A Mutation in *Paramecium tetraurelia* Reveals Functional and Structural Features of Developmentally Excised DNA Elements

Kimberly M. Mayer,* Kazuyuki Mikami† and James D. Forney*

**Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153 and †Research Institute for Science Education, Miyagi University of Education, Aoba-ku, Sendai 980, Japan*

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ABSTRACT

The excision of internal eliminated sequences (IESs) from the germline micronuclear DNA occurs during the differentiation of a new macronuclear genome in ciliated protozoa. In Paramecium, IESs are generally short (28–882 bp), AT rich DNA elements that show few conserved sequence features with the exception of an inverted-terminal-repeat consensus sequence that has similarity to the ends of *mariner*/Tc1 transposons (Klobutcher and Herrick 1995). We have isolated and analyzed a mutant cell line that cannot excise a 370-bp IESs (IES2591) from the coding region of the *51A* variable surface protein gene. A single micronuclear C to T transition within the consensus sequence prevents excision. The inability to excise IES2591 has revealed a 28-bp IES inside the larger IES, suggesting that reiterative integration of these elements can occur. Together, the consensus sequence mutation and the evidence for reiterative integration support the theory that Paramecium IESs evolved from transposable elements. Unlike a previously studied Paramecium IES, the presence of this IES in the macronucleus does not completely inhibit excision of its wild-type micronuclear copy through multiple sexual generations.

PROGRAMMED DNA rearrangements occur in a wide variety of eukaryotic organisms. Examples range from mating type switching in the yeast, to the recombination of immunoglobulin genes in mammals. Among all the eukaryotic phyla, some of the most dramatic and extensive examples of DNA rearrangement occur in ciliated protozoa. These organisms undergo massive developmentally controlled DNA rearrangements as a normal part of their sexual reproductive cycle (reviewed in Prescott 1994; Coyne *et al.* 1996; Klobutcher and Herrick 1997).

Like other ciliates, *Paramecium tetraurelia* contains two morphologically and functionally distinct types of nuclei. The micronuclei are transcriptionally silent and contain the germline genome. In contrast, the amplified DNA in the macronucleus is transcriptionally active and, thus, determines the phenotype of the cell. During sexual reproduction, the old macronucleus is destroyed and a new macronuclear genome is created from the micronuclear DNA. Several types of developmentally regulated DNA rearrangements occur as part of this process, including fragmentation of the germline chromosomes, *de novo* addition of telomeres, and DNA splicing events that eliminate thousands of non-coding sequences called IESs (internal eliminated sequences).

In Paramecium, IESs consist of 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). No significant open reading frames have been found inside the eliminated regions. In the micronuclear DNA, IESs frequently interrupt coding regions and their precise removal is required to maintain an open reading frame in the transcriptionally active macronuclear genes. Based on the number of IESs found so far, it is estimated that as many as 65,000 per haploid genome are removed during macronuclear development (Duharcourt *et al.* 1995).

The determination of the macronuclear and micronuclear DNA sequences for different variable surface protein genes has allowed the comparison of evolutionarily related IESs in Paramecium. Variable surface protein genes are a family of related genes that encode abundant, high molecular weight (250–300 kD) surface proteins which are mutually exclusive in their expression (reviewed in Preer 1986; Caron and Meyer 1989). Alignment of the micronuclear *A* and *B* surface protein gene sequences revealed that the positions of at least three IESs are conserved between the two genes. Comparisons between the sequences show that only the first 4 or 5 nucleotides at each end are identical, and the remaining sequence shares little recognizable similarity (Scott *et al.* 1994a). Even the size of the eliminated DNA is not conserved; IES4578 in the *A* gene is 882-bp in length but its corresponding IES in the *B* gene is only 416-bp.

Despite this general lack of conserved sequence fea-

Corresponding author: James D. Forney, Purdue University, Department of Biochemistry, 1153 Biochemistry Bldg., West Lafayette, IN 47907. E-mail: forney@biochem.purdue.edu

tures, a detailed statistical analysis revealed that Paramecium IESs share a defined, albeit poorly conserved, terminal-inverted-repeat consensus sequence of 8-bp at each end of the IES (Klobutcher and Herrick 1995). Interestingly, the consensus sequence is closely related to the terminal-inverted-repeats of *mariner*/Tc1 transposons (Klobutcher and Herrick 1995), a large family of transposable elements found in organisms ranging from insects to fish (reviewed in Robertson 1995). This observation provided the first indirect evidence that Paramecium IESs evolved from a specific class of transposons. The transposon evolution theory assumes that the ciliate host appropriated control of the excision activity which it supplies in *trans*; thus, the sequence of each element is constrained only to maintain the *cis*-acting sequences required for excision (Klobutcher and Herrick 1997). Additional support for this theory is provided by studies of *Euplotes crassus*, another ciliate with IESs bordered by 5′-TA-3′ dinucleotides. In this organism, micronuclear specific transposons called Tec1 and Tec2 share identity with the terminal-inverted-repeat consensus of Paramecium IESs as well as the *mariner*/Tc1 transposon family (Klobutcher and Herrick 1995). Despite considerable data from comparative sequence analysis, no study has provided direct evidence that the terminal-inverted-repeat consensus in Paramecium or Euplotes is functionally important for IES excision.

In addition to *cis*-acting signals in the micronuclear DNA, there is increasing evidence that DNA rearrangements in the new macronucleus are influenced by the content of the old macronucleus. The first evidence for this effect was obtained from cell lines that have a macronuclear deletion of the *A* surface protein gene but contain wild-type micronuclei (Epstein and Forney 1984; Kobayashi and Koizumi 1990). Despite the presence of normal micronuclei, the macronuclear deletion is inherited by the new macronucleus after sexual reproduction. The effect is gene specific, and it is controlled though the cytoplasm by a *trans*-acting signal originating in the old macronucleus. More recently, the effect of the old macronucleus has been observed for Paramecium IESs. Duharcourt *et al.* (1995) showed that the presence of an IES in the old macronucleus inhibits the excision of the corresponding micronuclear IES during the next round of sexual reproduction. This inhibition is IES specific and copy-number dependent; macronuclei containing more copies of the IES are better able to inhibit developmental excision. The remarkable specificity of the effects of the old macronucleus have led to models that propose nucleic acid interactions between RNA or DNA from the old macronucleus and the DNA in the developing macronucleus (Meyer and Duharcourt 1996a,b; Forney *et al.* 1996). It is unclear whether the inhibitory effect of IESs in the macronucleus is a general property shared by most Paramecium excised elements or only a small subset.

