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Deletion endpoint allele-specificity in the developmentally regulated elimination of an internal sequence (IES) in *Paramecium*

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ABSTRACT

Ciliated protozoa undergo thousands of site-specific DNA deletion events during the programmed development of micronuclear genomes to macronuclear genomes. Two deletion elements, W1 and W2, were identified in the *Paramecium primaurelia* wild-type 156 strain. Here, we report the characterization of both elements in wild-type strain 168 and show that they display variant deletion patterns when compared with those of strain 156. The $W1^{168}$ element is defective for deletion. The $W2^{168}$ element is excised utilizing two alternative boundaries on one side, both are different from the boundary utilized to excise the $W2^{156}$ element. By crossing the 156 and 168 strains, we demonstrate that the definition of all deletion endpoints are each controlled by *cis*-acting determinant(s) rather than by strain-specific *trans*-acting factor(s). Sequence comparison of all deleted DNA segments indicates that the 5′-TA-3′ terminal sequence is strictly required at their ends. Furthermore the identity of the first eight base pairs of these ends to a previously established consensus sequence correlates with the frequency of the corresponding deletion events. Our data implies the existence of an adaptive convergent evolution of these *Paramecium* deleted DNA segment end sequences.

INTRODUCTION

Ciliate cells contain two types of nuclei throughout their vegetative life. Macronuclei are transcriptionally active and govern the cell phenotype. Micronuclei are transcriptionally inert. In the course of sexual processes, macronuclei degenerate while micronuclei undergo meiosis, providing genetic continuity between sexual generations. Upon fecondation, some mitotic products of the zygotic nuclei differentiate into new macronuclei through extensive DNA amplification and DNA rearrangements. These include site-specific DNA deletion, chromosome fragmentation and *de novo* telomere addition (1–4).

Little is known about the factors involved in the site-specific DNA deletion reactions of *Paramecium aurelia* species although more than 65 000 such events have been estimated to occur per haploid genome (5). The internal eliminated sequences (IES) of *P.aurelia* are short unique DNA sequences of 26–882 bp (6–11). They are bounded by 5′-TA-3′ terminal repeats, one copy of which is retained at the macronuclear chromosome junctions. These sequences do not show any internal sequence conservation but clearly exhibit nucleotide preference within the first eight base pairs at their ends, the consensus (which includes the 5′-TA-3′ repeat) being 5′-TA(C/T)AG(C/T)N(A/G)-3′ (12). Thus, it has been suggested that ends of the deleted DNA segments are functionally related. Since their consensus sequence resembles the conserved sequence at the ends of the Tec transposons from the ciliate *Euplotes crassus* (12), *P.aurelia* IESs have been proposed to originate from a common ancestor despite the absence of overall sequence relatedness (12).

The W1 and W2 micronuclear sequences of *Paramecium primaurelia* strain 156 undergo site-specific deletion during macronuclear development (6,10). We characterised their alleles in *P.primaurelia* strain 168 and determined that the W1 element is constitutively retained within the macronuclear genome while the W2 element is excised utilising two alternative boundaries on one side. Analysis of crosses between the 156 and 168 strains, as well as sequence comparison between the ends of the eliminated DNA segments, provide information on the *Paramecium* IES excision *cis*-acting determinant(s) and on their evolution.

MATERIALS AND METHODS

Culture strains

Paramecium primaurelia strains 156 and 168 are wild-type strains of different geographical origins which have entirely homozygous micronuclear genomes (13). Strains 156 and 168 display important restriction fragment length polymorphisms (RFLPs) that have been used extensively in genetic studies (14,15). The macronuclear mutant of the 168 strain has a wild-type micronuclear genome and a mutant macronuclear genome that lacks many copies of the *G* gene 3′ end flanking region (16). All the cell clones used for molecular and genetic analysis derive from a karyonidal cell (or karyonide) that harbours a macronuclear genome produced by a single developmental event. Cell cultures and cell meiosis were as described (17).

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DNA analysis

The extraction and restriction of the whole-cell DNA were carried out as described (16). The amplification products were electrophoresed in 1× TBE (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3) and blotted onto Hybond N+ (Amersham). Oligonucleotides were labelled using [γ-^{32}P]ATP in a kinase reaction. Hybridization was carried out overnight in 7% sodium dodecyl sulfate (SDS), 0.5 M sodium phosphate, 1% bovine serum albumin (BSA) at 60°C. Membranes were washed at 63°C in 0.1% SDS and 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M Na citrate, pH 7.0). Sequencing reactions were performed using the Sequenase kit version 2.0 (United States Biochemical Corp.). Alignment between the 156 and 168 sequences was performed with the GAP/GCG program (version 8) (gap weight: 5.0, length weight: 0.0). We used a Molecular Dynamics phosphorimager to quantify hybridization of the 1.15 and 1.20 kb fragments with the p3 oligonucleotide and a FUGIS BAS1000 phosphorimager to quantify autoradiography densitometric traces of the 134, 139 and 140 bp end-labelled restriction fragments harbouring deletion junctions.

