

Homology-Dependent Maternal Inhibition of Developmental Excision of Internal Eliminated Sequences in *Paramecium tetraurelia*

SANDRA DUHARCOURT,¹ ANNE-MARIE KELLER,² AND ERIC MEYER^{1*}

Laboratoire de Génétique Moléculaire, Ecole Normale Supérieure, Paris,¹ and Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette,² France

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Thousands of single-copy internal eliminated sequences (IESs) are excised from the germ line genome of ciliates during development of the polygenomic somatic macronucleus, following sexual events. *Paramecium* IESs are short, noncoding elements that frequently interrupt coding sequences. No absolutely conserved sequence element, other than flanking 5'-TA-3' direct repeats, has been identified among sequenced IESs; the mechanisms of their specific recognition and precise elimination are unknown. Previous work has revealed the existence of an epigenetic control of excision. It was shown that the presence of one IES in the vegetative macronucleus results in a specific inhibition of the excision of the same element during the development of a new macronucleus, in the following sexual generation. We have assessed the generality and sequence specificity of this transnuclear maternal control by studying the effects of macronuclear transformation with 13 different IESs. We show that at least five of them can be maintained in the new macronuclear genome; sequence specificity is complete both between genes and between different IESs in the same gene. In all cases, the degree of excision inhibition correlates with the copy number of the maternal IES, but each IES shows a characteristic inhibition efficiency. Short internal IES-like segments were found to be excised from two of the IESs when excision between normal boundaries was inhibited. Available data suggest that the sequence specificity of these maternal effects is mediated by pairing interactions between homologous nucleic acids.

Ciliates are unicellular eukaryotes in which germ line and somatic functions are assumed by two kinds of nuclei coexisting in the same cytoplasm. The diploid micronuclei are transcriptionally silent during vegetative growth; their main function is to provide gametic nuclei upon meiosis. Vegetative transcription takes place in the polygenomic, somatic macronuclei that divide amitotically and are lost soon after meiosis. Following fertilization, both kinds of nuclei differentiate from copies of the diploid zygotic nucleus. Macronuclear development involves extensive rearrangements of the germ line genome: chromosomes are fragmented into shorter, acentric molecules, and tens of thousands of internally eliminated sequences (IESs) are removed from coding and noncoding sequences, in a reproducible and often highly precise manner. In addition, the genome is amplified to the final ploidy level, ~1,000n in *Paramecium aurelia* complex species (for general reviews, see references 5 and 34; for a review of IESs, see reference 19).

All known *Paramecium* IESs are short (26 to 882 bp), AT-rich, single-copy elements flanked by 5'-TA-3' direct repeats in the germ line sequence. Excision invariably leaves one of the TA repeats in the rearranged macronuclear sequence, suggesting that the same basic mechanism is used for all IESs. Although the excision mechanism is currently unknown, reaction products and intermediates have been identified in other ciliate species. In *Euplotes crassus*, the structure of free circles generated by the excision of IESs with very similar characteristics (TA IESs) is best explained by a mechanism involving staggered double-strand breaks at both ends (17). In the more closely related *Tetrahymena thermophila*, the mapping of tran-

sient DNA breaks suggests a transposition-like mechanism, in which a staggered double-strand break at one end of the element is followed by a transesterification step that joins the flanking sequences together, releasing the IES as a linear molecule (37). *Tetrahymena* IESs, however, differ from those of *Paramecium* and *Euplotes* in that they lack invariant TA boundaries.

The analysis of some 44 kb of *Paramecium* germ line sequences has revealed a high density of IESs, one every 1,300 bp on average, with no significant difference between coding and noncoding sequences (7, 22, 38, 41–43). Although most of the sequences analyzed (35 kb) belong to one multigene family encoding alternative surface antigens, the density of IESs is higher in the remainder of the sequence sample. Thus, the total number of different IESs in the haploid genome is likely to be greater than 50,000. One of the most puzzling questions raised by this massive excision program is how such a large number of different elements can be specifically recognized and excised during macronuclear development. Indeed, no absolutely conserved sequence motif, other than the TA repeats, has been identified within or outside IESs.

A partial answer came from a statistical analysis of the sequence of IES ends, which showed that base composition is not random over at least six nucleotides immediately internal to each of the TA repeats. The sequence of preferred bases forms a degenerate inverted repeat consensus with striking similarity to the ends of the inverted repeats of Tc1/*mariner* transposons (18). Several large, multicopy elements present in the germ line genome of *E. crassus* are clearly members of this transposon family; like IESs, they are precisely excised between TA direct repeats during macronuclear development and generate free circles with a similar structure (14). These similarities have led to the hypothesis that present-day IESs are mutated and internally deleted remnants of ancient transposon insertions;

* Corresponding author. Mailing address: Laboratoire de Génétique Moléculaire, ENS, 46 rue d'Ulm, 75005 Paris, France. Phone: 33 (1) 44 32 39 49. Fax: 33 (1) 44 32 39 41. E-mail: emeyer@wotan.ens.fr.

the observed consensus could reflect the conservation of minimal *cis*-acting determinants required to direct excision (19).

The functional significance of the *Paramecium* IES consensus is supported by the fact that mutations in the TA repeats or at other positions can abolish excision (6, 24, 25). However, the comparison of natural allelic variants of IESs located in non-coding sequences shows that excision boundaries can be displaced over evolutionary time, implying that mutations can also result in the recruitment of novel TA boundaries in adjacent sequences (6). The degenerate consensus may therefore reflect a process of convergent evolution adapting IES ends to mechanistic constraints of the excision machinery. It should be emphasized that only a small fraction of IES ends conform exactly to the consensus, while many perfect matches are found in sequences that are not excised during development. Thus, it is likely that other, unidentified determinants are involved in IES recognition.

Recent evidence suggests that some of these determinants are epigenetic in nature, i.e., that the excision pattern is not entirely determined by the germ line sequence itself (reviews in references 27 and 28). Sequence-specific maternal effects were first revealed by the selective transformation of the vegetative macronucleus with a plasmid containing a 222-bp IES located in the gene for surface antigen G. Following the induction of autogamy (a self-fertilization sexual process), the maternal macronucleus progressively stops replicating its DNA and is eventually lost when vegetative growth resumes; its genetic material, including the transforming plasmid, is not transmitted to sexual progeny. Surprisingly, the presence of the IES in the maternal macronucleus was shown to inhibit specifically the excision of the homologous IES from the germ line genome in the developing macronucleus (8). The efficiency of this transnuclear effect, as measured by the fraction of *G*-gene copies retaining the IES in the new macronucleus, was further shown to increase with the copy number of the IES in the maternal macronucleus and with the length of IES flanking sequences present on the plasmid. The retention of the IES on the endogenous macronuclear chromosomes has an even stronger effect during the following sexual cycle, resulting in an ever-increasing fraction of IES-retaining copies in subsequent sexual generations. Thus, it is possible to establish stable cell lines in which this particular IES is never excised, although the germ line genome remains entirely wild type.

