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A Mutation in the Flanking 5′-TA-3′ Dinucleotide Prevents Excision of an Internal Eliminated Sequence From the *Paramecium tetraurelia* Genome

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ABSTRACT

The germline chromosomes in Paramecium and other ciliated protozoa contain regions of DNA that are excised and eliminated during the development of a new macronuclear genome. *Paramecium tetraurelia* internal eliminated sequences (IESs) are invariably flanked by a 5′-TA-3′ dinucleotide sequence that is part of a larger 8-bp terminal inverted-repeat consensus sequence. Both features, the absolutely conserved 5′-TA-3′ and the remaining 6-bp terminal inverted repeat, are shared with the *mariner*/Tc1 class of transposons. In this article we describe a mutant cell line (AIM-2) defective in excision of a single IES from the coding region of the *A51* surface antigen gene. Excision of the 370-bp IES6649 is prevented by a single A to G transition in the invariably conserved 5′-TA-3′ dinucleotide. Failure to excise IES6649 also revealed a 29-bp IES located inside IES6649. Additional experiments with the previously isolated AIM-1 mutant, which also contains an internal IES, shows that alternate excision using the wild-type end of IES2591 with an end from the internal IES is extremely rare or nonexistent. These results indicate that IESs are discrete elements whose excision depends upon nucleotides located within the consensus sequence, but also suggest that additional information is required to match one end of an IES with its excision partner.

TRANSPOSABLE elements are a common feature of eukaryotic genomes. Ciliated protozoa are no exception. While functional transposable elements have not yet been found in *Paramecium tetraurelia*, elements that bear sequence homology to the *mariner*/Tc1 class of elements have been found in the micronuclear genome of the ciliate *Euplotes crassus* (Krikau and Jahn 1991; Jahn *et al.* 1993). The *mariner*/Tc1 class of elements is widely dispersed in nature and is present in organisms ranging from invertebrates to mammals (reviewed in Robertson 1995; Plasterk 1996; and Hartl *et al.* 1997). *Mariner*-like elements (MLEs) share a terminal inverted-repeat consensus sequence at each end of the element that is flanked by an invariably conserved 5′-TA-3′ dinucleotide derived from duplication of the insertion site. MLEs transpose by a cut-and-paste mechanism, excising themselves from one position in the genome and inserting at another (reviewed in Hartl *et al.* 1997). While the transposase function can be provided by another closely related element *in trans*, most MLEs are inactive and do not transpose because they contain only a pseudogene for transposase (Hartl *et al.* 1997). MLEs that do transpose have a tendency to do so in somatic tissues, implicating a developmental control over the transposase function. In Euplotes, all MLEs are excised during the formation of the somatic macronuclear genome from the germline micronuclear DNA (Baird *et al.* 1989).

The DNA of both Euplotes and Paramecium contains short eliminated regions called internal eliminated sequences (IESs). These IESs are precisely excised and the surrounding macronucleus-destined DNA is ligated back together during sexual development when the old macronucleus is destroyed and a new macronuclear genome is created from the micronuclear DNA (reviewed in Prescott 1994; Coyne *et al.* 1996; Klobutcher and Herrick 1997).

In Paramecium, IESs are short (28–882 bp), AT-rich, single copy sequences that are invariably flanked by 5′-TA-3′ dinucleotides, only one copy of which remains in the macronuclear DNA after excision (Steele *et al.* 1994). The only conserved sequence feature discovered to date in Paramecium IESs is an 8-bp terminal inverted-repeat consensus sequence (Klobutcher and Herrick 1995) that has similarity to the consensus sequence at the termini of the *mariner*/Tc1 superfamily of transposable elements. Klobutcher and Jahn (1991) and Klobutcher and Herrick (1997) have formally proposed that the TA class of IESs evolved from the *mariner* class of transposons. In this model, the ciliate host assumed control of the excision activity that it supplies *in trans*, and then each IES was constrained to maintain only the *cis*-acting sequences required for excision (Klobutcher and Herrick 1997). Our success in isolating a mutant cell line that cannot excise one of the IESs in the *A51* surface antigen gene provided the first evidence for the functional importance of the consensus sequence (Mayer *et al.* 1998).

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This article describes a second mutation that prevents IES excision. Molecular and genetic analysis of the *A51* **I**ES **M**utant (AIM)-2 cell line demonstrates that a micronuclear mutation consisting of a single base pair change within the absolutely conserved flanking 5′-TA-3′ dinucleotide is responsible for the defect in excision. In addition, this mutant cell line has revealed the presence of a 29-bp IES inside IES6649. The small internal IES has ends that match the inverted consensus sequence, including the flanking 5′-TA-3′ repeats, and it is removed from the mutant during macronuclear development even though the remaining portion of IES6649 is not excised.

Each AIM mutation results in a cell line that has one wild-type IES end as well as an internal IES. Experiments designed to detect alternate excision products involving the wild-type end and an internal IES end showed that these events rarely if ever occur. Because alternate excision junctions do not occur, the *cis*-acting signals regulating IES excision must include additional information that matches one terminal inverted-repeat consensus sequence with its corresponding partner. The possible relationship between Paramecium IESs and *mariner*/Tc1 transposons is discussed in light of the findings from these studies.