Amplification reactions and cloning of the reaction products

Polymerase chain reactions (PCRs) (25 µl) comprised 200 ng DNA, 1× PCR buffer supplied by the manufacturer (Epicentre, Tebu), 50 µM of each dNTP, 1 µM of each oligonucleotide and 0.8 U *Tfl* enzyme. Reactions were performed in capped 0.5 ml Sigma polypropylene microcentrifuge tubes in a Perkin Elmer Cetus thermocycler. They involved 25–30 cycles of 92°C for 1 min, 63°C for 1 min 15 s and 70°C for 1 min 30 s, followed by a final extension at 72°C for 3 min. The amplification reaction products were purified by using the Geneclean procedure (Bio 101, Inc).

The following oligonucleotides (5′ to 3′) were used either in PCR or in hybridization reactions as indicated:

p1, CCAACCATTCTCTTCTAAATTAAATCATACTCA;
p2, ATATTTAAATTATGGACCTCACCTCTA;
p3, GGGATGCAGAAATGCTTGAAATGAAATCTG;
p4, TTAATTCTTTAAGAGCAATTCTATTTAAGACTTC;
p5, GAAAAAAGTAGCAGAATTCGCCTGCTAAATTA;
p6, CTAAACAAAGGCAAATTTAAATCAATGAAAC;
p7, GAAAAAAATAGCAGTATTCACCTGCTAAATGA.

The PCR products of 1.15 and 1.20 kb were separated by electrophoresis on a 1.5% agarose gel. They were identified as macronuclear and micronuclear products of the 168 strain, respectively, since they were obtained with highly different ratios from semi quantitative PCRs performed on DNA of wild-type and macronuclear mutant cells. The conditions of semi-quantitative PCR were ensured by monitoring the products of a reaction performed with a 1:1 mixture of the micronuclear and macronuclear DNA templates of the 156 strain.

The PCR products of 1.15 and 1.20 kb were purified from the reactions performed on DNA from the wild-type and macronuclear mutant cell clones, respectively. Both PCR products were unstable in the pGEM2 vector. They were restricted with the *Ase*I and *Sfa*NI enzymes to facilitate cloning as five fragments, based on the sequence of a macronuclear fragment (cloned in the λEMBL4 vector) that overlaps the first 800 bp of the PCR products and on the micronuclear allelic sequence of the 156 strain. The fragments were filled in using T4 DNA polymerase, phosphorylated with kinase and inserted into a *Sma*I-dephosphorylated M13mp18 vector.

The sequence of the 1.20 kb product was derived by sequencing the five fragments; the sequence of the W2^{168} element was checked in an independent PCR trial.

When the 1.15 and 1.20 kb PCR products were used directly as templates for sequencing reactions, only the W2 element of the 1.20 kb appeared missing from the 1.15 kb product. The *Ase*I/*Sfa*NI fragment harbouring the corresponding macronuclear junction was therefore the only one to be sequenced. The 156 and 168 micronuclear sequences have GenBank accession nos U75900 and U75901, respectively.

RESULTS

Sequence comparison of the W1 and W2 elements between strains 156 and 168

Two micronuclear sequences of 76 and 67 bp are eliminated during the development of the micronuclear genome to a macronuclear genome in *P.primaurelia* strain 156 (6,10). These deletion elements are located downstream from the 3′ end of the *G* gene, within a DNA region exhibiting 75% A+T base composition, a characteristic of non-coding DNA (Fig. 1). They are separated by 0.7 kb of macronucleus-destined sequence and hereafter named elements W1^{156} and W2^{156}, respectively. To examine potential variation of elimination patterns between strains, we analysed the W1 and W2 micronuclear elements and the macronuclear sequences downstream from the *G* gene 3′ end in *P.primaurelia* strain 168.

We performed PCR by using the p1 and p2 oligonucleotide primers derived from the sequence of *P.primaurelia* strain 156 on whole-cell DNA of two *P.primaurelia* strain 168 cell clones (Fig. 1). The reaction products were electrophoresed and then hybridized with the p3 internal oligonucleotide, also derived from the 156 sequence (Fig. 1). PCR on whole-cell DNA of wild-type cells was expected to generate a lot of macronuclear product but little micronuclear product as a consequence of the ratio of 800 that characterizes the macronuclear to haploid micronuclear DNA content of *P.aurelia* (18), this ratio resulting in unfavorable competition of the micronuclear DNA template with the macronuclear DNA template. However, PCR on whole-cell DNA of macronuclear mutant cells, in which most of the copies of the *G* gene 3′ end flanking region are missing in the macronuclear genome (16) (see Materials and Methods), was expected to generate large amounts of both micronuclear and macronuclear products due to a balanced competition between their respective DNA templates.