A similar parental control of developmental IES excision has also been reported in *T. thermophila* for two different elements (2), raising the prospect that the study of epigenetic regulation will bring valuable insight into basic mechanisms of recognition and excision of ciliate germ line-specific elements in general. We have tested the generality and sequence specificity of the maternal inhibition of excision by analyzing the effects of macronuclear transformation with 13 different *Paramecium* IESs. We show that five of them can be maintained in the zygotic macronucleus in response to maternal transformation. The effect is completely sequence specific, and each IES appears to inhibit its own excision with a different efficiency relative to maternal copy number.

MATERIALS AND METHODS

Cell lines and cultivation. *Paramecium tetraurelia* 51 and d4.2 are well-characterized homozygous stocks carrying the *A*⁵¹ and *A*²⁹ alleles of the gene for surface antigen A, respectively (40). Strain d4.2 was obtained by repeated backcrosses with strain 51 and is therefore largely isogenic; this study confirmed that it carries the same allele of the gene for surface antigen G (*G*⁵¹) by restriction mapping analysis and the sequencing of over 2,800 bp from both strains, including the central and most variable region of the coding sequence. Cells were grown in a wheat grass powder (Pines International Co., Lawrence, Kans.) infusion medium bacterized the day before use with *Klebsiella pneumoniae* and

supplemented with 0.8 mg of β -sitosterol (Merck, Darmstadt, Germany) per liter, at 27°C. Basic methods of cell culture have been described previously (39).

Recombinant λ phages and plasmids. The λ 51Gmic and λ 51Amic phages, containing the micronuclear *G*⁵¹ and *A*⁵¹ genes, were isolated from the library of micronuclear DNA from strain 51 constructed by C. J. Steele et al. (41) in the λ GEM11 vector, by using IES 51G4404 (29) and a 210-bp *ScaI* fragment from the central part of the coding sequence (31) as probes, respectively. The λ 51Gmic insert was entirely sequenced (12,326 bp). The *G*⁵¹ coding sequence was identified by comparison with known alleles of the *G* gene from *Paramecium primaurelia* (33); it is 88% identical with *G*¹⁵⁰. IES excision boundaries were confirmed by sequencing the relevant parts of the macronuclear version from the macronuclear phage λ 51Gmac, which was cloned from a library of total DNA from strain d4.2 in the λ EMBL3 vector (29). Plasmids p51A-712mic and p51A-712mac are *Sall-SpeI* fragments of λ 51Amic and the macronuclear clone λ SA1 (9), respectively, subcloned into vector pUC18. Plasmid p29A6649mic was obtained by PCR amplification of micronuclear DNA from strain d4.2 with primers 6435A and 6649-3' (see below).

Microinjections. Young cells (less than eight divisions after autogamy) were injected in mineral water (Volvic Co., Volvic, France) containing 0.2% bovine serum albumin, under an oil film (Nujol), while being visualized with a phase-contrast inverted microscope (Axiovert 35M; Zeiss). CsCl-purified λ phage DNA was cut within the vector arms with *SmaI*, leaving 3,380 and 728 bp of λ sequences on either side of the insert for λ 51Gmic and λ 51Amic and 3,362 and 728 bp for λ 51Gmac. Column-purified (Qiagen) plasmid DNA was cut within the vector sequence with *XmnI*. Restriction digests were extracted with phenol, filtered on a 0.22- μ m-pore-size Ultrafree-MC filter (Millipore), and precipitated with ethanol. Approximately 5 μ l of a 5-mg/ml solution in water was delivered into the macronucleus. Injected DNA molecules replicate autonomously at stable copy numbers in the vegetative macronucleus, forming linear monomers and multimers to which telomeres are added (11, 12, 15, 16).

Dot blot analyses. For each sample, 100 cells were pipetted from depression slide cultures and transferred to 400 μ l of 0.4 N NaOH–50 mM EDTA. The lysates were incubated for 30 min at 68°C and loaded on a Hybond N⁺ membrane (Amersham, Little Chalfont, United Kingdom) with a dot blot apparatus. Genomic DNA samples were treated in the same way. The membrane was kept wet with 0.4 N NaOH for 15 min, washed in 2 \times SSC (1 \times SSC is 150 mM NaCl–15 mM sodium citrate), and subsequently treated as a Southern blot.

Copy number quantification. The copy numbers of phage inserts in transformed clones were determined after hybridization of dot blots with a 3,359-bp *SmaI-SacI* fragment from the small arm of phage λ GEM11, which is linked to the inserts in all *SmaI*-digested phage DNAs, with a Fuji Bas 1000 imager. Measured signals were normalized by rehybridizing the same dot blots with an oligonucleotide specific for macronuclear telomeric repeats, as previously described (8). Probes specific for IESs 51A2591 and 51G4404 were used to check relative variations of IES copy numbers. The normalized λ probe signals were then translated for all clones into copy numbers per haploid genome (phg), by using a *G*-gene probe to compare the signals obtained for clones transformed with *G*-gene phages to those obtained for reference samples (genomic DNA from clones transformed with *G*-gene plasmids) loaded on the same dot blots, for which copy numbers had previously been determined (8). The reference value of one copy phg was arbitrarily defined as the average copy number of the *G* gene in uninjected clones. Plasmid copy numbers were determined on Southern blots of preautogamous DNA samples digested with *PvuII* after hybridization with a 322-bp *PvuII* fragment from pUC18, by using the same reference samples.

Autogamy. Autogamy was induced by starving the cells after they had reached the appropriate clonal age (30 vegetative divisions) and assessed by staining with a 15:1 (vol/vol) mix of carmine red (0.5% in 45% acetic acid) and fast green (1% in ethanol). For karyonidal analyses, cells were isolated from depressions showing 100% autogamous cells. After the first cellular division, one of the two karyonides was isolated and cultivated. For mass autogamies, about 20 autogamous cells (i.e., 40 karyonidal clones) were transferred to bacterized medium and grown collectively.

Genomic DNA extraction. Cultures of exponentially growing cells (400 ml) at 10⁵ cells/ml were centrifuged. After being washed in 10 mM Tris (pH 7.0), the cell pellets were resuspended in 0.5 volume of the same buffer and quickly added to 3 volumes of lysis solution (0.44 M EDTA [pH 9.0]–1% sodium dodecyl sulfate [SDS]–0.5% *N*-laurylsarcosine [Sigma]–1 mg of proteinase K [Merck] per ml) at 55°C. The lysates were incubated at 55°C overnight, gently extracted once with phenol, and dialyzed twice against TE (10 mM Tris-HCl–1 mM EDTA, pH 8.0) containing 25% ethanol and once against TE.