MATERIALS AND METHODS

Cell lines, media, and growth conditions: *P. tetraurelia* stock 51 is homozygous for the *A51* surface antigen gene. Line 51ND was derived from stock 51 and contains a Mendelian mutation that prevents trichocyst discharge but is wild type at the *A51* locus. Line d12(−1300) was originally derived from stock 51 and contains macronuclear and micronuclear deletions of the *A51* gene starting at base −1300 (Rudman *et al.* 1991). Line AIM-1 contains a single base mutation inside IES2591, resulting in retention of this IES in the macronucleus (Mayer *et al.* 1998). All cells were cultured in a 0.25% wheat grass medium buffered with 0.45 g/liter sodium phosphate and supplemented with 0.25 mg/liter stigmasterol. The medium was inoculated with a nonpathogenic strain of *Klebsiella pneumoniae* 1–2 days prior to use. All cell lines were maintained at 27° and cultured as described by Sonneborn (1970).

Mutagenesis and screening: The AIM-2 cell line was isolated from nitrosoguanidine-treated cells using the procedure previously described in Mayer *et al.* (1998).

Total DNA isolation: Total genomic DNA was isolated using the Boehringer Mannheim high pure PCR template preparation kit (Boehringer Mannheim, Indianapolis). The cell culture (200 ml, at 1000 cells/ml) was pelleted, resuspended in 0.3 ml of culture fluid, and then quickly squirted into binding buffer. Proteinase K was added and the samples were incubated at 72° for 10 min; then RNase was added and the samples were incubated at room temperature for 5 min. The samples were run through glass fleece columns and washed twice with washing buffer before being eluted into 200 μl of warm (70°) 10 mm Tris, pH 8.5.

Southern blot analysis: Southern blot analyses were performed according to the method of Sambrook *et al.* (1989). In Paramecium, macronuclear DNA is ∼250 times more abundant than micronuclear DNA; thus a Southern blot of total genomic DNA is essentially an analysis of macronuclear DNA. Total genomic DNA was digested with *Ssp*I and then run on agarose gels. The gels were blotted onto Nytran filters (Schleicher & Schuell, Keene, NH), which were UV-cross-linked and washed in a solution containing 10× Denhardt's, 0.2 m phosphate buffer, 0.1% SDS, and 5× SET (1× SET is 0.15 m NaCl, 30 mm Tris, and 2 mm EDTA) at 65° for 1 hr. The filters were incubated in hybridization solution (1× Denhardt's, 0.02 m phosphate buffer, 5× SET, 0.25% SDS) for 1 hr at 65° before the labeled probe (pSA8.8R, a plasmid clone containing the *Eco*RI fragment of the wild-type macronuclear *A51* gene from −1052 to +7026; see Figure 1) was added. After incubating at 65° overnight, the filters were washed three times for 30 min each in a solution containing 0.2× SET, 0.1% SDS, 0.1% sodium pyrophosphate, and 25 mm phosphate buffer at increasingly stringent temperatures (65°, 68°, and 72°, respectively).

PCR amplification: Macronuclear amplification products of the sequence surrounding IES2591 in the *A51* gene were obtained using primers 2460 (5′-GGCATGTAGAAGTGCAA 3′) and 2638 (5′-GGCATTAAGCTTGTGTC-3′). Micronuclear amplification products were obtained using the 2460 primer plus a primer (d28) overlapping part of the 28-bp deletion (5′-GCTTTTAAACTTATGAATCAAG-3′; Figure 1). Amplification products from the AIM-1 macronuclear copies of IES2591 were obtained using the mut-1 primer (5′-GCACTGGGATGT GTATTT-3′) and primer +3021. Macronuclear amplification products of the area surrounding IES6649 in the *A51* gene were obtained using primers 6568 (5′-GATGTCGCTTGTA CAACTGCC-3′) and 6771 (5′-GCAAGCTGCTCTTACGGT GG-3′). Micronuclear amplification products were obtained using the 6568 primer plus a primer (d29) overlapping part of the 29-bp deletion (5′-GAAATAATTGATTCAACG-3′; Figure 1). Amplification products of the macronuclear junction after excision of either IES2591 and IES6649 were obtained using primers 2578 (5′-GCACTGGGATGTGTACC-3′) and 3021 (5′-GCAGGTTGCTGGAGAGG-3′) or 6645 (5′-GAGA TAAAGATTGCTAGG-3′) and 6955 (5′-AACATAATGTAATT CCTAC-3′), respectively.

Whole-cell PCR was performed using approximately five cells (5 μl), which were added to 5 μl of 1.0% NP40 and placed at 65° for 10 min. All other PCR reactions were performed using ∼4 ng of DNA. Reactions of either type were placed at 92° for 3 min, and then 10 μl 10× buffer (15 mm $MgCl_2$, 250 mm KCl, 100 mm Tris pH 8.8), 10 μl 2.0 mm dNTPs, 2 μl each 66.7 μm primer, 5 U Taq DNA polymerase, and 65 μl H_2O were added to give a 100-μl reaction. PCR consisted of 30–35 cycles of 92° for 1 min, 45–55° for 1 min, and 72° for 1 min, followed by a final elongation cycle of 72° for 5 min.