We took a classical genetics approach to identify mutations in *cis*-acting elements which disrupt IES excision. In theory, our approach could also isolate mutations in *trans*-acting elements important in the effect of the old macronucleus on DNA rearrangements. The *A* surface protein gene, which contains 7 IESs within its coding region, is an excellent target for mutagenesis of IES excision. Failure to remove any IES will create either a frameshift or a stop codon and prevent A surface protein expression. We selected mutants that were unable to express the A surface antigen, then analyzed the resulting collection of A− cell lines for mutants defective in the removal of IESs from the *A* gene coding region.

The first mutant we identified fails to remove IES2591 from the *A* gene. Molecular and genetic analysis of this cell line demonstrated that a micronuclear mutation consisting of a single base-pair change within the terminal-inverted-repeat sequence is responsible for the defect in excision. In addition, this mutant cell line has revealed the presence of a 28-bp IES inside IES2591. The small internal IES has ends which 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 IES2591 is not excised. Evidence for internal IESs in Paramecium supports the transposon evolution hypothesis because it implies that IESs are mobile elements that can integrate into other IESs. This is analogous to the reiterative integration of some classes of transposons (San Miguel *et al.* 1996; Bryan *et al.* 1990). Although the presence of either the wild-type or the mutant version of IES2591 in the macronucleus has a partial inhibitory effect on the excision of its wild-type micronuclear copy, the inhibition is not maintained through several sexual generations as observed for the previously studied example of IES inhibition (Duharcourt *et al.* 1995). The combination of evidence for internal IESs and a functionally important base pair in the consensus sequence shared with the *mariner*/Tc1 transposon family supports the hypothesis that Paramecium IESs have evolved from transposable elements.

MATERIALS AND METHODS

Cell lines, media, and growth conditions: *P. tetraurelia* stock 51 is homozygous for the *51A* 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 *A* locus. Line d12 was originally derived from stock 51 and contains macronuclear and micronuclear deletions of the *51A* gene (Rudman *et al.* 1991). All cells were cultured in a 0.25% wheat-grass medium buffered with 0.45 g/L sodium phosphate and supplemented with 0.25 mg/L 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).

Isolation of mutants: Approximately 400,000 well-fed cells

expressing A surface protein were concentrated by centrifugation into a volume of 50 ml. The cells were added to an equal volume of 0.15 mg/ml nitrosoguanidine (1-methyl-3-nitro-1-nitrosoguanidine) dissolved in Dryl's solution (2 mm sodium citrate, 1.0 mm Na_2HPO_4, 1.0 mm NaH_2PO_4, 2 mm $CaCl_2$). After mutagenesis, the cells were divided into 24 different tubes and starved to induce autogamy. The resulting homozygous cells were cultured for 8–10 fissions at 34° to induce A expression and then treated with anti-A serum. The surviving cells were grown an additional 8–10 fissions at 34°, and again treated with anti-A serum. Finally, clonal lines were isolated and scored for A expression.

Total DNA isolation: Two-hundred ml of cell culture (1000 cells/ml) were pelleted in pear-shaped flasks, re-suspended in 0.4 ml of culture fluid, then quickly squirted into 0.8 ml of low EDTA lysing solution (10 mm Tris pH 9.5; 50 mm EDTA; 1% SDS; 0.1 μg/ml proteinase K) and incubated at 65° for 10 min. The DNA was then extracted once with phenol:chloroform (1:1) and once with chloroform before being precipitated with 0.1 volume 3 m NaOAc and 2.5 volumes 95% EtOH. The DNA pellets were re-suspended in 100 μl 1× TE (10 mm Tris pH 8.0, 1 mm EDTA).

Southern blot analysis: Southern blot analyses were performed according to the method of Sambrook *et al.* (1989). In Paramecium, macronuclear DNA is approximately 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 *Xba*I and *Eco*RI, then run on a 1.2% agarose gel. The gel was blotted onto a nitrocellulose filter (Schleicher & Schuell, Keene, NH), which was dried, then 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 filter was 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 *A* gene from −1052 to +7026; see Figure 1) was added. After incubating at 65° overnight, the filter was 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°, 72°, respectively).

PCR amplification: Macronuclear amplification products of the area surrounding IES2591 in the *A* 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; Figure 1). Approximately 5 cells (5 μl) were added to 5 μl of 1.0% NP40. This was placed at 65° for 10 min and 92° for 3 min, 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 primer, 5 U Taq DNA polymerase, and 65 μl H_2O were added to give a 100 μl reaction. PCR consisted of 30 cycles of 92° for 1 min, 50° for 1.5 min, and 72° for 2 min, followed by a final elongation cycle of 72° for 5 min.

Sequencing: The PCR product from AIM-1 was amplified in two separate reactions and purified on an agarose gel. The DNA was extracted from the agarose using a QIAGEN gel purification kit (QIAGEN, Chatsworth, CA). The purified PCR product from each reaction was cloned into pUC119, then sequenced using the Sequenase dideoxy kit (version 2.0; United States Biochemical, Cleveland, OH). The PCR products from two F2 mutant lines were amplified, cloned, and sequenced at the Purdue Sequencing Center. The PCR products from 6 other F2 mutant lines, the micronuclear PCR products from AIM-1 and the PCR products from post-autogamous cells injected with wild-type IES2591 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 F1 exconjugant clones with identical micronuclear genotypes. Homozygous F2 lines are obtained by inducing autogamy in the F1 clones. For each gene locus, half of the resulting F2 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 segregate with a 1:1 ratio in the F2 generation. The Mendelian marker ND (non-discharge) was used to distinguish between the two parents and to indicate the proper exchange of nuclei. True conjugation was confirmed by the appearance of the trichocyst discharge trait in F1 cells from both sides of the cross and by its 1:1 segregation in the F2 (verified by chi-square analysis). Expression of A surface protein was used as a marker for identification of the parental cytoplasm of the F1 progeny as previously described (Epstein and Forney 1984).