Southern blotting. DNA restriction and electrophoresis were carried out according to standard procedures (36). DNA was transferred from agarose gels to Hybond N⁺ membranes (Amersham) in 0.4 N NaOH after depurination in 0.25 N HCl. Hybridization was carried out according to the procedure described in reference 3 in 7% SDS–0.5 M sodium phosphate–1% bovine serum albumin–1 mM EDTA (pH 7.2) at 63°C. Probes were labeled with a random priming kit (Boehringer, Mannheim, Germany) to a specific activity of 3 \times 10⁹ cpm/ μ g. Membranes were washed for 30 min in 0.2 \times SSC–0.5% SDS at 60°C prior to autoradiography or image plate quantification.

PCR amplification and sequencing. Macronuclear DNA was amplified with primers located about 25 bp away from each deletion junction, from total genomic DNA of caryonidal clones, or from λ phage DNA. PCRs were carried

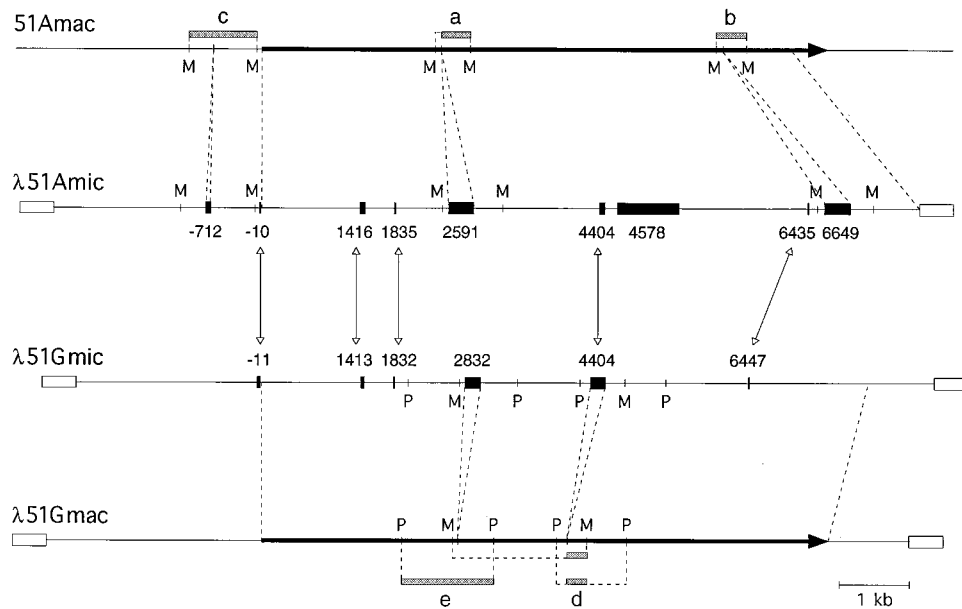


FIG. 1. Maps of the macronuclear A^{51} gene (51Amac) and of the inserts of phages $\lambda 51Amic$, $\lambda 51Gmic$, and $\lambda 51Gmac$. Coding sequences are represented by thick arrows in the macronuclear sequences. IESs are shown as black boxes in the micronuclear sequences. Double-headed arrows indicate paralogous IES pairs (IESs occurring at homologous positions in the two genes). Open boxes at the ends of the phage inserts symbolize the vector arms. The positions and lengths of probes a, b, c, d, and e are shown. Restriction sites: M, *MnII*; P, *PstI* (only sites relevant to Fig. 2 and 3 are shown).

out in capped 0.5-ml Sigma polypropylene tubes with reaction mixtures (25 μ l) containing 100 ng of total genomic DNA or 1 ng of cloned DNA, 1 \times PCR buffer (Promega), 80 μ M (each) deoxynucleoside triphosphate, 2 μ M (each) oligonucleotide, and 0.8 U of *Tfl* polymerase. Amplifications were run for 20 cycles (92°C, 1 min; 63°C, 1 min 15 s; 74°C, 30 s) in a Perkin-Elmer Cetus thermocycler. Micronuclear DNA was amplified from total genomic DNA with one primer located within an IES and the other in the macronuclear sequence. PCRs were run for 30 cycles under the same conditions as for macronuclear amplification, except that the annealing temperature was 55°C and the extension time was increased to 1 min. PCR products were cloned into plasmid pCRScript (Stratagene) and sequenced with the Sequenase sequencing kit (U.S. Biochemical Corp.). At least two clones arising from PCR products of two different karyonidal clones were sequenced to check for PCR-induced substitutions. The sequenced portions of the micronuclear A^{29} allele were amplified with primer pairs 1835A/2591-3' and 6435A/6649-3'. The same primer pairs were used to check the micronuclear sequence in some postautogamous karyonidal clones.

Sequences of primers (all sequences are written 5' to 3'; the numbers in parentheses indicate the positions of the 5' end of the primers in the A^{51} or G^{51} micronuclear sequence [accession no. L26124 and AJ010441, respectively]) are as follows: 51A-712-5', (57) GTCATTTGTTTATGAAAAAATTCATCAA CTAAG; 51A-712-3', (416) GGGTTCCAAACAAGAAATTTCCAT; 51A2591-5', (3747) ACACCAAGCGAAACATGCACAGTCG; 51A2591-3', (4222) TTT TATGGCATTAAAGCTTGTGTCAT; 51A6649-5', (9156) AAATGGTACTGT TTGTGCTTGGGATAGTGC; 51A6649-3', (9644) CAGCAGTACATCCAGC TCTCTAAGTTAGC; 51A1835A, (3016) ATAGATGGATTGTTTTCCAAG TATCTATATC; 51A6435A, (8971) GTATCGATAAATATTGTTATTAATATA TTATAC; 156G2835-5', (5524) AAACAGGATCAGGTTTGACATTTGCAG ATTG; 51G2832-3', (5948) GTCACACAAGTAGAAGAACCATTTAATGC GC; 51G4404-5', (7325) CAACATGTGCTGCATATAATGTAGG; 51G4404-3', (7678) ATGAAAGGGAACCAGTTGATTATGCAGAGC. The underlined T in primer 156G2835-5', which is based on the G^{156} allele, is an A in G^{51} .

Nucleotide sequence accession number. The complete sequence of the $\lambda 51Gmic$ insert was submitted to the EMBL database (AJ010441).