Sequencing: The macronuclear and micronuclear PCR products from AIM-2 were amplified in two separate reactions each and purified on an agarose gel. The DNA was extracted from the agarose using a gel purification kit (QIAGEN, Chatsworth, CA). Macronuclear PCR products from 8 F_2 mutant lines were amplified and gel purified. Micronuclear PCR products from 10 F_2 lines (5 each of wild type and mutant) were amplified and gel purified. These purified PCR products were sequenced directly using the ThermoSequenase dideoxy kit (Amersham, Arlington Heights, IL).

Genetic crosses: Mating and the induction and scoring of autogamy were carried out as described by Sonneborn (1970). A cross between two Paramecium cell lines produces heterozygous F_1 exconjugant clones with identical micronuclear genotypes. Homozygous F_2 lines are obtained by inducing autogamy in the F_1 clones. For each gene locus, half of the resulting F_2 lines are homozygous for the allele found in the one parent and the other half are homozygous for the allele found in the other parent. Hence, a normal Mendelian mutation would

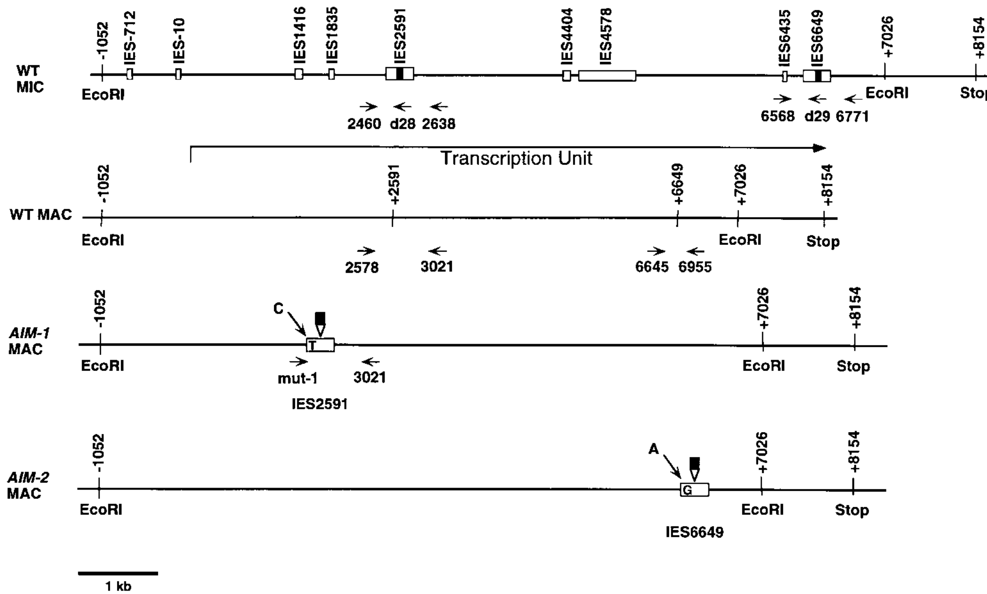

Figure 1.—Maps of wild-type, AIM-1, and AIM-2 micronuclear and macronuclear copies of the *A51* surface antigen gene. The wild-type micronuclear *A51* gene contains seven IESs in the coding region plus two in the 5′ noncoding region. The position of each IES is indicated by a number corresponding to the macronuclear sequence numbered from the start of translation of the *A51* gene. The AIM-1 version of IES2591 contains a single C-to-T base pair mutation that renders it incapable of excision, shown as a C-T in the AIM-1 macronuclear map. The AIM-2 version of IES6649 contains a single A-to-G base pair mutation that renders it incapable of excision, shown as an A-G in the AIM-2 macronuclear map. The *Eco*RI sites designated the ends of the macronuclear probe used in diagnostic Southern blots discussed in the text. The black boxes inside IES2591 and IES6649 represent IESs that are excised from both AIM and wild-type cells during macronuclear development. The arrows represent PCR primers used in analyses. Primers 2578 and 6645 overlap the junctions that are formed if IES2591 and IES6649 are properly excised from the macronucleus. Primer mut-1 begins at +2578 and its most 3′ base is the AIM-1 mutation.

segregate with a 1:1 ratio in the F_2 generation. The Mendelian marker ND (nondischarge) was used to distinguish between the two parental cell lines and to indicate the proper exchange of nuclei. True conjugation was confirmed by the appearance of the trichocyst discharge trait in F_1 cells from both sides of the cross and by its 1:1 segregation in the F_2 (verified by chi-square analysis). Expression of A51 surface protein was used as a marker for identification of the parental cytoplasm of the F_1 progeny as previously described (Epstein and Forney 1984).

Scoring for A51 serotype and trichocyst discharge: Expression of the A51 serotype was scored by mixing 100 μl of cells (~100 cells) and 100 μl of anti-A51 serum diluted 1:100 in Dryl's solution (2 mm sodium citrate, 1.0 mm Na_2HPO_4, 1.0 mm NaH_2PO_4, 2 mm $CaCl_2$).