PCRs performed on the two DNAs yielded 1.15 and 1.20 kb products (Fig. 2). The wild-type reaction generated a lot of 1.15 kb product and little 1.20 kb product, with a ratio of 18.3:1.0. Although shorter DNA templates are more efficiently amplified than longer ones, a 0.05 kb difference between two DNA templates was not sufficient, in our hands, to generate products with such different efficiencies (see Materials and Methods). The ratio of 18.3:1.0 should, therefore, mainly reflect the different amounts of the 1.15 and 1.20 kb templates in DNA of wild-type cells. It should be noted that this ratio was very different from the ratio of 800:1 characterizing the macronuclear to haploid micronuclear DNA content of *P.aurelia* (18). This apparent discrepancy was caused by the fact that the macronuclear molecules harbouring the *G* gene end at different nucleotides within the

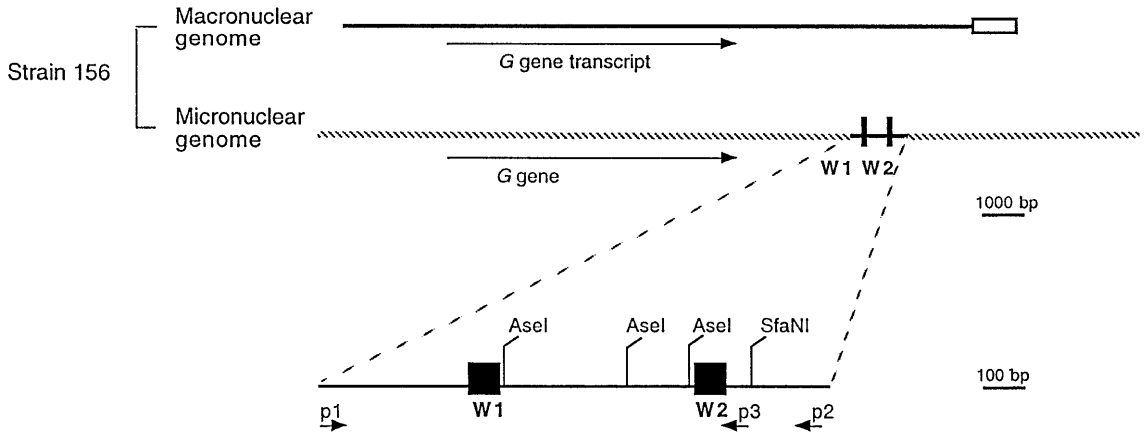

Figure 1. Map of the region flanking the *G* gene in *P.primaurelia* strain 156. The upper and the middle parts of the figure show the macronuclear and micronuclear *G* gene-flanking regions. Macronuclear genome formation involves site-specific excision of the W1 and W2 DNA sequences and chromosome breakage 1.4–2.2 kb downstream from the W2 element (the middle breakage position has been represented in the figure). The hatched lines indicate regions of unknown micronuclear structure. The open rectangle represents the stretch of telomeric repeats added to the new ends. The lower part of the figure shows an enlargement of the W1 and W2 element-containing region. The p1 and p2 oligonucleotide primers used in PCR, the p3 oligonucleotide used for hybridization of the amplification products and the *Ase*I and *Sfa*NI restriction sites used for cloning the amplification products are indicated.

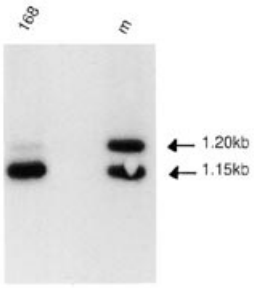

Figure 2. Analysis of the region flanking the *G* gene 3′ end in *P.primaurelia* strain 168. PCR was performed on DNA from two cell clones of *P.primaurelia* strain 168 by using the p1 and p2 oligonucleotide primers derived from the sequence of *P.primaurelia* strain 156 as primers (Fig. 1). The amplification products were separated on a 1.5% agarose gel and hybridized with the p3 internal oligonucleotide also derived from the 156 sequence. Two products of 1.15 and 1.20 kb were identified with different relative intensities in the reactions performed on DNAs of wild-type (lane 168) and of macronuclear mutant (lane m) cell clones.

region encompassing the p1 and p2 oligonucleotides in *P.primaurelia* strain 168 (15). As a consequence, only a fraction of the *G* gene-harbouring chromosomes of the macronuclear genomes of the 168 wild-type cells, i.e. those that end downstream from the p2 oligonucleotide, could provide templates for PCR. The fraction of the *G* gene-harbouring chromosomes that end downstream from the p2 oligonucleotide was even lower in cells of the mutant 168 strain. Indeed, the mutant reaction generated both the 1.15 and 1.20 kb products with a ratio of 0.9:1.0. Altogether, these results suggest that the 1.15 and 1.20 kb products are not generated from different macronuclear templates that result from various rearrangements, but were generated from the micronuclear and macronuclear templates, respectively.

The 1.20 kb product obtained from DNA of the macronuclear mutant cells was purified then restricted with the enzymes *Ase*I and *Sfa*NI and cloned as fragments for sequencing (see Materials and Methods). The 1.20 kb sequence obtained was aligned with the micronuclear sequence of strain 156 (Fig. 3). The 1.20 kb nucleotide sequence of strain 168 differed by 6% with the micronuclear sequence of strain 156, excluding 71 nt that were absent in strain 168. In strain 156, these nucleotides included the first eight positions of the left end of the W1 element and 63 nt into its flanking region.