RESULTS

Experimental design. We chose to test IESs from two surface antigen genes, one of which contains the previously studied 222-bp IES (8). The A and G genes are related, nonessential members of a family of at least 11 genes showing mutually exclusive expression, so that selection against macronuclear genomes containing nonfunctional forms of these genes is not expected to bias the results. Recombinant λ phages containing the germ line versions of these genes were isolated from a

library of micronuclear DNA from strain 51 (Fig. 1). The 12.4-kb insert from $\lambda 51Amic$ contains all but the last 500 bp of the A -gene coding sequence, as well as about 2.9 kb of 5' flanking sequences. Nine IESs (51A-712 through 51A6649) have previously been identified, seven of them located within the coding sequence and two in the upstream region (41). Sequencing of the 12.3-kb insert from $\lambda 51Gmic$ showed that it contains the complete micronuclear G gene and revealed the presence of six IESs, four of which are studied here (51G1413, 51G1832, 51G2832, and 51G4404, the previously studied 222-bp IES; IESs are named according to the positions of their insertion sites in the macronuclear sequence, relative to the translation start).

The transformation of the macronucleus of vegetative cells with the $\lambda 51Gmic$ insert is expected to inhibit the excision of IES 51G4404 during development of a new macronucleus, after induction of autogamy in transformed clones. Previous quantitative analyses (8) predict that inhibition will be observed even at low copy numbers of the transforming molecule (~1 copy phg, i.e., about 1,000 copies per macronucleus), providing a positive control for the experiment. The retention of other IESs can be examined in the same postautogamous samples. Because all G -gene IESs will be present in the maternal macronucleus at the same copy number, the efficiencies of inhibition can be directly compared by measuring the fraction of new macronuclear copies of the gene retaining each IES. As a negative control for the effect of IES sequences, a λ phage containing the complete macronuclear G gene was used ($\lambda 51Gmac$ [Fig. 1]).

Similarly, the excision of each A -gene IES can be examined in the sexual progeny of clones transformed with $\lambda 51Amic$. Should any of them be maintained in the new macronuclear genome, $\lambda 51Amic$ and $\lambda 51Gmic$ will serve as specificity controls for each other. The A and G coding sequences are very similar and can be aligned with no ambiguity (78% identity at the DNA level, 80% at the protein level). An additional point of interest is that some of the IESs occur at exactly the same

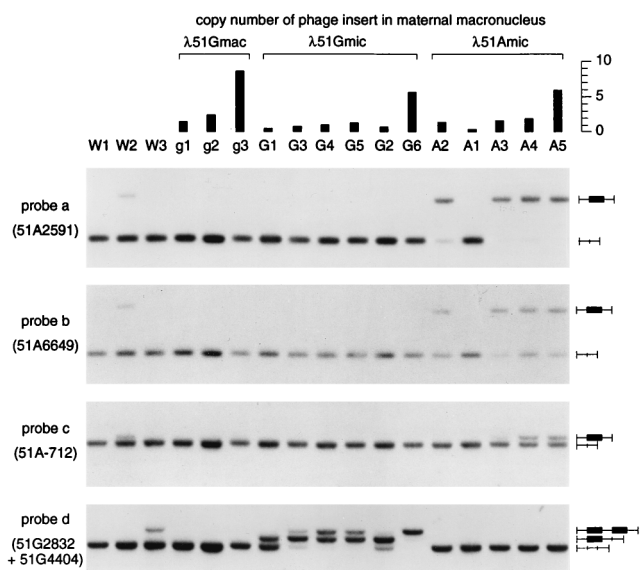


FIG. 2. Southern blot analysis of the macronuclear genome of sexual progeny of clones transformed with λ 51Gmac, λ 51Gmic, and λ 51Amic. Total DNA from mass-autogamy samples was digested with *MnII*. Because the mic/mac ploidy ratio is $\sim 1/250$, only macronuclear DNA is visible. For each sample, the copy number of the phage insert in the maternal macronucleus is indicated by a vertical bar above the lane. Lane W1 is control DNA from uninjected cells. Lanes W2 and W3 contain the same DNA as in lane W1, mixed with a small amount of λ 51Amic and λ 51Gmic DNA, respectively, prior to digestion with *MnII*. Symbols on the right indicate the positions of IES-retaining and wild-type macronuclear fragments. The same blot was hybridized successively with probes a, b, c, and d (Fig. 1).

positions in the two genes. In contrast to their flanking sequences, however, paralogous IESs (51A-10/51G-11, 51A1416/51G1413, 51A1835/51G1832, 51A4404/51G4404, and 51A6435/51G6447) show no significant sequence similarity and have different lengths. IESs 51A-712, 51A2591, 51A4578, 51A6649, and 51G2832 are gene specific, having no paralogous counterparts.

Gene-specific maternal effects of A- and G-gene micronuclear sequences. DNA from each of the λ phages was microinjected into the macronucleus of vegetative cells from strain 51. Injected cells were cultured separately, and the copy numbers of phage inserts were precisely measured after about 20 divisions by a dot blot procedure (see Materials and Methods). Selected clones maintaining between 0.1 and 10 copies phg were then allowed to undergo autogamy. To examine the new macronuclear genome of sexual progeny, about 20 autogamous cells for each clone were refed and grown collectively for DNA extraction. Postautogamous DNA samples were analyzed on a Southern blot after restriction with *MnII*. There are many sites for this enzyme in the A- and G-gene coding sequences but none within IESs; the retention of any IES can thus be detected by a size increase of the corresponding *MnII* fragment.

In the top panel of Fig. 2, the Southern blot was hybridized with a probe specific for the *MnII* fragment containing 51A2591, a 370-bp A-gene IES (probe a [Fig. 1]). Lane W1 is a control sample from uninjected cells and shows only the 501-bp macronuclear fragment. In lane W2, the same control DNA was mixed with a small amount of phage λ 51Amic DNA prior to digestion with *MnII*, to show the position of the corresponding 871-bp micronuclear fragment. The sexual progeny of clones transformed with λ 51Gmac (lanes g1 to g3) or λ 51Gmic (lanes G1 to G6) show only the 501-bp fragment,

indicating that IES 51A2591 was correctly excised during macronuclear development. In contrast, the sexual progeny of clones transformed with λ 51Amic (lanes A1 to A5) show mostly the IES-retaining fragment, except for A1, a postautogamous sample derived from a clone maintaining only 0.1 copies phg.

The blot was then stripped and rehybridized successively with probe b, which is specific for the *MnII* fragment containing IES 51A6649, and probe c, specific for the *MnII* fragment containing IES 51A-712 (Fig. 2, second and third panels from top). As with IES 51A2591, the excision of these IESs was affected in the sexual progeny of clones transformed with λ 51Amic, in which both the micronuclear and the macronuclear fragments are observed, but not in the progeny of clones transformed with G-gene phages. In all three cases, the fraction of IES-retaining copies increases with the copy number of λ 51Amic in the maternal macronucleus. However, for a given copy number, this fraction differs for each IES (see quantitative analysis below). The blot was also hybridized with probes specific for the *MnII* fragments containing each of the other six A-gene IESs (51A-10, 51A1416, 51A1835, 51A4404, 51A4578, and 51A6435), one after the other. These IESs were fully excised in all samples (data not shown).