Micronuclear transplantation: Wild-type micronuclei were transplanted into amicronucleate AIM-1 cells using the methods of Koizumi (1974) and Mikami and Koizumi (1982) as summarized in Scott *et al.* (1994b). The successfully transplanted recipient cells were cultured for several days, and then autogamy was induced to create a new macronucleus. Presence of the ND phenotype confirmed that the AIM-1 amicronucleate cell was successfully renucleated with a micronucleus that contained a wild-type *A* gene. Transplanted cells were examined by whole-cell PCR to detect excision of the IES. A second autogamy was induced in each cell line to detect increased levels of excision of the IES during macronuclear development. After several days of growth, the cells were again scored for presence of the ND mutation and examined by whole cell PCR.

Macronuclear transformation: Macronuclear transformation was performed as described by Godiska *et al.* (1987). DNA was dissolved in 1× TE (10 mm Tris-HCl pH 8.0, 1 mm EDTA) at a concentration of about 1 mg/ml. Cells were coinjected with p947-18 (containing an *Sph*I-*Bgl*II fragment of the wild-type micronuclear *A* gene from +2393 to +2971) and pPVX-neo, containing the aminoglycoside 3′-phosphotransferase-II gene under the control of the Paramecium calmodulin gene promoter (Haynes *et al.* 1995).

Scoring for A serotype and trichocyst discharge: Expression of the A serotype was scored by mixing 100 μl of cells (approximately 100 cells) and 100 μl of anti-A 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 or ND.

RESULTS

Isolation of a cell line unable to excise the A gene IES2591: The seven IESs within the coding region of the *A* 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 se-

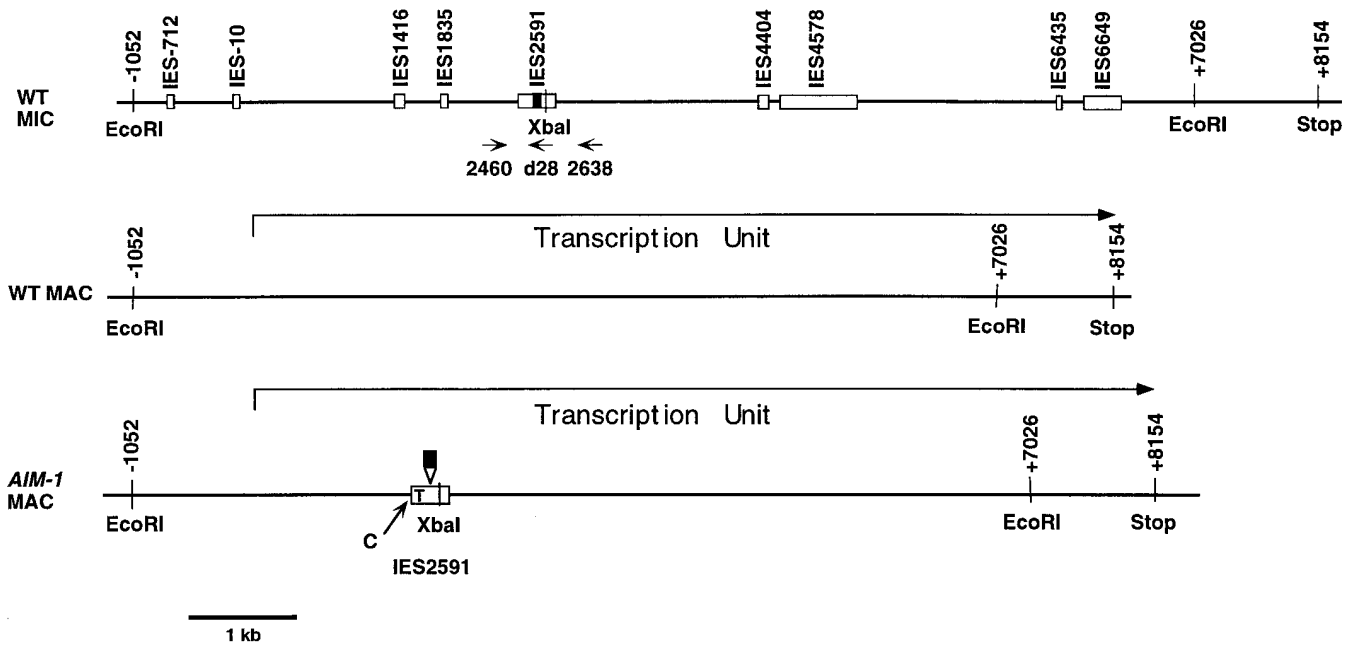

Figure 1.—Maps of wild-type and AIM-1 micronuclear and macronuclear copies of the *A* surface antigen gene. The wild-type micronuclear *A* gene contains seven IESs in the coding region, plus two in the 5′ non-coding region. The position of each IES is indicated by a number corresponding to the macronuclear sequence numbered from the start of translation of the *A* 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 *Eco*RI and *Xba*I sites are used in diagnostic Southern blots discussed in the text. The black box inside IES2591 represents a newly identified 28-bp IES that is excised from both the AIM-1 and wild-type cells during macronuclear development. The arrows represent PCR primers used in analyses.

quence of the gene. Our genetic selection for IES mutations was based on the observation that failure to excise any IES from the *A* gene will inhibit expression of the A protein because the IES will introduce either a frameshift or a stop codon.

A population of *Paramecium tetraurelia* stock 51 (hereafter referred to as wild-type) cells was mutagenized with nitrosoguanidine, and then induced to create new homozygous micronuclei and macronuclei by the self-fertilization process called autogamy. Cells were cultured at 34° to induce A expression, and then treated with anti-A serum, killing the A-expressing cells and selecting for A− cell lines. We analyzed each of the resulting 30 cell lines by genomic Southern blot (data not shown). The Southern hybridization pattern of one of these cell lines was consistent with the presence of IES2591 in the macronuclear genome (Figures 1 and 2), and it was subsequently named AIM-1 (**A** gene **I**ES **M**utation).