Trichocyst discharge was scored by mixing 20–30 cells with an equal volume of saturated picric acid (~10 μl of each). The discharge of the trichocysts creates a fuzzy halo surrounding the cell when observed under ×400 magnification (Sonneborn and Schneller 1979). Cells were scored as either D (discharge) or ND.

RESULTS

AIM-2 contains a single base change in the conserved TA dinucleotide and reveals a new IES: A collection of cell lines unable to express the A51 surface protein was isolated and then screened by Southern hybridization to detect mutants whose phenotype was the result of the inability to excise one of the IESs in the *A51* gene. The seven IESs within the coding region of the *A51* surface protein gene provide numerous targets for mutagenesis of nucleotides important for IES excision (Figure 1). Each IES is named according to the site of its insertion within the macronuclear coding sequence of the gene. We performed genomic Southern blots using *Ssp*I as the restriction enzyme (data not shown). The Southern hybridization pattern of one of these cell lines was consistent with the presence of IES6649 in the macronuclear genome (Figure 1), and it was subsequently named AIM-2.

Whole-cell PCR was used to amplify the region containing IES6649. Primers on either side of IES6649 (6568-6771) amplified a single band 203 bp in size from wild-type cells, but the same primers produced a fragment of ~550 bp (203 bp + 370 bp of IES) when used with AIM-2 cells. We sequenced two separate whole-cell PCR products from AIM-2. Comparison of the mutant sequence to the known wild-type sequence revealed a single A-to-G transition mutation within the conserved terminal repeat sequence (Figure 2A). This base change is located in the invariably conserved 5′-TA-3′ direct repeat in the consensus (Figure 2B).

In addition to the single base change, the macronuclear DNA of AIM-2 contains a deletion of 29 bp inside IES6649. This deletion is flanked by 5′-TA-3′ repeats and the ends have a reasonable match to the terminal inverted-repeat consensus (Figure 2B). The sequence features of this deletion suggest that it is an IES located inside IES6649. It was revealed only because excision of the larger 370-bp IES was inhibited.

Using one primer located outside IES6649 and one primer that overlapped the 29-bp macronuclear deletion, we amplified the micronuclear DNA from IES6649 in the AIM-2 line. The sequence of this segment confirmed that the 29 bp missing from the macronucleus

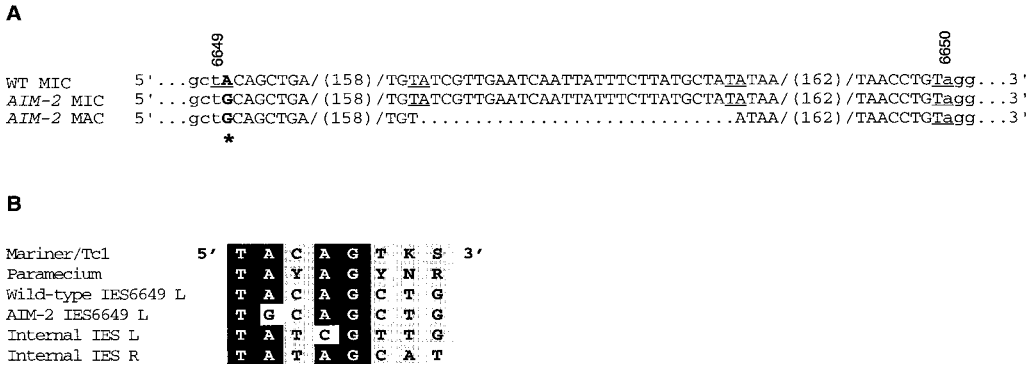

Figure 2.—(A) Sequence of IES6649 in wild-type and AIM-2 micronuclear DNA and AIM-2 macronuclear DNA. The IES sequence is uppercase and the macronuclear sequence is lowercase. A base at either end of IES6649 is numbered for reference. The base change is bold and starred; the deletion is shown as a series of dots in the AIM-2 macronuclear sequence. The numbers in parentheses indicate the number of bases not included in the figure. The micronuclear sequence of AIM-2 was directly determined from beyond the left end of IES6649 into the 29-bp internal IES. The remainder of the sequence was deduced from the AIM-2 macronuclear DNA. (B) The terminal inverted-repeat consensus sequences (5′-3′) for *mariner*/Tc1 transposons and Paramecium IESs are shown along with the corresponding terminal sequences from wild-type IES6649, AIM-2 IES6649, and the internal 29-bp IES. Left and right indicate the ends of the deleted elements. The black and gray shading indicates nucleotides that are identical and similar to the Paramecium IES consensus, respectively. K, G or T; S, C or G; N, any base; Y, C or T; R, G or A.

of AIM-2 is present in the micronuclear DNA. The PCR product contained the same base change found in macronuclear DNA, confirming that the DNA was amplified from the AIM-2 strain. Although we cannot conclusively demonstrate that the product was amplified from micronuclear DNA as opposed to a nonexcised macronuclear copy, the results clearly indicate that the 29-bp deletion is the result of a DNA processing event and not a micronuclear deletion of the sequence. Therefore, the 29-bp sequence inside IES6649 meets the criteria for a Paramecium IES.