Rehybridization of the same blot with a G-gene probe gives a different picture. Probe d is specific for a *MnII* fragment containing two IESs, 51G2832 and 51G4404 (Fig. 1). In the postautogamous progeny of clones transformed with λ 51Gmic (lanes G1 to G6), two different fragments are revealed in addition to the macronuclear fragment (Fig. 2, fourth panel from top). The largest has the same size as the micronuclear fragment visible in control lane W3, which contains W1 DNA mixed with a small amount of λ 51Gmic phage DNA, indicating that both IESs can be maintained in the new macronuclear genome. The intermediate fragment suggests that some G-gene copies retain only one of the two IESs. Excision of these IESs was not affected in the sexual progeny of clones transformed with λ 51Gmac or λ 51Amic, even at high copy numbers. Thus, the effect depends on the presence of the IESs themselves in the maternal macronucleus and is strictly gene specific. Hybridization with probes specific for the fragments containing IESs 51G1413 and 51G1832 showed that these IESs were always fully excised (data not shown).

To look at each of the maintained IESs separately, the postautogamous samples derived from λ 51Gmic-transformed clones were also analyzed on a different Southern blot after digestion with *PstI*, which cuts the coding sequence in between the two IESs (Fig. 1). In Fig. 3, this blot was hybridized successively with probe d, which reveals a *PstI* fragment containing only 51G4404, and probe e, which reveals a fragment containing only 51G2832. If one considers only the mass-autogamy samples G1 to G6, it can be seen that for both IESs the average fraction of IES-retaining copies increases with the copy number of the λ 51Gmic phage insert in the maternal macronucleus. It is also very clear that, for a given copy number, the excision of 51G4404 is inhibited more efficiently than that of 51G2832.

Karyonidal variability of maternal inhibition of excision. Many of the postautogamous DNA samples, which were prepared from mass autogamies of transformed clones, contained both rearranged and IES-retaining molecules. This heterogeneity could mean that IES excision was inhibited only on a fraction of the $\sim 1,000$ copies of the genome in each new macronucleus or that only a fraction of the ~ 40 new macronuclei analyzed in each sample were affected. To address this question, a number of autogamous cells were isolated from the same populations that were used for the mass-autogamy sam-

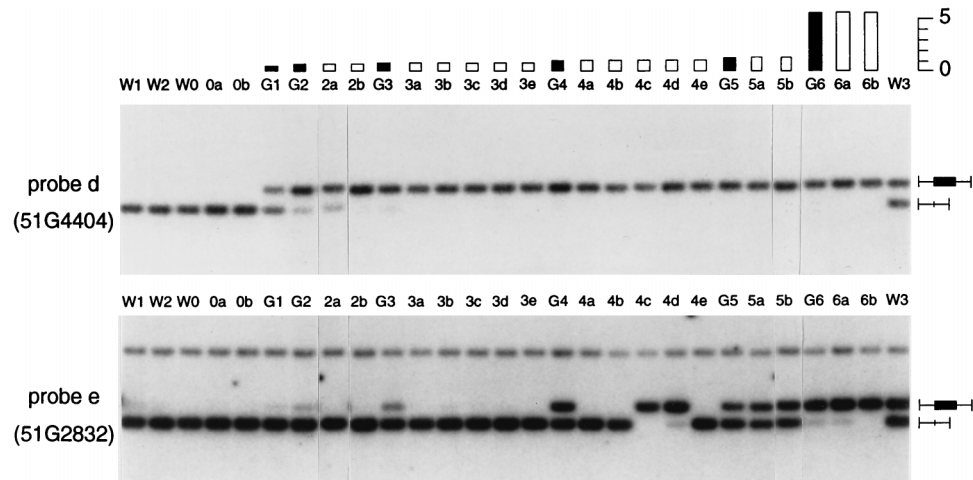


FIG. 3. Southern blot analysis of the excision of IESs 51G4404 and 51G2832 in sexual progeny of clones transformed with λ 51Gmic. Samples G1 to G6 and W1 to W3 are the same as in Fig. 2. Each of the mass-autogamy samples G2 to G6 is followed by a series of karyonidal samples representing individual clones isolated from the same mass autogamy. Vertical bars above the lanes indicate the copy numbers of phage inserts in maternal macronuclei. W0 is a control mass autogamy from an uninjected cell; 0a and 0b are karyonidal clones from this mass autogamy. All samples were digested with *Pst*I. The same blot was hybridized successively with probes d and e (see Fig. 1). In addition to the *Pst*I fragment containing IES 51G2832, probe e cross-hybridizes with the central *Pst*I fragment from the *G* gene (upper band in lower panel), due to the repeated structure of this region of the coding sequence.

ples. In each autogamous cell, two new macronuclei develop independently from mitotic products of the zygotic nucleus; during the first vegetative division, they segregate without dividing to the two daughter cells, which are called karyonides. To prepare postautogamous DNA samples corresponding to single events of macronuclear development, only one of the two karyonides from each isolated cell was cultured.

Karyonidal samples from mass autogamies G2 to G6 are shown in Fig. 3. When the mass-autogamy sample contains both forms, striking differences in the relative amounts of these forms can be observed in individual karyonidal clones. This is most clearly seen with the G4 mass-autogamy sample, in which IES 51G2832 is excised on about 60% of molecules (lane G4, probe e). Of five karyonidal clones analyzed (lanes 4a to 4e), one shows only IES-retaining copies, a second one shows a small fraction of rearranged copies, and the other three contain only the rearranged form. Most of the karyonidal clones analyzed for G2 and G3, which showed only a small fraction of IES-retaining copies, appear to be pure for the rearranged form. Intranuclear heterogeneity is also observed: karyonidal clones 5a and 5b contain roughly equal copy numbers of the two forms, like the G5 mass-autogamy from which they were isolated. A similar analysis of the progeny of clones transformed with λ 51Amic also revealed a high karyonidal variability in the inhibition of excision of *A*-gene IESs (data not shown). Thus, excision inhibition appears to be a stochastic process in single developing macronuclei, occurring with a probability that depends on the copy number of micronuclear sequences in the maternal macronucleus.

Sequence specificity of the maternal effects of single IESs.

To test the specificity of the effect among the three *A*-gene IESs showing inhibition, plasmids containing only one of them were constructed. IESs 51A-712 and 51A6649 were chosen to make the test stringent, as the absence of any effect on 51A2591, which was the most readily maintained IES in the λ 51Amic experiment, would be more conclusive. Plasmid p51A-712mic contains a 1.5-kb segment of the germ line sequence upstream of the *A* gene, including IES 51A-712 but not IES 51A-10; a negative control is provided by plasmid p51A-712mac, which contains the homologous segment from the

rearranged macronuclear sequence (see map in Fig. 4). To study IES 51A6649, we used plasmid p29A6649mic, which contains a segment of the micronuclear *A*²⁹ allele including the IES as well as 301 bp of flanking sequences (242 bp upstream, which also includes IES 51A6435, and 59 bp downstream; see map in Fig. 5). Within this segment, the *A*²⁹ allele differs from *A*⁵⁷ by only a single substitution within the IES (see below). The maternal effects of these plasmids were tested as in the λ phage experiment, except that plasmid copy numbers in the macronucleus of transformed clones were determined by Southern blotting of preautogamous DNA samples (see Materials and Methods).