AIM-1 contains a single base change in the terminal-inverted-repeat and reveals an internal IES: Whole-cell PCR was used to amplify the region containing IES2591. Primers on either side of IES2591 (primers 2460 and 2638) amplified a single band 178-bp in size from wild-type cells, but the same primers produced a fragment of about 550-bp (178-bp + 370-bp of IES) when used with AIM-1 cells. We sequenced two clones made from separate whole-cell PCR amplifications of AIM-1. Comparison of the mutant sequence to the known wild-type sequence revealed a single C to T transition mutation within the conserved terminal repeat sequence (Figure 3A). This base change is located in a position that is generally conserved as G in the consensus (Figure 3B) and although A and C residues are found in this position in other IESs, only one of the published Paramecium IES sequences contains a T in this position (Vayssie *et al.* 1997).

Figure 2.—Southern blot of wild-type and AIM-1 total genomic DNA digested with *Eco*RI and *Xba*I. Wild-type shows only the 8.1-kb *Eco*RI fragment (lane 1); AIM-1 shows the two *Eco*RI-*Xba*I fragments at 3.9-kb and 4.6-kb (lane 2). The blot was probed with the wild-type 8.1-kb macronuclear *Eco*RI A gene fragment contained in the plasmid pSA8.8R described in the text.

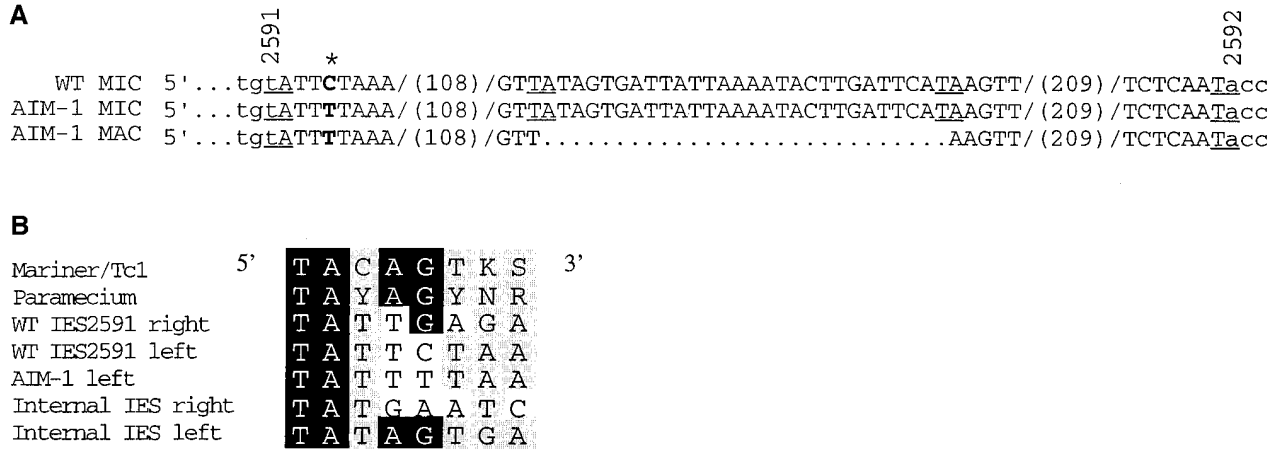

Figure 3.—(A) Sequence of IES2591 in wild-type and AIM-1 micronuclear DNA and AIM-1 macronuclear DNA. The IES sequence is uppercase, the macronuclear sequence is lower case. A base at either end of IES2591 is numbered for reference. The base change is bold and starred; the deletion is shown as a series of dots in the AIM-1 macronuclear sequence. The numbers in parentheses (108 and 209) indicate the number of bases not included in the figure. The micronuclear sequence of AIM-1 was directly determined from beyond the left end of IES 2591 into the 28-bp internal IES. The remainder of the sequence was deduced from the AIM-1 macronuclear DNA. (B) The terminal inverted repeat consensus sequence (5′–3′) for *mariner*/Tc1 transposons and Paramecium IESs are shown along with the corresponding terminal sequences from wild-type IES2591, AIM-1 IES2591, and the internal 28-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.

In addition to the single base change, the macronuclear DNA of AIM-1 contains a deletion of 28-bp inside IES2591. This deletion is flanked by 5′-TA-3′ repeats and the ends have a reasonable match to the terminal-inverted-repeat consensus (Figure 3B). The sequence features of this deletion suggest that it is an IES located inside IES2591. It was revealed only because excision of the larger 370-bp IES was inhibited. Using one primer located outside IES2591 and one primer that overlapped the 28-bp macronuclear deletion, we amplified the micronuclear DNA from IES2591 in the AIM-1 line. The sequence of this segment confirmed that the 28-bp missing from the macronucleus of AIM-1 is present in the micronuclear DNA (Figure 3A). The PCR product contained the same base change found in macronuclear DNA, confirming that the DNA was amplified from the AIM-1 strain. Although we cannot conclusively demonstrate that the product was amplified from micronuclear DNA as opposed to a non-spliced macronuclear copy, the results clearly indicate that the 28-bp deletion is the result of a DNA processing event and not a micronuclear deletion of the sequence. Therefore, the 28-bp sequence inside IES2591 meets the criteria for a Paramecium IES.

The AIM-1 mutation shows Mendelian segregation: Micronuclear mutations are expected to show typical Mendelian inheritance in a genetic cross. Nevertheless, due to effects of the old macronucleus on the DNA rearrangements in the developing macronucleus the macronuclear and micronuclear genotypes do not always correspond. The only previous example of a mutation effecting IES excision, called *mtF*^{E}, does not exhibit simple Mendelian inheritance because the presence of a 222-bp IES in the macronucleus inhibits the excision of the corresponding micronuclear IES whether the micronuclei are mutant or wild-type (Meyer and Keller 1996). This results in a non-Mendelian maternal pattern of inheritance in which all the F1 from the mutant parent are unable to excise the IES. A cross between AIM-1 and wild-type cells was performed to (1) demonstrate that the nucleotide mutation is correlated with defective IES excision and (2) determine whether the presence of the IES in the macronucleus of AIM-1 inhibits the excision of its corresponding wild-type micronuclear copy.