The W1 element is not deleted in strain 168

In parallel with sequencing of cloned products, we used both micronuclear and macronuclear PCR products directly as templates for sequencing reactions (data not shown). The sequence of the 1.15 kb macronuclear product appeared to lack the $W2^{168}$ element and to retain the $W1^{168}$ element. Therefore, unlike the $W1^{156}$ element that is efficiently eliminated during macronuclear genome differentiation (6), the $W1^{168}$ element appears to be constitutively retained in all or the majority of the macronuclear chromosomes.

Deletion of the $W1^{156}$ element and retention of the $W1^{168}$ element in macronuclear genomes could be a consequence of the strain specificity of either *cis*- or *trans*-acting factor(s). To distinguish between these alternatives, we looked for the deletion/retention patterns of the $W1^{156}$ and $W1^{168}$ elements in whole-cell DNAs from four F1 heterozygous cell clones. These clones were obtained from mating (or conjugation) of one cell of strain 156 with one cell of strain 168. Ciliate conjugation is a reciprocal process that results in the formation of a genetically identical zygotic nucleus in each of the two ex-conjugant cells. Further development gives rise to four karyonidal cells (or karyonides) from which cell clones are derived; a karyonide harbours a macronuclear genome that has arisen from a single developmental event. Two of the karyonides harbour macronuclear genomes formed in the context of the 156 parental ex-conjugant cell; the other two harbour macronuclear genomes formed in the context of the 168 parental ex-conjugant cell. Conjugation features therefore allow us to test, in a single cross, the influence of the parental and progeny genotypes on the deletion of the W1 element. In cases where a *trans*-acting factor(s) encoded by the parental genome determines the deletion patterns, deletion should

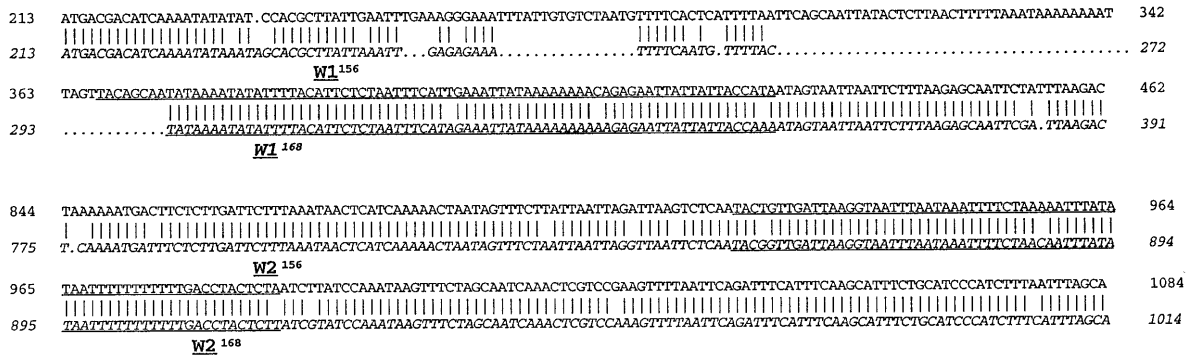

Figure 3. Alignment of the micronuclear sequences of *P.primaurelia* strain 156 (upper line) and *P.primaurelia* strain 168 (lower line) around the W1 and W2 elements. The W1^{156} and W2^{156} elements that are deleted from the macronuclear genomes, and their W1^{168} and W2^{168} corresponding sequences are underlined. Vertical bars between the two sequences indicate nucleotide identity.