The new macronuclear genome of sexual progeny was probed on a Southern blot of mass-autogamy samples digested with *Mn*II and *Bst*BI. The top panel of Fig. 4 shows hybridization of the blot with probe f, revealing the *Bst*BI-*Mn*II fragment containing 51A-712. The retention of this IES is observed only in the postautogamous progeny of the clone transformed with the highest copy number of p51A-712mic (9.9 copies phg, lane 10; the weak signals in lanes 7 and 10 are due to a different effect of high-copy-number transformation with cloned macronuclear or micronuclear sequences, which causes imprecise deletions of the homologous regions of the germ line genome during development of a new macronucleus [8, 26, 30]). Retention of 51A-712 is not observed in the progeny of clones transformed with p51A-712mac, even at 15.6 copies phg, or with p29A6649mic. Hybridization of the same blot with probe b reveals the *Mn*II fragment containing IES 51A6649. The excision of this IES is inhibited in the progeny of the clone transformed with p29A6649mic (2.1 copies phg) but not in any other sample. It can be noticed that the IES-retaining fragment in this sample migrates slightly faster than the control micronuclear fragment in lane W2; this difference will be discussed below. In all of these samples, the more readily blocked 51A2591 is fully excised, as shown by hybridization with probe a.

We took advantage of a previous experiment to examine the sequence specificity of the effect among *G*-gene IESs. A plasmid containing only IES 51G4404 (p51G4404mic; see map in Fig. 5) had previously been shown to inhibit the excision of this IES (8). Hybridization of a Southern blot of *Pst*I-digested post-

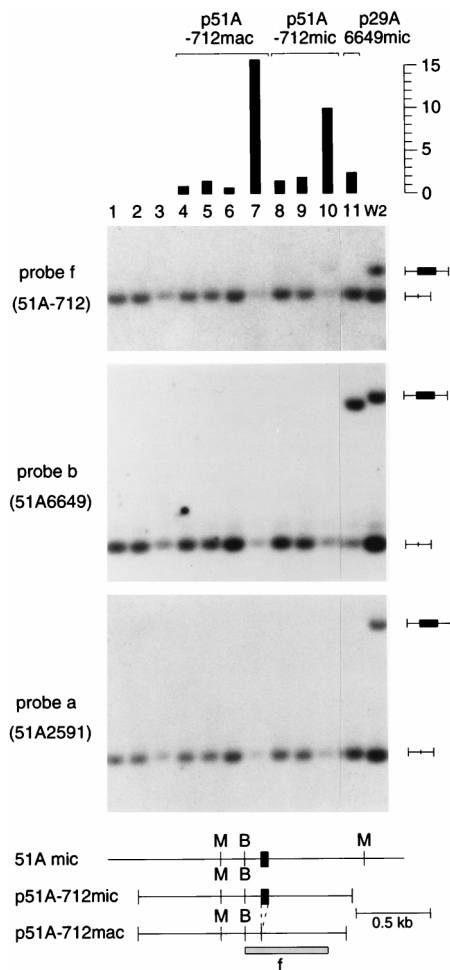


FIG. 4. Southern blot analysis of the excision of IESs 51A-712, 51A6649, and 51A2591 in sexual progeny of clones transformed with plasmids p51A-712mac, p51A-712mic, and p29A6649mic (see map in Fig. 5). Plasmid copy numbers in the maternal macronucleus are indicated by the vertical bars above the lanes. Mass-autogamy samples were digested with *MnlI* and *BstBI*. The same blot was successively hybridized with probes a, b (Fig. 1), and f (see map; M, *MnlI*; B, *BstBI*).

autogamous samples with probe e showed that the presence of p51G4404mic in the maternal macronucleus had no effect on the excision of 51G2832, even at a copy number 30-fold higher than that necessary to completely abolish excision of 51G4404 (data not shown). Thus, maternal inhibition of excision appears to be completely sequence specific, both between genes and between IESs in the same gene. These results are summarized in Fig. 5.

Quantitative analysis of excision inhibition. As noted above, the efficiency of excision inhibition, relative to the copy number of phage insert in the maternal macronucleus, differs for each IES. To quantify these differences, the relative intensities of the rearranged and IES-retaining versions of each fragment were determined, by using only mass-autogamy samples to average the karyonidal variability. Since the probes used contain only macronuclear sequences, relative intensities give an exact measure of the fraction of rearranged copies in new macronuclei. This fraction was then plotted as a function of the copy number of phage insert in the maternal macronucleus. The fairly regular aspect of the plots obtained for the two *G*-gene IESs (Fig. 6A) indicates that karyonidal variability is

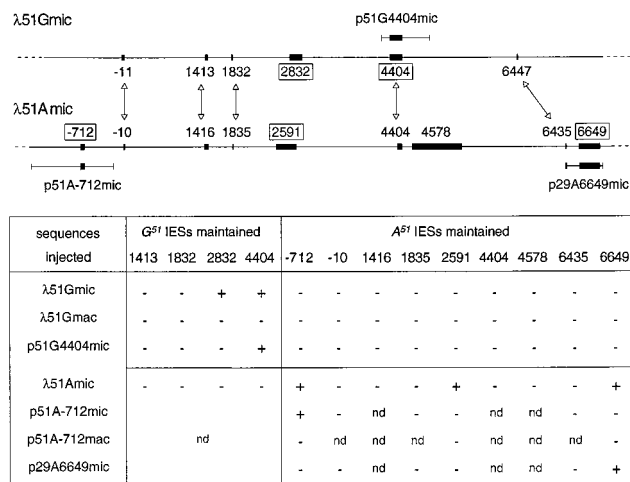


FIG. 5. Summary of the results showing the sequence specificity of the inhibition of IES excision by maternal sequences. The maps show the phage and plasmid inserts used. IESs are shown as black boxes. Paralogous IES pairs are indicated by double-headed arrows. The names of the five IESs showing inhibition are boxed. In the lower panel, the plus sign indicates that excision inhibition was observed. nd, not determined.

reasonably well averaged in pools of ~ 40 karyonidal clones and illustrates clearly the quantitative difference in the effects of the λ 51Gmic insert on the two IESs. Excision of 51G4404 is reduced by 50% with only 0.4 copies phg in the maternal macronucleus, while about 2 copies phg are necessary to achieve a similar inhibition of the excision of 51G2832. The plots obtained for the three *A*-gene IESs also show significant differences in the efficiency of inhibition by the λ 51Amic insert (Fig. 6B). Maternal copy numbers of λ 51Gmic and λ 51Amic can be directly compared because they were both measured by probing with a λ vector sequence which remains linked to the inserts in injected phage DNA. The blocking of 51A2591 is slightly less efficient than that of 51G4404, while blocking efficiencies of 51A6649 and 51G2832 are comparable. Only a weak effect is observed for 51A-712 ($\sim 30\%$ inhibition at the highest copy number tested, 5.9 copies phg).