The AIM-2 mutation shows Mendelian segregation: Micronuclear mutations are expected to show typical Mendelian inheritance in a genetic cross (Figure 3). A cross between AIM-2 and wild-type cells was therefore performed to demonstrate that the nucleotide mutation is correlated with defective IES excision.

We mated homozygous AIM-2 cells to a homozygous cell line that contained a recessive Mendelian marker called trichocyst nondischarge (51ND) and was wild type at the *A51* locus. F_1 cell lines from nine mated pairs were scored for *A51* gene expression and trichocyst nondischarge. Because conjugation between two cells results in F_1 progeny with identical micronuclear genomes, true exconjugants are heterozygous (ND/+) and therefore trichocyst discharge. Southern hybridization of total genomic DNA was performed to determine whether each F_1 contained the IES in its macronucleus. All F_1 lines expressed the A51 surface protein and contained both the wild-type and mutant versions of the

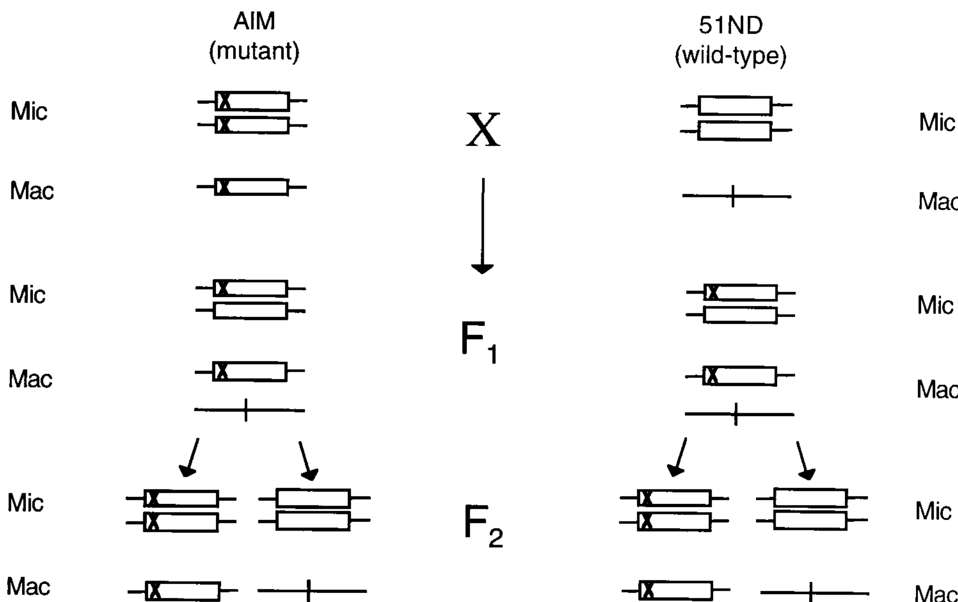

Figure 3.—Diagram of a mating between an AIM cell line and wild type (51ND). The base change (X) in the AIM parent causes retention of the IES (rectangle) in the macronucleus whereas the IES is excised in the wild-type parent. The F_1 are heterozygous for IES retention in both the macronucleus and micronuclei. The F_2 are generated by autogamy. Each F_1 cell produces a single haploid zygotic nucleus that it then replicates by mitosis. The two haploid products fuse and macronuclear development occurs. This creates the two possible classes of F_2 (AIM and wild type) in equal numbers (1:1 segregation).

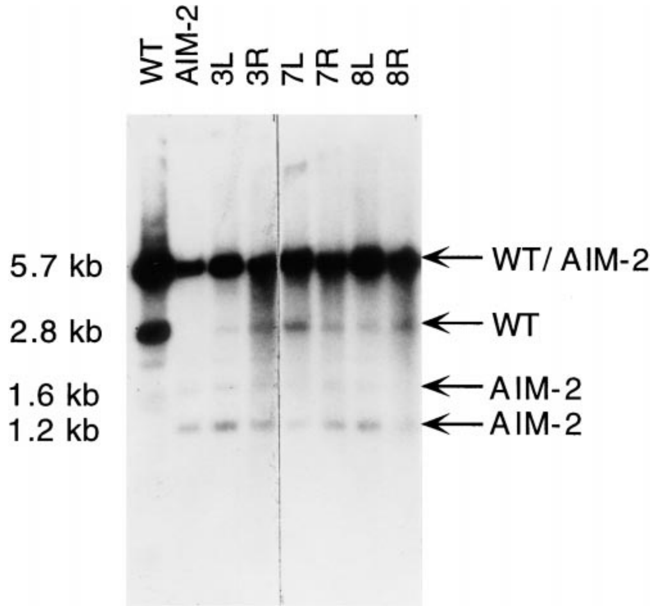

Figure 4.—Southern blot of F_1 cell lines from three separate AIM-2 to wild-type matings. Genomic DNA from each F_1 cell line was digested with *Ssp*I. The blot was probed with the wild-type 8.1-kb macronuclear *Eco*RI *A51* gene fragment contained in the plasmid pSA8.8R described in the text. Wild type shows two fragments of 5.7 kb and 2.8 kb, respectively. In AIM-2, the 2.8-kb fragment is further digested into two bands of 1.2 kb and 1.6 kb due to an *Ssp*I site within IES6649.