We mated homozygous AIM-1 cells to a homozygous cell line that contained a recessive Mendelian marker called trichocyst non-discharge (51ND) and was wild-type at the *51A* locus. F1 cell lines from twelve mated pairs were scored for *A* gene expression and trichocyst non-discharge. Because conjugation between two cells results in F1 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 F1 contained the IES in its macronucleus. Southern analysis of three pairs is shown in Figure 4. All F1 lines expressed the A surface protein and contained both the wild-type and mutant versions of the *A* gene in the macronucleus. The presence of both the wild-type and mutant bands was confirmed with whole-cell PCR (data not shown). This is consis-

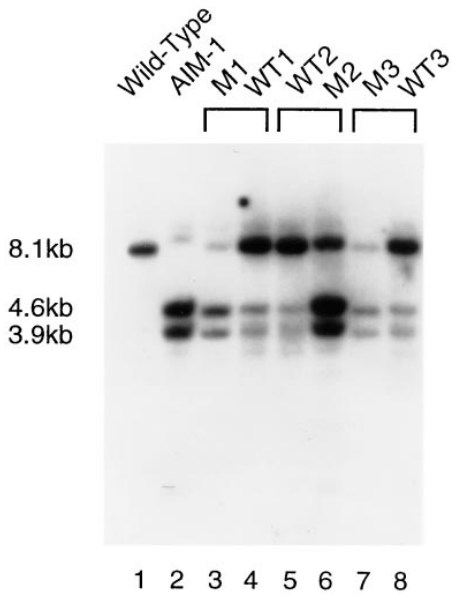

Figure 4.—Southern blot of F1 cell lines from three separate AIM-1 to wild-type matings. Genomic DNA from each F1 cell line was digested with *Eco*RI and *Xba*I. The blot was probed with the wild-type 8.1-kb macronuclear *Eco*RI A gene fragment contained in the plasmid pSA8.8R described in the text. "M" denotes AIM-1 exconjugant, "WT" denotes wild-type exconjugant.

tent with the Mendelian inheritance of a recessive micronuclear nucleotide change.

Although the Southern hybridization of F1 progeny indicates that all exconjugant lines contain both the wild-type (IES−) and mutant (IES+) bands, there are differences in the amounts of each product in different cell lines. These differences in the ratio of excised and non-excised IES from the F1 macronuclear genomes may result from an effect of the old macronucleus on DNA excision. It is possible to differentiate between F1 progeny from the AIM-1 parent and the wild-type (51ND) parent by scoring cells with anti-A serum a few cell divisions after conjugation. Heterozygotes from the AIM-1 parent tend to remain A− and those from the wild-type parent remain A+. Examination of the Southern shows that those F1 which originate from the wild-type (51ND) parent have similar amounts of excised and non-excised product, but those from the AIM-1 parent generally contain much less of the wild-type product (for example, Figure 4, compare lanes 3 and 4). Although not conclusive, the results are consistent with the ability of the IES in the macronucleus to partially inhibit the excision of the wild-type allele. Such an inhibitory effect has been demonstrated for a 222-bp IES in the *G* surface protein gene (Duharcourt *et al.* 1995), but in those experiments the IES completely inhibited excision of its micronuclear counterpart. Evidence presented in the next two sections provides additional support for the conclusion that the AIM-1 version of IES2591 does not completely inhibit excision of its wild-type micronuclear copy.

Selected crosses were followed into the F2 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 F2. Each F2 line was scored for trichocyst non-discharge, and then analyzed using PCR to determine the presence or absence of IES2591 in the macronucleus. The results of three representative crosses are shown in Table 1. Both the presence of the IES in the macronucleus and trichocyst non-discharge segregated 1:1. To determine whether the nucleotide mutation segregates with the inability to excise IES2591, we selected eight independent mutant F2 lines, amplified the IES region using PCR, and sequenced the PCR products. All eight lines contained

TABLE 1

Inheritance of phenotypes in the F2 generation from three representative AIM-1 (mutant) to wild-type (51ND) matings

Pair no. and descendant cytoplasm	No. of descendants with PCR band					Marker segregation			
	Mutant	Wild-type	Both	χ^2	*P*	D	ND	χ^2	*P*
1									
Mutant	6	5	2	0.09	>0.5	4	9	1.92	>0.1
Wild-type	3	3	0	0.00	>0.995	4	2	0.66	>0.1
2									
Mutant	2	1	3	0.33	>0.5	3	3	0.00	>0.995
Wild-type	4	8	0	1.33	>0.1	8	4	1.33	>0.1
3									
Mutant	8	2	1	3.60	>0.05	6	5	0.09	>0.5
Wild-type	2	6	2	2.00	>0.1	7	3	1.60	>0.1

The number of F2 cell lines exhibiting each phenotype is given, along with the chi-squared value based on a 1:1 segregation for each trait.

the mutant base change, confirming that the inability to excise IES2591 segregates with the base change.

Some F2 lines contained both wild-type and mutant PCR bands. These were taken through autogamy to the F3 generation, at which point many resolved to a single macronuclear genotype. Cell lines that did not resolve by the F3 generation were not included in the chi-square analysis but are listed in Table 1. The reason for these "mixed" lines is not clear. Some may result from partial inhibition of wild-type IES excision due to macronuclear copies of the IES; alternatively, under some conditions the mutant allele may be excised with a low efficiency. The complex nature of the F2 data was illustrated by the results from one unusual mated pair (data not shown) which showed 1:1 segregation of excised and non-excised products in the F2 generation from progeny derived from the wild-type parent (51ND), yet only non-excised products were obtained from the mutant parent (AIM-1). This non-Mendelian effect occurred even though the F1 progeny contained both the excised and non-excised products (data not shown). Despite some complexity within the F2 data, the results clearly demonstrate that the C to T base change in the IES is a micronuclear mutation which inhibits IES excision.