It was previously shown that the inhibition of 51G4404 is much more efficient when the maternal IES is present on endogenous macronuclear chromosomes (50% inhibition with ~ 0.3 copies phg) than when it is present on a plasmid with 629 bp of flanking sequences (50% inhibition with ~ 3 copies phg) (8). These copy numbers can be directly compared to those given in the present study because the same reference samples were used for quantification. It can thus be concluded that the efficiency of the IES in the context of the λ 51Gmic insert (12.3 kb) is very close to that of the IES-retaining macronuclear chromosome. To determine whether the weaker effects of other IESs can be enhanced when they are present on endogenous macronuclear chromosomes, postautogamous karyonidal clones retaining various copy numbers of each IES were allowed to undergo a second autogamy. The new macronuclear genome was analyzed on Southern blots of mass-autogamy samples (data not shown), and the fraction of rearranged copies was plotted as a function of the copy number of chromosome-borne IES in the maternal macronucleus, which cannot exceed 1 copy phg (Fig. 6C and D). Most of the points indicate that endogenous IESs indeed have slightly higher efficiencies than phage inserts; however, their effects also appear to be much more variable. To establish a stable cell line which never

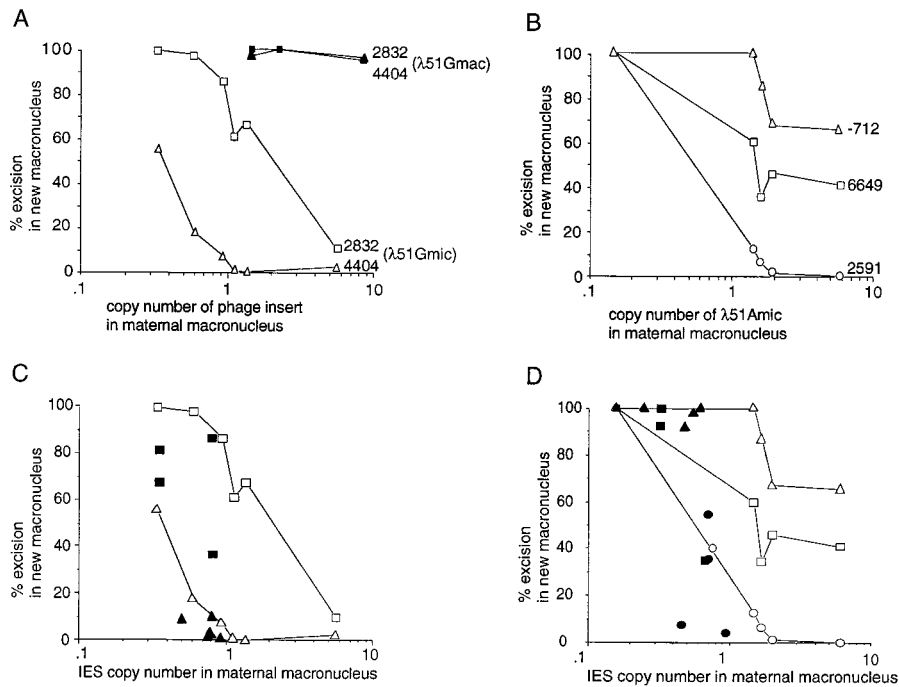


FIG. 6. Quantitative analysis of the excision of different IESs as a function of the copy number of specific sequences in the maternal macronucleus. “% excision in new macronucleus” is the fraction of rearranged copies in the new macronuclear genome, as measured in mass-autogamy samples. (A) Excision of IESs 51G4404 and 51G2832 after transformation of the maternal macronucleus with $\lambda 51G_{mac}$ or $\lambda 51G_{mic}$. (B) Excision of IESs 51A2591, 51A6649, and 51A712 after transformation of the maternal macronucleus with $\lambda 51Amic$. (C and D) Filled symbols show the quantification of the maternal effects of IESs present on the endogenous macronuclear chromosomes and are superimposed on the same graphs as in panels A and B, respectively, to compare the inhibition efficiencies of IES-retaining chromosomes with those of the phage inserts.

excises a given IES, the minimal efficiency required is 100% inhibition with ≤ 1 copy phg. Although a couple of second-generation samples still showed little or no rearranged copies for 51A2591, such an efficiency appears to be reproducibly attained only for 51G4404.

Macronuclear maintained IESs are not copied from maternal sequences. To test whether the maintenance of IESs in the macronuclear genome could be due to the repair of a gap left in the genomic sequence after constitutive excision of the IES, by a mechanism involving the copying of a homologous template from the maternal macronucleus, advantage was taken of the fact that two different alleles of the *A* gene, *A*⁵⁷ and *A*²⁹, can be distinguished by a number of substitutions. Partial sequencing of the micronuclear *A*²⁹ gene from strain d4.2 (see Materials and Methods) showed that the two alleles are ~99% identical in the regions around IESs 51A2591 and 51A6649. The macronucleus of vegetative cells from strain d4.2 was transformed with $\lambda 51Amic$ DNA. After induction of autogamy, the new macronuclear genome was analyzed as described for the experiment with strain 51. IES 29A2591 was maintained, with an efficiency similar to that of 51A2591 (data not shown). The macronuclear maintained IES was PCR amplified and cloned (see Materials and Methods). Its sequence was shown to be identical with the *A*²⁹ germ line sequence, which differs from the *A*⁵⁷ germ line sequence by one substitution and a 12-bp insertion (Fig. 7A). Thus, it can be concluded that the maintained IES originated from the d4.2 germ line genome and had not been converted by the $\lambda 51Amic$ sequence which had caused its retention. This extends an earlier result obtained for 51G4404, showing that the engineering of a novel restriction site within the injected IES does not alter the sequence of the IES recovered from the macronucleus of post-autogamous progeny (8).