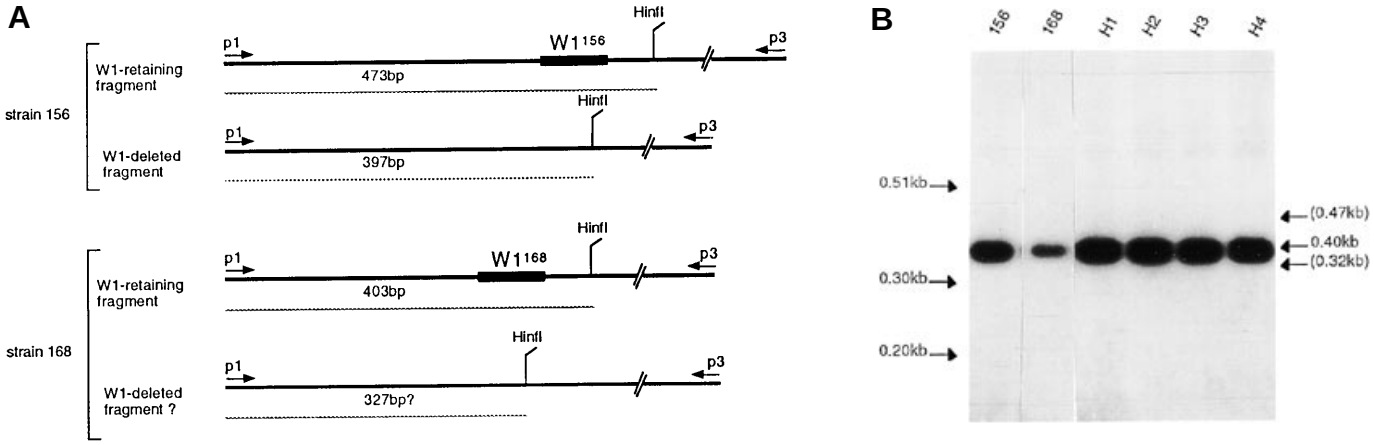

Figure 4. *En masse* characterisation of the patterns of elimination of the W1^{156} and W1^{168} elements. (**A**) Diagram of the amplification, restriction and hybridization reactions designed to analyse the 156 and 168 alleles. The oligonucleotides p1 and p3 were used to simultaneously amplify the 156 and 168 alleles by PCR. The oligonucleotide p1 was used to identify the *Hin*fI restricted fragments, their sizes allow distinction between W1-deleted and W1-retaining fragments for each allele. The question mark '?' in '327bp?' indicates that this band is not expected if the W1 segment is not excised from the 168 allele. (**B**) Hybridization of the amplification products after electrophoretic separation on a 1.5% agarose gel. Migration of '1 kb DNA ladder' fragments (Gibco BRL) are indicated on the left side of the figure. Migration expected for W1-retaining fragments in strain 156 (0.47 kb) and W1-deleted fragments in strain 168 (0.32 kb) are indicated in parentheses on the right side of the figure. Only one product of 0.40 kb was identified in PCRs performed on DNA from a 156 cell clone (lane 156), from a 168 cell clone (lane 168) and also from their four heterozygous progeny cell clones (lanes H1–H4). Macronuclar genomes of the H1 and H2 heterozygotes had differentiated in the 156 parental cell; those of the H3 and H4 heterozygotes in the 168 parental cell.

be dependent on the cytoplasmic context in which the macronuclear genome has developed. Where a *trans*-acting factor(s) encoded by the zygotic genome determines the deletion patterns, then deleted and non-deleted forms should be produced for each allele within the macronuclear genomes of all heterozygous clones. Finally, in the case where a *cis*-acting factor(s) determines the different deletion patterns, these patterns should be allele-specific.

We performed PCR on DNA from parental clones 156 and 168 and from the four progeny clones issued from mating one cell of each of them. Reactions were primed with the p1 and p3 oligonucleotides that are common to both alleles (Fig. 4A). We first checked the successful exchange of the gametic nuclei during the sexual events. Part of the PCR products were restricted with the *Alu*I enzyme, separated by electrophoresis then hybridized with the p3 oligonucleotide (data not shown). Restriction fragments of 174 and 355 bp, respectively, specific for the 156 and 168 alleles, were identified in the macronuclear genome of all heterozygous clones. The same PCR products were then restricted with the *Hin*fI enzyme, electrophoresed and hybridized with oligonucleotide p1 (Fig. 4A). Restriction of the W1^{156}-deleted and W1^{156}-retaining fragments were expected to produce fragments of 397 and 473 bp, respectively; restriction of the W1^{168}-deleted and W1^{168}-retaining fragments were expected to produce fragments of 327 and 403 bp, respectively. Restriction fragments ~0.40 kb (Fig. 4B), representative of a mixture of W1^{156}-deleted and W1^{168}-retaining fragments, were identified from DNA of the F1 progeny whose macronuclar genomes have differentiated in the 156 parental cell (lanes H1 and H2) and in the 168 parental cell (lanes H3 and H4). This indicated that *cis*-acting elements were responsible for the deletion of the W1^{156} element and for the retention of the W1^{168} one during macronuclear genome development.

The W2 element exhibits distinct deletion endpoints in strains 156 and 168

While performing the direct sequencing of the 1.15 kb PCR product obtained from DNA of wild-type cells, we observed a

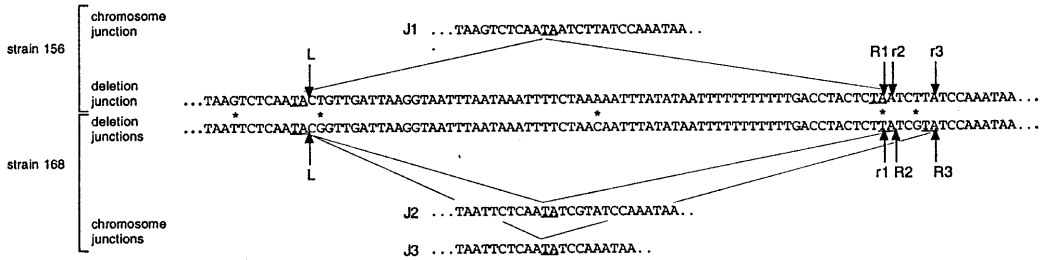

Figure 5. Chromosome junctions resulting from the deletion of the W2^{156} and W2^{168} elements. The sequences of the W2^{156} and W2^{168} elements and their flanking nucleotides are aligned. Asterisk indicate nucleotide differences between the alleles. The upper cases R1, R2 and R3 denote deletion boundaries that were detected experimentally. The lower cases r1, r2 and r3 denote the corresponding positions in the other strain. These positions could not be experimentally detected as deletion boundaries. The J1 junction identified in the macronuclear genome of *P.primaurelia* strain 156 is indicated in the upper part of the figure. The J2 and J3 junctions identified in the macronuclear genome of *P.primaurelia* strain 168 are indicated in the lower part of the figure.