Excision of the 28-bp IES inside IES2591 is detected in wild-type cells: The Mendelian inheritance of AIM-1 contrasts with the strong macronuclear effect reported for cell lines containing the 222-bp IES from the *G* surface protein gene (Duharcourt *et al.* 1995). Two possibilities to explain this difference were considered. Either wild-type IES2591, when present in the macronucleus, is not capable of completely inhibiting the excision of its micronuclear counterpart, or the 28-bp that are missing from the macronuclear copies of IES2591 in AIM-1 prevent the inhibitory effect. To distinguish between these possibilities we attempted to create a stable IES+ cell line from a wild-type cell by injecting cloned wild-type copies of IES2591 into the old macronucleus and inducing autogamy to create a new macronucleus. Cells were coinjected with pPXV-NEO, which provides resistance to G418 (Haynes *et al.* 1995), and a plasmid containing IES2591 plus 198-bp and 380-bp of DNA from the left and right flanking regions, respectively. After autogamy whole-cell PCR was performed to examine the macronuclear DNA products. Each cell line contained both the wild-type (IES−) and mutant (IES+) PCR bands indicative of incomplete removal of the IES during macronuclear development (Figure 5). After a second autogamy, all of the cell lines contained the wild-type band (some also retained a fraction of IES+ copies), signifying that the epigenetic effect of the IES in the macronucleus is unstable (Figure 5). In other words, IES2591 is able to inhibit its own excision, but is unable to maintain this inhibition over several cycles of sexual reproduction.

Interestingly, when the PCR products from the cells after one autogamy were run on a high-percentage agarose gel, we were able to discern at least two distinct bands running between 500–550-bp in size (Figure 5). The most abundant band matched the size of the wild-type IES2591 plus flanking sequence from +2460 to +2638 (548-bp). This product may be amplified from plasmid DNA contained in fragments of the old macronucleus that are still present in exconjugants. The plasmid DNA in the old macronuclear fragments is easier to detect by PCR because it is present in higher copy number than endogenous macronuclear DNA. A minor band occasionally observed above the most abundant product was not investigated. We believed that the band below the most abundant represented IES2591 without the 28-bp internal IES; in other words, the smaller IES was excised from a fraction of the macronuclear copies of IES2591. Direct sequencing of the smaller PCR band confirmed that it was missing the 28-bp internal IES. Therefore, the internal 28-bp IES is not merely an aberrant splicing product in the mutant cell line; it also occurs (at least under some conditions) in wild-type cells.

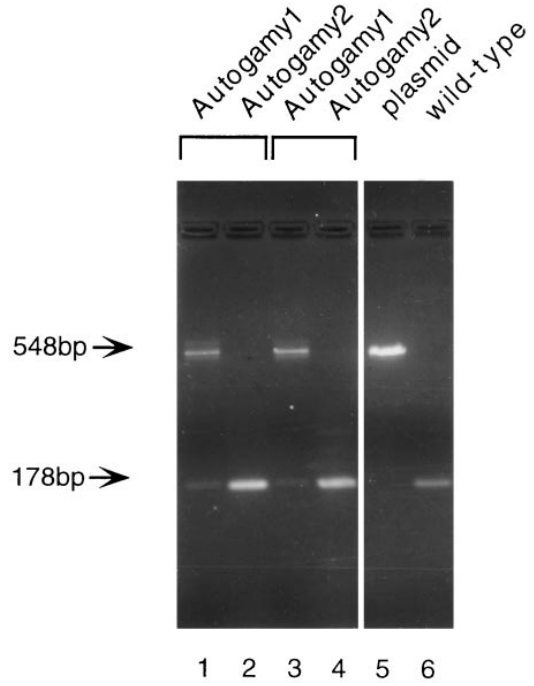

Figure 5.—PCR after the first and second round of autogamy of cells microinjected with wild-type IES2591. Lanes 1 and 2 represent a single injected cell line; lanes 3 and 4 represent another injected cell line. Lane 5 contains the plasmid DNA injected into the cells. Lane 6 is the amplification product obtained from wild-type cells using the same primers.

Transplantation of a wild-type micronucleus into AIM-1 does not result in stable inhibition of IES2591 excision: Micronuclear transplantation is an alternative method used to determine the effect of the old macronucleus on DNA rearrangements in the developing

macronucleus. The idea is to remove the micronuclei from the mutant cell and replace them with a wild-type micronucleus. After induction of autogamy, the new macronucleus should be wild-type unless the old macronucleus inhibits the normal excision events. We used 51ND as the micronuclear donor and an amicronucleate AIM-1 cell line as the recipient. The first autogamy after transplantation resulted in cell lines (T1) that showed the non-discharge phenotype of the donor and were able to express A, signifying IES removal in the macronucleus (Table 2). However, when these cells were analyzed by PCR, they showed both wild-type and mutant bands in their macronucleus, indicating that IES removal was incomplete (data not shown). Because the micronuclear IES sequence is wild-type in these cells, the retention of some copies of the IES in the new macronucleus must be caused by the presence of the IES in the recipient cell macronucleus (the AIM-1 macronucleus). After the second autogamy (T2 lines), each cell line contained only the wild-type band (data not shown). We conclude that IES2591 in AIM-1 (which lacks the 28-bp internal IES) can partially inhibit its own excision; however, this effect is neither complete nor stable.

DISCUSSION

Significance of the inverted-terminal-repeat consensus sequence: The elimination of internal regions of DNA followed by rejoining of the flanking sequences is a common event during ciliate macronuclear development. Although DNA splicing is common to all ciliates, there is increasing evidence that different types of eliminated elements have different sequence requirements for excision. Thus far, all the eliminated elements examined in Paramecium and Euplotes are bounded by 5′-TA-3′ repeats and are therefore referred to as TA IESs. TA elements share several common features, and they are rather different from the features of the best-studied IESs, those in Tetrahymena (discussed below).