A51 gene in the macronucleus (Figure 4). The presence of both the wild-type and mutant bands was confirmed with whole-cell PCR (data not shown). This is consistent with the Mendelian inheritance of a recessive micronuclear nucleotide change.

Selected matings were followed into the F_2 generation by the induction of autogamy, which results in a completely homozygous micronuclear genome. A typical micronuclear mutation in Paramecium should show 1:1 segregation in the F_2 (see Figure 3). Each F_2 line was scored for trichocyst nondischarge and then analyzed using PCR to determine the presence or absence of IES6649 in the macronucleus. The results of a representative mating are shown in Table 1. The presence of the IES in the macronucleus segregated 1:1. More importantly, micronuclear IES sequences of 10 F_2 lines from this mating showed that those with the mutant PCR band contained the base change in the micronucleus while those F_2 with the wild-type PCR band contained a wild-type micronucleus. Additional matings were done that also were consistent with a 1:1 segregation and in which mutant F_2 lines were sequenced to ensure they contained the base change (data not shown). However, in these matings, our PCR assay occasionally showed a mixture of both wild-type and mutant products, which we now believe were due to the presence of old macronuclear fragments that were not sufficiently diluted by cell divisions in the F_2 generation.

Each mutation is sufficient to eliminate excision of its respective IES: Recent research has shown that IES2591 and IES6649 in the old macronucleus are capable of inhibiting the excision of their corresponding micronuclear copy during macronuclear development (Duharcourt *et al.* 1998; Mayer *et al.* 1998); therefore, the old macronuclear copies of IES2591 or IES6649 could contribute to the retention of these IESs in the AIM cell lines. To determine whether IES retention in the AIM macronuclei was due to the micronuclear base change or to inhibition by the old macronucleus, we mated each AIM mutant to d12. This cell line contains a total deletion of the *A51* gene in both the micronucleus and the macronucleus; thus, in the F_1 progeny derived from the d12 parent, the IES mutation was placed into a cytoplasmic environment where inhibition of excision by old macronuclear copies could not occur. Furthermore, any excision that occurs must be of the mutant version of the IES. No excision was detectable by Southern blot in any of the F_1 progeny from 10 matings of each AIM mutant to d12 (data not shown).

To determine whether a limited number of excision events occurred, we analyzed the DNA from each F_1 cell line by PCR using one primer that overlaps the macronuclear junction formed after proper excision of each IES and another primer inside the *A51* coding region. By performing PCR on a series of dilutions of wild-type total genomic DNA, we determined that the primers for IES2591 could detect 1 excised copy in 100, while the primers for IES6649 could detect 1 excised

TABLE 1

Inheritance of phenotypes in the F_2 generation from a representative AIM-2 (mutant) to wild-type mating

Descendant cytoplasm	No. of descendants with PCR band			Marker segregation		
	Mutant	Wild type	χ^2	D	ND	χ^2
Mutant	15	8	2.13	6	17	5.26[a]
Wild type	12	8	0.8	11	9	0.2

The number of F_2 cell lines exhibiting each phenotype is given, along with the chi-square value based on a 1:1 segregation for each trait.

[a] Significant values, $P > 0.950$.

copy in 1000. The agarose gel containing PCR products from the IES2591 assay was blotted and probed with the *Eco*RI fragment of the macronuclear *A51* gene, which increased detection levels to 1 in 1000. None of the F_1 lines from any of the matings produced a detectable PCR product, suggesting that fewer than 1/1000 copies of either IES2591 (Figure 5) or IES6649 (data not shown) are excised. Although imprecise excision would not be detected with this assay, genomic Southern blots of the same F_1 progeny only detect the unexcised version of each respective IES. Each F_1 cell line contains two independently formed macronuclei that segregate as caryonides. Because each macronucleus contains ~1000 copies of each gene, this assay should detect any accurately excised molecule in the F_1 population. The lack of PCR products suggests that excision is extremely low or nonexistent.

No alternate excision of IES2591 is detected: A question was posed in our first article on the AIM-1 mutant. We considered whether the base change in IES2591 could create alternate excision products utilizing one of the ends of the internal IES along with the wild-type end of IES2591. To investigate the possibility that this alternate type of excision was occurring in AIM-1, we analyzed F_1 DNA from the AIM-1 to d12 matings by PCR using one primer overlapping the left edge of IES2591 (mut-1 primer) and one primer outside the right edge of IES2591 (+3021; see Figure 1). Primer mut-1 contains the AIM-1 mutation as the most 3′ base, and tests showed that it will amplify only the mutant copy of IES2591 (data not shown). This primer pair results in a band of ~800 bp in AIM-1 cells and no band in wild-type cells. If an alternate form of excision using either edge of the internal IES with the wild-type edge of IES2591 had occurred, two additional amplification products could have been produced (592 bp and/or 565 bp). No such bands were visible on the agarose gel when stained with ethidium bromide. The gel was blotted and probed with the macronuclear *A51* gene (Figure 6). Again, no bands of the correct size were visible, suggesting that any alternate excision must occur at a very low frequency.