mixture of two sequences around the site of deletion of the W2^{168} element (data not shown). This suggested that two macronuclear junctions were produced by alternative deletion events. The 1.15 kb product was purified and restricted with the enzymes *Ase*I and *Sfa*NI. Fragments indicative of a chromosome junction were selected by hybridization with oligonucleotide p3 and sequenced. Two chromosome junctions, J2 and J3, were characterised (Fig. 5); they both differed from the J1 chromosome junction identified in the macronuclear genome of strain 156. The J2 and J3 junctions, like the J1 junction, resulted from the deletion of DNA segments bounded by 5′-TA-3′ terminal repeats, one copy of which was retained in macronuclear chromosomes. The three deleted DNA segments share a common left endpoint but had distinct right endpoints, R1 in strain 156 and R2 and R3 in strain 168 (Fig. 5). The r1 corresponding position in strain 168 and the r2 and r3 corresponding ones in strain 156 are also indicated in Figure 5. The R2 and R3 endpoints are located 1 and 6 bp, respectively, distal to the r1 position in strain 168.

We examined the strain specificity of the J1, J2 and J3 chromosome junctions by performing *en masse* analysis of the macronuclear genome of 156 and 168 clones. Allele-specific amplification reactions were performed (Fig. 6A) by using oligonucleotides p4 and p5 in the 156 allele-specific reaction and oligonucleotides p6 and p7 in the 168 allele-specific reaction. The reaction products were end-labelled, restricted with the *Ase*I enzyme and separated on a sequencing gel (Fig. 6B). A 140 bp restriction fragment characteristic of the J1 chromosome junction was detected only in the 156 allele-specific reaction. Indeed, no 140 bp fragment could be detected in the 168 allele-specific reaction that would have been indicative of the deletion of a DNA segment ending at the r1 position of allele 168. On the other hand, 134 and 139 bp restriction fragments characteristics of the J2 and J3 chromosome junctions were detected only in the 168 allele-specific reaction. No 134 bp nor 139 bp fragments, that would have been indicative of the deletion of a DNA segment ending at the r2 and r3 positions of allele 156, were detected in the 156 allele-specific reaction. However, there was a weak band ladder resulting from the use of crude oligonucleotides. Further analysis of the PCR products showed that the use of crude oligonucleotides was also responsible for the 120 bp minor fragment in the 168 allele-specific reaction (data not shown); the end-labelled PCR products were restricted with the *Hin*fI enzyme. We observed fragments of 175 and 170 bp, corresponding to the J2 and J3 chromosome junctions, respectively, and fragments of 83 and 82 bp, corresponding to the opposite labelled end primed either with 30mers or 29mers, respectively. The J1, J2 and J3 chromosome junctions therefore appeared to be strain-specific and to represent most, if not all, chromosome junctions in these strains.

To characterise the genetic determinants responsible for the three deletion patterns of the W2^{156} and W2^{168} elements, we performed the 156 and 168 allele-specific reactions on DNA from the four progeny clones issued from mating one cell of strain 156 with one cell of strain 168 derived from the cell clones studied in this section. The J1 chromosome junction characterised by a restriction fragment of 140 bp was detected in DNA of the four progeny clones but in the 156 allele-specific reaction only (Fig. 6B). The J2 and J3 chromosome junctions characterised by restriction fragments of 139 and 134 bp, respectively, were detected in DNA of the four progeny clones but in the 168 allele-specific reaction only. *Cis*-acting elements were thus responsible for the strain-specific patterns of deletion of the W2^{156} and W2^{168} elements during macronuclear development.

We quantified the 134 and 139 bp fragments of this autoradiography by taking densitometric traces. Although autoradiographic saturation could impede accurate quantification, the two fragments showed similar respective proportions in all DNAs (Table 1). The J2 chromosome junction in the 134 bp restriction fragment was more prominent than the J3 junction in all clones.

DISCUSSION

Deletion endpoint allele specificity

Earlier work had demonstrated the presence of the W2 deletion element in the micronuclear genome of *P.primaurelia* strain 156 (6). We show here that deletion of the W2 element produces a unique type of junction on the macronuclear chromosomes in this strain but two types of macronuclear chromosome junction in *P.primaurelia* strain 168. These two junctions result from the alternative deletion of two DNA segments defined by the R2 and R3 endpoints on the right side of the W2^{168} element, both of which differ from the r1 position that is homologous to the R1 endpoint limiting the W2^{156} element. We demonstrate that the deletion patterns of strain 156 and 168 are each controlled by *cis*-acting determinant(s). To our knowledge, this is the first report of a strain-specific deletion pattern, as well as the first report of the use of alternative deletion endpoints for IES elimination in *P.aurelia* species.