In Euplotes, the TA IESs include both large 5.3-kb elements, called Tec1 and Tec2, that have the characteristics of transposons (Jahn *et al.* 1993), and short (31–539-bp) generally unique sequences that have no significant open reading frames. The presence of 5′-TA-3′ direct repeats, frequent inverted repeats, and large IESs that appear to be transposable elements led to early suggestions that short IESs in Euplotes and other hypotrich ciliates are related to transposable elements (Herrick *et al.* 1985; Klobutcher and Jahn 1991). Although only short (28–882-bp) IESs have been identified in Paramecium, the availability of numerous sequences from these elements has allowed a statistical analysis that identified the inverted-terminal-repeat consensus (Klobutcher and Herrick 1995). The similarity of this sequence to the terminal repeats of *mariner*/Tc1 transposons argued that the two types of elements are related, but the lack of an *in vivo* or *in vitro* excision assay has prevented a direct experimental investigation of the *cis*-acting sequences required for TA IES excision. The AIM-1 mutant has now provided a link between the conserved inverted-terminal-repeat sequence and its function in IES excision.

The AIM-1 mutation, a C to T transition in the fifth position of the consensus sequence, is consistent with our expectations based on the analysis of Paramecium IESs. None of the known Paramecium IESs match the mutant allele of IES2591. Although A and C residues are occasionally found in the fifth position of the consensus (predominantly G), none of the original 20 IESs analyzed by Klobutcher and Herrick (1995) had T in the fifth position. Interestingly, a recent report showed that an IES inside the intron of a Paramecium centrin gene does contain a T in the fifth position (Vayssie *et al.* 1997). This suggests that there is no absolute prohibition of T and that other nucleotides in the consensus may compensate for a poor consensus match at position 5.

Effect of the AIM-1 mutation on DNA excision: The single base mutation on the left end of IES2591 in the AIM-1 cell line results in retention of the IES in the macronuclear DNA even though the right end of the IES has a wild-type sequence. The alternative outcome, a broken end healed by the addition of telomere sequence, is clearly not the predominant product, despite the precedence for such healing events in Paramecium. For example, the *A* gene chromosome is broken and healed in the d48 strain (Forney and Blackburn 1988). A connection between DNA elimination and telomere formation in ciliates has been suggested previously (Blackburn and Karrer 1986; Amar 1994). Although in Tetrahymena it is clear that a 15-bp chromosome breakage sequence specifies chromosome fragmentation (Yao *et al.* 1990), the connection between IES excision and telomere formation remains a possibility in Paramecium. It is interesting to note that *in vitro* excision assays of Tc1 transposons show that mutations which prevent double-strand cleavage at one end of an element do not effect cleavage at the wild-

TABLE 2

Phenotypes of strains for micronuclear transplantation

Cell line	Marker	Serotype	PCR
Stock 51ND (micronucleus donor)	ND	A+	Wild-type
AIM-1 Mutant (amicronucleate recipient)	D	A−	Mutant
T1 Lines (3 recipients after 1st autogamy)	ND	A+	Both wild-type and mutant
T2 Lines (3 recipients after 2nd autogamy)	ND	A+	Wild-type

type end (Vos *et al.* 1996). If cleavage at one end of an IES is prevented by a mutation but occurs at the other end of the IES, it should result in double-strand breaks. Although we have no evidence for broken ends in AIM-1, Paramecium may be a useful organism to identify such events *in vivo.* The developmentally controlled excision of IESs means that large numbers of excision events can be examined easily as opposed to the relatively rare excision events of transposons. Also, the ability to heal broken DNA ends during macronuclear development makes it likely that broken ends would be maintained. If double-strand breaks cannot be found it may suggest models for the mechanism of IES excision in Paramecium.

Currently, we do not have any quantitative measure of the AIM-1 mutation's effect on excision of IES2591. Although we cannot detect any spliced product in AIM-1 (K. Mayer, unpublished results), the inhibition by copies of the IES in the old macronucleus may enhance the effect of the nucleotide mutation. In fact, this inhibition may have allowed us to isolate the mutant line. Even if the first macronucleus formed after mutagenesis contained some correctly excised products, the non-excised copies of the IES could increase the inhibition of IES excision after the next round of autogamy, thus strengthening the A− phenotype. The analysis of F1 progeny (Figure 4) shows that without the influence of macronuclear inhibition (progeny from wild-type parent) excision is still inhibited, but it cannot determine whether a portion of the excised product came from the mutant allele. Measurement of the amount of mutant allele that can be excised will require a cross between AIM-1 and the d12 cell line which has a deletion of the *A* gene in the macronucleus and micronucleus (Rudman *et al.* 1991).

Reiterative integration of IES elements and evolution: The AIM-1 mutation revealed the presence of a 28-bp IES inside IES2591. We do not consider this a cryptic splice product because two internal splice sites are used rather than a deletion involving one internal site paired with the non-mutant end of IES2591. In addition, we showed that the internal 28-bp IES can be excised independently in wild-type cells. This result has also been obtained by S. Duharcourt and E. Meyer (personal communication). In a series of quantitative experiments that examine the ability of different IESs to inhibit excision of their micronuclear counterpart they demonstrated independent excision of the 28-bp IES inside IES2591. Their results indicate that, in fact, all detectable macronuclear copies after autogamy are missing the 28-bp internal IES. This demonstrates that excision of the internal IES cannot be inhibited by copies in the old macronucleus, and also suggests that the presence of the PCR product containing the entire IES in our experiments (Figure 5) may be the result of amplification of injected plasmid DNA present in old macronuclear fragments.

Although previous examples of Tec transposons within other Tec transposons have been described in Euplotes (Baird *et al.* 1989; Krikau and Jahn 1991), the presence of small internal IESs presents additional questions regarding the evolution and function of these DNA elements. The most explicit model for IES evolution has proposed that transposons initially spread throughout the micronuclear genome, and then began to shrink in size after the excision function was assumed by the host (Klobutcher and Herrick 1997). The existence of the internal 28-bp IES cannot be explained easily by this model. There is no obvious selective pressure for an internal IES to maintain its *cis*-acting excision sequences since it is already inside an eliminated region. Therefore, if the 28-bp IES was originally an ancient insertion of a large transposon when it inserted into IES2591, it should have lost the ability to be excised from the genome long before it reached its current size of 28-bp.

