kb bands appear in developing macronuclear DNA beginning at 26 h. The presence of linear forms in these samples is most likely due to the CTAB treatment and repeated phenol-chloroform extractions needed to render these DNA samples susceptible to restriction enzyme digestion. In parallel experiments in which an *E. crassus* cell lysate is phenol-chloroform extracted only once and then ethanol precipitated, only the circular form is observed. Because slight variations could exist between DNA samples isolated in different time courses, the nylon membrane containing the Southern-blotted DNA samples in Fig. 6 was stripped of radioactivity and rehybridized with a labeled restriction fragment specific for the inverted repeats of Tec1. The resulting autoradiograph verified that extrachromosomal forms of the Tec1 element also appear at 26 h in this time course (data not shown). Thus, extrachromosomal circular forms of both Tec2 and Tec1 appear at the same time point during macronuclear development.

DISCUSSION

The characterization of a repetitive element interrupting the precursor to a 0.85-kb macronuclear gene has led to the identification of a second element family in the micronuclear genome of *E. crassus*. DNA sequence analysis has revealed that this element family structurally resembles transposons in that it possesses 705-bp inverted terminal repeats flanked by a direct repeat of the dinucleotide TA. Only one copy of this direct repeat is present within the mature 0.85-kb macronuclear gene at the exact point of the interruption, indicating that this direct repeat may be the result of a target site duplication. Although similar in length, the DNA sequence of this element's inverted repeats showed significant identity to the inverted repeats of the previously described Tec1 element for only the first 120 bp. Internal restriction fragments from the interruption also failed to cross-hybridize with internal restriction fragments of Tec1. Because this repetitive element structurally resembles a transposable element but fails to cross-hybridize with the Tec1 element, it has been named Tec2.

A wide variety of eukaryotic systems have been found to contain multiple transposable elements. For example, over 20 different transposable element families have been identified in the *Drosophila* genome (3). Because heterogeneity has been found to exist among members of a particular element family, subfamilies of transposable elements have been designated. Examples of such subfamilies include the Tc1 through Tc5 elements in *Caenorhabditis elegans* (28), the Ty1, Ty2, and Ty3 elements in *Saccharomyces cerevisiae* (4), and the Tam1 through Tam5 elements in *Antirrhinum majus* (7). Although these subfamilies differ in primary sequence, their overall structures are generally similar, and in most cases the extreme terminal portions of their terminal repeats share a significant degree of sequence identity. The Tec1 and Tec2 element subfamilies of *E. crassus* also possess these features. The preservation of terminal repeat sequences that are known to be important recognition signals for transposition suggests that the mobility of multiple element subfamilies within one organism may be under the same type of regulation. The recent observation that the *mut-4* gene can activate Tc2 transposition in *C. elegans* in addition to Tc1 transposition provides some initial evidence that two element subfamilies within the same organism may be coregulated (26).

The similarities in both the timing of excision and the structure of the excised product suggest that Tec1 and Tec2 may be under the same developmental regulation. Both elements appear as extrachromosomal forms in DNA isolated from cells undergoing macronuclear development at 26 h after mixing of cells of opposite mating type. The structures of the extrachromosomal forms of Tec1 have previously been shown to be circles generated by an element's inverted terminal repeats coming together in a head-to-head configuration (19). Unique restriction fragment sizes present in restriction digests of developing macronuclear DNA probed with a Tec2-specific inverted repeat probe are absent from similar digests of vegetative DNA, indicating the presence of extrachromosomal circular forms of Tec2 with structures similar to those of Tec1.