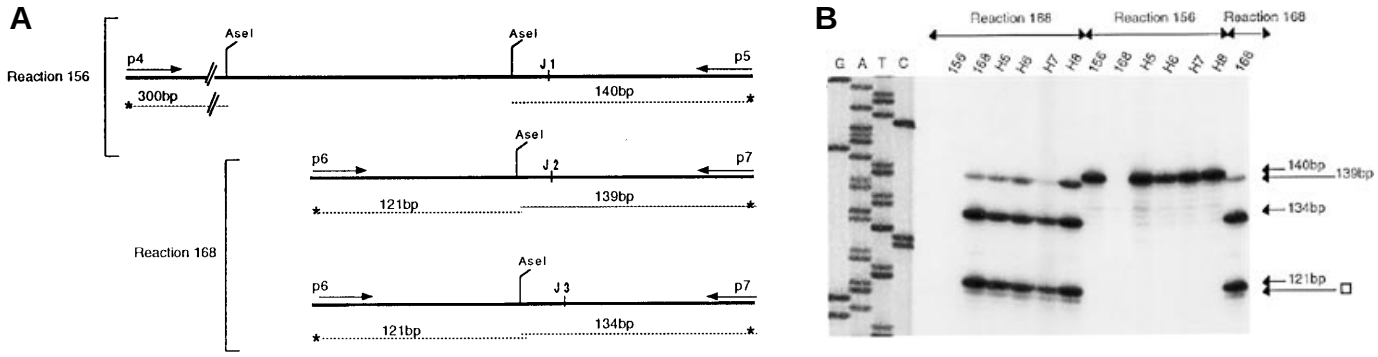

Figure 6. *En masse* characterisation of the pattern of elimination of the W2^{156} and W2^{168} elements. (**A**) Diagram of the amplification and restriction reactions designed to analyse separately the 156 and 168 alleles. Oligonucleotide primer sets p4 and p5, and p6 and p7, were used to selectively amplify the macronuclear versions of the 156 and 168 alleles, respectively, by PCR. The amplification products were end-labelled with [γ-^{32}P]ATP (identified by an asterisk) and restricted with *Ase*I. (**B**) Autoradiography of the amplification products end-labelled with [γ-^{32}P]ATP, restricted with *Ase*I, and separated on an 8 M urea–6% polyacrylamide gel. The 156- and 168-reactions were performed on DNA from a 156 cell clone (lane 156), a 168 cell clone (lane 168), their four heterozygous progeny cell clones whose macronuclar genomes had differentiated either in the 156 parental cell (lanes H5 and H6) or in the 168 one (lanes H7 and H8). The 134, 139 and 140 bp restriction fragments were identified by their sizes, according to the scale defined by sequencing an*Ase*I–*Sfa*NI macronuclear fragment (harbouring the J3 junction) primed with the p7 oligonucleotide. The 121 bp restriction fragment corresponded to the other end-labelled*Ase*I restriction fragment of the 168-specific reaction. The use of crude oligonucleotides was responsible for the weak band ladder observed under the 140 bp fragment and for the 120 bp minor fragment indicated by the □ symbol.

Table 1. Analysis of the ends sequences of the segments eliminated from the W2^{156} and W2^{168} elements

Element	Deletion endpoint[a]	End sequence[b]	Match to the end consensus[c]	Deletion frequency (%)
W2^{156}	L	TACTGTTG	6/7	100
	R1	TAGAGTAG	6/7	100
W2^{168}	L	TACGGTTG	6/7	1000
	R2	TAAGAGTA	3/7	21–43
	R3	TACGATAA	5/7	57–79
W2^{156}	r2	TtAGAGTA	2/7	0
	r3	TAaGATAA	4/7	0
W2^{168}	r1	aAGAGTAG	5/7	0

[a]Positions r2 and r3 in strain 156 correspond to R2 and R3 deletion endpoints in strain 168. Position r1 in strain 168 corresponds to R1 deletion endpoint in strain 156. Nucleotide differences between the end sequences and their corresponding sequences (R1/r1, R2/r2 and R3/r3) are indicated in lower case.
[b]Nucleotides that are identical between the IES end sequences and the IES end consensus sequence 5′-TA(C/T)AG(C/T)N(A/G)-3′ consensus (12) are underlined.
[c]Sequence matches do not take into account the seventh 'free' position of the 5′-TA(C/T)AG(C/T)N(A/G)-3′ consensus.

Alternative chromosome junctions generated by the deletion of the M and R elements were described in the ciliate *Tetrahymena thermophila* (19–21). These junctions correspond closely to the staggered double-strand breaks detected at both ends of the M and R elements and appear to result from the alternative processing of both ends of each element (22). Alternative chromosome junctions in *P.primaurelia* are strain-specific, strongly suggesting that they could be caused by alternative primary events at the right end of the W2 element rather than by alternative processing of both its ends.