Nevertheless, there are several possible explanations for the presence of the small internal IES. It could represent a random sequence that is utilized due to its similarity with the inverted-terminal-repeat consensus. Although this remains a possibility, many close matches to the consensus can be identified within regions of macronuclear DNA that are never excised (Klobutcher and Herrick 1995). Therefore, if IESs can be created by chance, it is probably a special property of those located within a functional IES, perhaps due to the recruitment of the excision machinery to the larger IES.

Alternatively, the internal IES could have originated as a full-length transposon that inserted into another transposon, disrupting an important *cis*-acting element (an internal promoting sequence). This model predicts that over evolutionary time the internal IES has conserved its excision function because it is required for excision of the larger IES. Excision of the internal IES regenerates the required site. This explanation is compatible with the Klobutcher and Herrick (1997) model for IES evolution and predicts that the 28-bp IES is removed prior to excision of the larger IES. Unfortunately, at this time we have no detailed information about the excision intermediates for this or any other Paramecium IES.

Finally, it is possible that the 28-bp IES is a recent insertion into IES2591. Small (28-bp) mobile IESs could in part explain the rapid evolution of these excised elements (reviewed in Prescott 1994; Klobutcher and Herrick 1997). Of the four IESs common to both the *A* and *B* surface protein genes, only one is the same size in both genes (Scott *et al.* 1994a). Insertion or excision of IESs into or out of one another could account for these size differences. It is not clear whether reiterative IES integration will be found frequently in Paramecium. One consequence of the mobile IES model is that the internal IESs which are found will represent

the most recent integrations, the older insertions having diverged so that they are no longer capable of independent excision.

Klobutcher and Herrick (1995) have noted that Paramecium IESs are approaching a minimum size of 28–29-bp. The AIM-1 cell line illustrates how mutations which prevent IES excision could increase the frequency of small IESs in the genome. Regardless of how the internal 28-bp IES originated, the mutation causes the incorporation of additional DNA into the macronuclear genome, and the resulting elimination event is 28-bp rather than 370-bp. Although this is unlikely to be common in coding regions, it could occur with reasonable frequency within intergenic regions.

Comparison of Paramecium IES to other types of IES: The best-studied eliminated elements in any ciliate are those in Tetrahymena (reviewed in Coyne *et al.* 1996). Unlike Paramecium and Euplotes, Tetrahymena IESs are neither bounded by 5′-TA-3′ repeats nor do they contain an inverted-terminal-repeat consensus. They are generally flanked by 4–8-bp direct repeats and range in size from a few hundred base pairs to over 13-kb. Elimination of the M− element is dependent on two types of *cis*-regulatory elements: a polypurine tract (A_5G_5) located about 45-bp outside of the deleted region which specifies the deletion border, and internal promoting sequences which are required for excision (Godiska *et al.* 1993). Analysis of an element called mse2.9 has also provided evidence that a short sequence outside the excised element is required for excision (Li and Pearlman 1996). These elements do not resemble any known transposons and do not appear to be related to the TA IESs in Paramecium. Although Tetrahymena IESs undergo alternative deletion events, the products result from joining different sequences to one constant end (Austerberry and Yao 1988). There are no reports of internal IESs.

Macronuclear inhibition of IES excision: The mechanism responsible for the inhibition of IES excision by macronuclear copies of the element is unknown. Duharcourt *et al.* (1995) showed that a 222-bp IES in the *51G* surface protein gene inhibited the excision of its corresponding micronuclear copy, but did not inhibit any other IESs examined. This macronuclear mutation (IES+) shows stable inheritance through multiple rounds of sexual reproduction (autogamy) even though the micronuclear genome is wild-type. Mating this cell line with a normal (IES−) cell line results in F1 progeny that are IES+ if they originate from the IES+ parent and IES− if they originate from the IES− cell line.

The presence of IES2591 in the macronucleus does not have such a dramatic influence on excision. Some excision of the IES occurs in F1 progeny from the AIM-1 parent. In addition, substitution of a wild-type micronucleus for the AIM-1 micronucleus does not result in stable inheritance of the IES+ phenotype in the macronuclear genome. Finally, transformation of the old macronucleus with cloned wild-type IES2591 was able to inhibit IES excision during formation of the next macronucleus, but the trait was not stable into the next sexual generation. S. Duharcourt and E. Meyer have performed a comprehensive study of the ability of various IESs to inhibit excision of their micronuclear counterparts (personal communication). Their results also indicate that inhibition of IES2591 is not as stable as inhibition of the 222-bp IES in *51G*. One possible explanation for the difference in stability is the presence of the internal 28-bp IES. Duharcourt *et al.* (1995) showed that an internal deletion of 147-bp from the 222-bp IES eliminated the macronuclear inhibition of excision. Thus, the AIM-1 macronuclear copy of IES2591, which contains a 28-bp deletion, may be less efficient in macronuclear inhibition. If, in addition, the 28-bp IES does not efficiently inhibit itself (perhaps due to its small size), it would be difficult to keep a stable IES2591+ cell line.

The only other known micronuclear mutation affecting IES excision is called mtF^E (Meyer and Keller 1996). This pleiotropic mutation affects mating type determination as well as several other phenotypic characteristics and inhibits the excision of the 222-bp IES in the *51G* gene. Although the molecular defect in this strain is not known, it has been suggested that mtF^E encodes a *trans*-acting factor required for excision of a subset of IESs, at least one of which is involved in mating-type determination.

In lieu of an *in vivo* or *in vitro* excision assay, the isolation of mutations that prevent IES excision may provide the best opportunity to investigate the structure and function of IESs in Paramecium. The apparent flexibility of the inverted-terminal-repeat consensus suggests that it may be possible to isolate intragenic suppressors of the AIM-1 mutation. These suppressors could be mutations in other positions of the consensus that compensate for the original mutation. We may also identify genetic loci encoding *trans*-acting factors that are required for excision of one or a few IESs as proposed for mtF^E. These would appear as IES+ *A* gene mutants in which the mutation is unlinked to *A*.

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