Hybridization experiments and DNA sequence analysis demonstrate that the interruption of the 0.85-kb micronuclear precursor is composed of two Tec2 elements, one inserted into the other. The element-inside-an-element structure observed for this interruption appears to be a common feature among Tec elements. As mentioned previously,

another study has shown that repetitive DNA interruptions of two different macronuclear genes are composed of at least two internally interrupted Tecl elements (1). Hybridization experiments in our laboratory have revealed that the previously characterized Tecl element in micronuclear phage clones EC5 interrupts a Tec2 element (23). Sequence analysis of the regions immediately adjacent to the inverted repeats of this Tecl element confirms that it inserted into a Tec2 element at a 5'-TA-3' corresponding to a 5'-TA-3' located 55 bp downstream of the putative TA target site used by Tec2-1 to insert into Tec2-2. A wide variety of eukaryotic systems, such as the *Drosophila* (9), *Dictyostelium* (5), sea urchin (16), and maize (8) systems, contain transposable elements that appear to be hot spots for the insertion of additional mobile genetic elements. This preferential insertion of one element into or nearby another element implies that DNA sequences important for element insertion should be present at a high frequency within the element into which the insertion occurs. In the slime mold *Dictyostelium discoideum*, for example, the preferential insertion of the Tdd-3 element into the Tdd-2 element in two examples appears to involve a 22-bp region that is conserved between members of the same element family (31). Although multiple combinations of elements within elements have been observed in the case of Tecl and Tec2, a comparison of the sequences flanking each side of the TA target site used in the three examples of Tec element insertions available in our laboratory shows no additional sequence identity. Thus, the apparent preference of one Tec element to insert into another at a TA target site may be attributed to the simple fact that both Tecl and Tec2 are very A+T rich (24).

One of the primary differences between the two Tec elements and previously described transposons is in their unusually high copy number. The detailed study of several transposons, such as P elements in *Drosophila melanogaster* (25, 32), the Spm element family in maize (10), and Tn*10* (IS*10*) elements in *E. coli* (33, 36), has revealed complex mechanisms that tightly regulate the transposition of each element. Such mechanisms have been suggested to exist to prevent increased frequencies of genetic rearrangements due to increases in the transposon's copy number. In the case of Tecl and Tec2, the apparent lack of transcriptional activity within the micronuclear genome could allow these elements to insert into the precursors of macronuclear genes without any detrimental effect to the organism. As a result, Tec elements could proliferate as long as they possessed the ability to excise precisely during macronuclear development. The similarity in copy number between Tecl and Tec2 does suggest that one element could be regulating the other or that both elements are under the same type of regulation in *E. crassus*. Structural features such as the overall size of the elements and the length of their terminal inverted repeats have apparently been conserved, suggesting that these features may play important roles in the regulation of Tec elements. In addition to the length of the inverted repeats, the sequence comparison in Fig. 3C shows that the first eight nucleotides of each element's inverted repeats are 100% conserved. This high degree of conservation suggests that the extreme termini of the inverted repeats could serve as important recognition signals for the elements' elimination during macronuclear development. Further analysis of the inverted repeat sequence comparison in Fig. 3C shows that the sequence 5'-TTGAA-3', beginning 17 bp from the end of each inverted repeat, is also 100% conserved. This sequence is found in the same location in more than 15 different inverted repeat sequences of various Tec elements in our laboratory (24), suggesting that it may also serve as a recognition signal for excision. Interestingly, this same 5'-TTGAA-3' forms part of a larger conserved sequence that closely resembles a putative consensus sequence that is believed to define the sites of chromosome breakage and subsequent telomere addition during macronuclear development in *E. crassus* (2). As part of the consensus chromosome fragmentation sequence, the 5'-TTGAA-3' is located 17 bp away from the actual fragmentation site; this is the same distance that it is found from the inverted repeat ends of Tec elements. Although chromosome fragmentation occurs during the vesicle stage (50 to 65 h after mixing of cells), which is approximately 30 h after Tec element excision, the observed similarities in both primary sequence and location suggest that this consensus sequence may be involved in both Tec element excision and chromosome fragmentation. The difference in timing of these two events implies that multiple factors would be involved in their developmental programming, some perhaps Tec element encoded. We are currently analyzing the complete DNA sequence of representative Tecl and Tec2 elements from *E. crassus* for any extended open reading frames. Such an analysis not only may provide the putative identity of any element encoded proteins and their functions but will allow us to further study the evolutionary relationships between the two Tec element subfamilies and how their developmentally coordinated excision has come to play an essential role in the life cycle of this organism.

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