IES ends as *cis*-acting determinant(s) for excision

The allelic *cis*-acting elements that determine the strain specificity of the R1, R2 and R3 right deletion endpoints (Fig. 5) could arise from one of the nucleotide differences lying outside the W2^{156} and W2^{168} elements, or from that lying inside the elements, or from the one lying at each end and participating in the previously established consensus of 5′-TA(C/T)AG(C/T)N(A/G)-3′ (12) identified at the ends of *P.aurelia* IESs. The eight base pairs from the ends of the eliminated DNA segments and from their allelic counterparts are compared in Table 1 with the reported IES end consensus sequence. In the hypothesis that the nucleotide differences at the ends of the eliminated sequences are responsible for the allele-specificity of the deletion patterns, sequence end comparison indicates that the 5′-TA-3′ dinucleotide—which has been characterised at the ends of all described *P.aurelia* IESs (6–11)—does not only reflect nucleotide preference for the deletion process but corresponds to a strictly defined nucleotide requirement. This hypothesis also supports the proposition (12) that IES ends constitute *cis*-acting determinants for deletion and that IES ends of different sequences share a functional relationship since the recruitment of the deletion boundaries increase in frequency as the end sequences of the deleted segments more closely fit the consensus sequence (Table 1). The R2- and R3-ending segments of W2^{168} are eliminated with characteristic efficiencies which are proportional to the match of their last nucleotides to the described IES end consensus sequence in the cell clones used in this study and in additional cell clones (data not shown). All end sequences associated with unique deletion junctions match the IES end consensus sequence better than the end sequences associated with alternative deletion junctions (Table 1). The deletion of a unique W1^{156} segment (data not shown) also agrees with this observation since its right and left end sequences match six and seven base pairs of the IES end consensus, respectively (Fig. 3). Competition between two sequences having different affinities for the same machinery could also account for the fact that the r3 sequence fails to define the end of a deleted segment in strain 156, as a result of unfavourable competition with the R1 sequence.

Additional determinants for IES excision

Our data suggest that IES ends are not defined *per se*, but rather that other(s) *cis*-acting element(s) define a short stretch of DNA, in which 5′-TA-3′ sequences are alternatively recruited to act as

deletion boundaries with efficiencies dependent on their adjacent nucleotides. Determinant(s) other than IES ends also appeared necessary for identifying IESs from the profile analysis of *P.aurelia* micronuclear sequences. Based on the 5′-TA(C/T)AG (C/T)N(A/G)-3′ consensus, this analysis identified IES ends but also many non-IES ends (12). *Cis*-acting determinant(s) lying outside IESs have been reported necessary for correct IES excision in the ciliate S*tylonychia lemnae* (23). The *cis*-acting element(s) that are involved in the identification of *P.aurelia* IES ends other than IES ends themselves could be external and/or internal to IESs, as seen in the case of the M and R regions of *T.thermophila* (24,25). Since several 5′-TA-3′ dinucleotides are present at the ends of the $W1^{168}$ element that could define deletion boundaries, it is probable that such *cis*-acting element(s) lie within the 71 nt absent in strain 168 and that their absence is responsible for the constitutive retention of the $W1^{168}$ element in macronuclear genomes.

A *trans*-acting effect was shown to be exerted by the parental macronuclear DNA content on IES deletion in *P.aurelia* differentiating macronuclei (5), in addition to the *cis*-acting element(s) we described above. The presence of a 222 bp IES in the parental macronucleus of *Paramecium tetraurelia* cells results in the specific maintenance of this 222 bp sequence on the macronuclear chromosomes of their sexual progeny (5). This maternal effect is homology-dependent as excision of other IESs is not affected. A similar effect has been described in *T.thermophila* (26). Our analysis shows that the deletion of the $W1^{156}$ element in the differentiating macronucleus fails to be affected by the presence of the $W1^{168}$ sequence in the parental macronucleus (Fig. 4). This could result from nucleotide sequence differences between the 156 and 168 alleles of the W1 element. Alternatively, the deletion of some IESs could escape the *trans*-acting effect exerted by the parental macronucleus DNA content.

IES ends and IES evolution

On the basis of the sequence similarity between their end consensus sequence and the sequence of the ends of the *E.crassus* Tec transposons, *P.aurelia* IESs have been proposed to be derived from ancestor Tc1/mariner transposon elements that have invaded the genome and evolved through various internal deletions (12). This evolutionary scheme has also been supported by sequence comparison of two *P.tetraurelia* paralogous genes that harbour three pairs of paralogous IESs (7,12). The IES pairs present highly divergent internal sequences but show similarity of some of their terminal sequences. Our identification of the allele-specificity of three deletion endpoints clearly indicates that sequence similarity between the ends of DNA segments regularly deleted during macronuclear genome development can also be the consequence of an adaptive evolutionary convergence in *Paramecium.* The recruitment of these alternative deletion endpoints appears to be similar to the utilization of a pseudo-end by a transposon wild-type end in distantly related organisms (27–30).

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