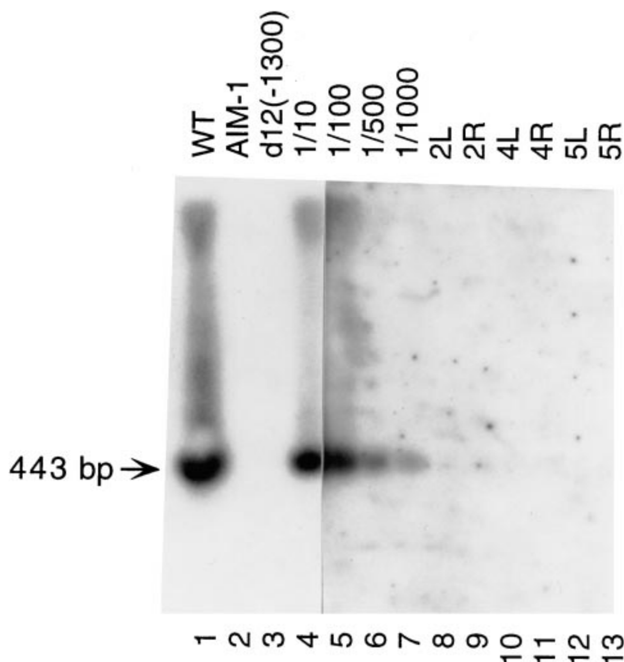

Figure 5.—Genomic DNA from F_1 cell lines from three separate AIM-1 to d12 matings, AIM-1, d12, wt, and wt diluted with d12 in the ratios indicated was amplified with primer +2578 and +3021 to detect junctions formed after correct excision of IES2591. The PCR products were run on a 1.8% agarose gel that was then blotted and probed with the wild-type 8.1-kb macronuclear *Eco*RI *A51* gene fragment contained in the plasmid pSA8.8R described in the text. Lanes 1–4 are from a 24-hr exposure of the blot; lanes 5–13 are from a 10-day exposure of the same blot. No bands were detected in the F_1, even after a 10-day terminal exposure.

DISCUSSION

Sequence requirements for IES excision: The micronuclear genomes of both *P. tetraurelia* and *E. crassus* contain IESs flanked by 5′-TA-3′ dinucleotides. Extensive sequence analysis of these so-called TA IESs revealed an 8-bp terminal inverted-repeat consensus sequence similar to that of *mariner*/Tc1 transposons (Klobutcher and Herrick 1995); however, the lack of procedures for germline transformation in these ciliates has prevented a systematic analysis of the sequence requirements for IES excision. Thus far, the isolation of Paramecium mutants unable to excise specific IESs provide the only functional analysis. The mutation in the AIM-2 cell line, an A-to-G transition in the flanking TA dinucleotide, prevents any detectable excision of IES6649, thus demonstrating the necessity of the TA for IES excision. Interestingly, Dubrana *et al.* (1997) previously found indirect evidence for the requirement of the flanking TA by studying various alleles of a single IES located downstream of the *G156* and *G168* surface antigen genes. They found that a base change from A to T in the terminal TA caused the IES boundary to be shifted to a new TA 1 bp downstream in a different allele. Both data are consistent with the necessity of a junction occurring at TA. It is possible that there is only a small region near the IES where alternate use of a TA can occur. Because IES6649 does not contain an alternate TA within the window, the mutation in AIM-2 completely prevents IES excision.

More surprising is our demonstration that the single base change in AIM-1 prevents any detectable excision of IES2591. Originally we anticipated that the presence of IES2591 in the old macronucleus contributed to the excision defect in AIM-1 and therefore we expected to detect some excised products when AIM-1 was mated to the *A51* deletion strain d12. The C-to-T mutation in the AIM-1 cell line occurs in the conserved but not

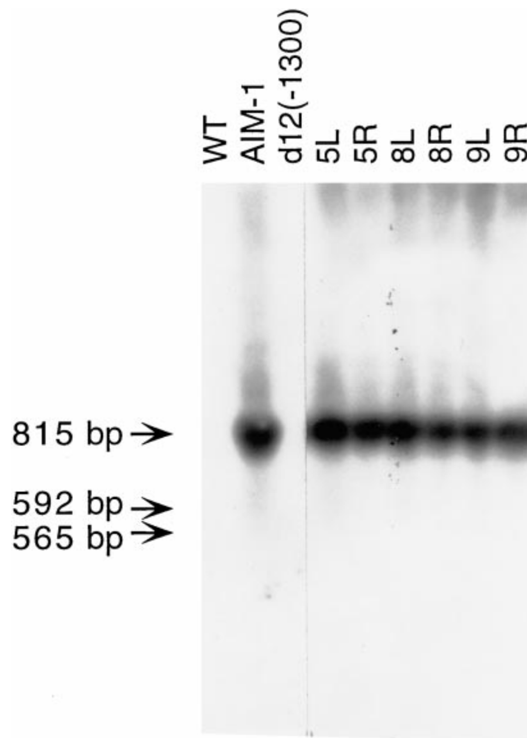

Figure 6.—Genomic DNA from F_1 cell lines from four separate AIM-1 to d12 matings was amplified with the mut-1 and +3021 primer, run on a 2.0% agarose gel, and then blotted and probed with the wild-type 8.1-kb macronuclear *Eco*RI *A51* gene fragment contained in the plasmid pSA8.8R described in the text. This primer pair amplifies only the AIM-1 mutant version of IES2591; thus the wild-type control is blank. If alternate excision were occurring as described in the text, additional bands would be present at 592 bp and/or 565 bp. No such bands are visible in this 19-hr exposure of the blot, nor in a 10-day terminal exposure of the blot (data not shown).

invariant position 5 of the inverted-repeat consensus. Although G appears at a frequency of 78% and most alternate sequences contain an A residue, C does appear in a few cases (including the wild-type IES2591 sequence) and one IES has been reported with a T in this position (Vayssie *et al.* 1997). The observation that T can function in position 5 within at least one Paramecium IES, but not in the AIM-1 IES2591, suggests that the context of specific IES sequences is critical for excision.

Relationship between Paramecium IESs and *mariner*/Tc1 transposons: The two strongest pieces of evidence for a link between TA IESs and transposable elements are the shared terminal inverted-repeat consensus sequence and the presence in Euplotes of somatically excised Tec transposons that share the TA IES consensus and also contain putative open reading frames for transposase (Jacobs and Klobutcher 1996). However, it is also clear that even if IESs are related to transposons, IESs and their excision factors have become quite different from their transposon ancestors. For example, the low abundance of the transcript encoding the putative Tec transposase makes it unlikely that it is responsible for the excision of the Euplotes short IESs (Jaraczewski *et al.* 1994). And, while the isolation of the AIM cell lines clearly supports the functional importance of the Paramecium terminal inverted-repeat consensus, the functional relationship between this consensus and the conserved sequences at the termini of *mariner*/Tc1 transposons remains unclear. The flanking 5′-TA-3′ dinucleotide is apparently required for excision of some MLEs but not for others. *In vitro* excision of Tc1 is eliminated when the flanking TA is changed to CG (Vos *et al.* 1996), while Tc3 transposition still occurs *in vivo* despite mutation of the flanking TA to CG (van Luenen *et al.* 1994). Whether these results reflect a real difference between the two transposons or the context of the experiments (*in vivo vs. in vitro*) remains to be resolved.

There are also differences between TA IESs and *mariner*/Tc1 transposons in both frequency and precision of excision. Both *mariner*/Tc1 transposons and TA IESs are somatically excised from the genome, which, in the case of Paramecium, means that as many as 65,000 excision events occur during macronuclear development. Furthermore, TA IES excision is evidently very precise. Only one example of alternate excision is known (Dubrana *et al.* 1997). One allele of the W2 IES located downstream of the *G156* surface antigen gene alternatively uses two different TAs at the 3′ end of the element, but the only remnant of the IES after excision is one of the original TAs. On the other hand, small "footprints" of a few base pairs generated by gap repair are common remnants of *mariner*/Tc1 excision (Bryan *et al.* 1990).

And, despite the interesting link between TA IESs and transposons, research on Tetrahymena IESs (reviewed in Coyne *et al.* 1996) does not support a common evolutionary connection between all IESs. The termini of Tetrahymena IESs do not contain the 5′-TA-3′ flanking dinucleotide, nor an inverted repeat, but instead are generally flanked by a 4- to 8-bp direct repeat. Excision of the best-studied eliminated element, M, requires internal promoting sequences as well as a polypurine tract (A5G5) about 45 bp outside the element to define the border for excision (Godiska *et al.* 1993). It is not clear yet whether Paramecium IESs require host flanking sequences for excision, but it is known that such sequences are not necessary for *mariner*/Tc1 transposition (Vos *et al.* 1996).

Mechanism of IES excision: The AIM-1 and AIM-2 mutants suggest that recognition of the termini of an IES must involve the consensus sequence, including the flanking TA dinucleotide. However, the lack of alternate excision involving the wild-type end the mutant IES2591 with its respective internal IES implies there must be additional *cis*-acting sequences that serve to define each IES as an independent unit of excision. Whether these additional sequences are located outside or inside the IES remains to be discovered. Interestingly, the *mariner*

element requires an internal site located near the *Sac*I (position +790) site for full mobility (Lohe and Hartl 1996). However, there is no evidence for such an internal site in Paramecium IESs as yet.

A second mechanistic implication arises from our inability to identify broken chromosome ends on a Southern blot (data not shown). Broken ends would indicate that the wild-type end of the mutant elements could be cut by the excision machinery, even though the mutant ends are not cut. It has been shown in previous studies of the d48 mutant that the *A51* gene locus can tolerate broken ends (Forney and Blackburn 1988); thus, the lack of broken ends in AIM-1 and AIM-2 suggests that both ends of an IES must be recognized by the excision machinery prior to cleavage. It has been suggested that such dimerization of the Tc3 transposase is necessary for binding and perhaps cleavage of the DNA (van Pouderoyen *et al.* 1997). Because we found no broken ends in the AIM cell lines, it is possible that Paramecium IESs also require dimerization of the excision machinery for binding or cleavage to occur.

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