Short IES-like segments are excised from some of the maintained IESs. Macronuclear maintained copies of IES 51A2591 were also cloned and sequenced after PCR amplification, by using postautogamous samples from the first experiment (progeny of strain 51 cells transformed with $\lambda 51Amic$). Quite unexpectedly, the maintained sequence proved to differ from the germ line sequence by the deletion of an internal 28-bp segment (Fig. 7A). The same size difference could be detected in postautogamous DNA samples by comparison with $\lambda 51Amic$ DNA on appropriate Southern blots, showing that all macronuclear maintained copies of IES 51A2591 carry the 28-bp deletion (data not shown). To check that the particular stock of strain 51 used did not carry the deletion in its germ line genome, micronuclear DNA was PCR amplified from total DNA samples of karyonidal clones containing the shortened version in their macronuclear genome, by using micronuclear sequence-specific primers located in other IESs. The micronuclear sequence was found to be identical with the published sequence present in $\lambda 51Amic$. Since micronuclei and macronuclei develop from mitotic products of the same zygotic nucleus, the 28-bp deletion must occur during macronuclear development. We also checked that the 28-bp segment was not deleted in the $\lambda 51Amic$ molecules replicating in the maternal macronucleus, by using preautogamous DNA samples from transformed clones.

The 28-bp internal segment has all the characteristics of a *Paramecium* IES. It is bounded by two TA direct repeats, one of which is left in the macronuclear maintained sequence, and its ends show a reasonable match to the IES end consensus (51A28 in Fig. 8). Thus, it can be viewed as a short IES within the larger 51A2591, which is excised during macronuclear development when the excision of 51A2591 is inhibited by the $\lambda 51Amic$ sequence in the maternal macronucleus. Remark-

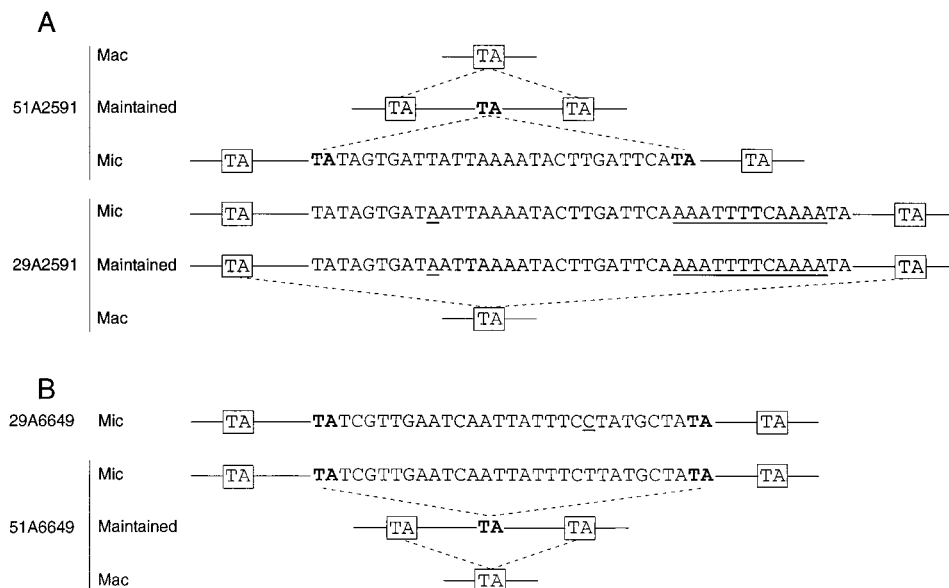


FIG. 7. Internal IES-like segments excised from the macronuclear maintained copies of 51A2591 (A) and 51A6649 (B). Only the sequences of internal segments are shown. Boldface TAs are the excision boundaries of internal segments. Boxed TAs are the excision boundaries of the whole IESs. The sequences of homologous segments of the A^{29} allele are also shown; differences with the A^{57} allele are underlined. The 40-bp segment in 29A2591 is not excised in the macronuclear maintained version.

ably, both of the mutations that distinguish 29A2591 from 51A2591 are located within the IES-like segment (Fig. 7A). The 12-bp insertion in the A^{29} allele is located immediately internal to the downstream TA boundary, disrupting the adjacent consensus; this may be related to the fact that no internal deletion occurs in IES 29A2591 when excision of the latter is inhibited by λ 51Amic.

The macronuclear maintained versions of the other four regulatable IESs were sequenced in the same way. The maintained 51A6649 sequence also lacked a 29-bp internal segment present in the micronuclear sequence (Fig. 7A), explaining the size difference already noticeable in Fig. 4. Like the 28-bp segment of 51A2591, the 29-bp segment has all the characteristics of an IES (51A29 in Fig. 8). PCR amplification and sequencing were again used to check that the segment was present in the micronuclei of clones maintaining the shortened version in their macronuclei. The only difference between allelic IESs 29A6649 and 51A6649 is a substitution located in the IES-like segment. To determine whether the 29-bp segment can also be independently excised in the A^{29} allele, the excision of 29A6649 was inhibited by transforming d4.2 cells with plasmid p29A6649mic, at a copy number which did not cause copy number reduction in the new macronucleus. Restriction mapping of the PCR-amplified maintained version of 29A6649 showed that it was about 29 bp shorter than the micronuclear sequence, consistent with the idea that the same segment as that in 51A6649 had been excised (data not shown). The deletion of short internal segments, however, is not a general feature of maintained IESs: the macronuclear versions of 51A-712, 51G4404, and 51G2832 were shown to be identical with the corresponding germ line sequences.

Characteristics of IESs showing inhibition. Only five of the tested IESs could be maintained in the new macronucleus, following maternal transformation with micronuclear sequences. They share no obvious feature that could clearly distinguish them from the other eight. Four of them are among the largest (222 to 370 bp), while none of the short ones (28 to

52 bp), including the short IES-like segments located within 51A2591 and 51A6649, could be blocked. However, maternal inhibition is not determined only by size, because only one of several 77-bp IESs shows the effect, and the largest IES in the sample (51A4578, 882 bp) does not. There appears to be nothing special about the location of IESs showing inhibition, as they are interspersed with other IESs in coding sequences; one of them (51A-712) is located in noncoding sequences. Since the only significant sequence feature identified in IESs is the degenerate consensus of end sequences, the ends of tested IESs were examined (Fig. 8). Internal IES-like segments, which were always excised from the maintained versions of 51A2591 and 51A6649, were included in the analysis. In IESs not showing inhibition, the degree of conservation of each of the eight positions of the consensus is similar to that observed in the whole sample of IESs from which the consensus was established. In IESs showing inhibition, the fourth position shows a T in 8 sequences of 10, whereas an A is preferred in 70% of sequences in the general sample. Although the number of sequences is limited, this deviation is interesting because all known IESs beginning with the sequence 5'-TATT-3' on both sides have now been shown to be subject to maternal inhibition. However, this sequence feature is not necessary for the effect, since both ends of 51A6649 show the more standard sequence 5'-TACA-3'.

DISCUSSION

This study has shown that the developmental excision of five different *Paramecium* IESs can be inhibited by transformation of the maternal macronucleus with specific micronuclear sequences. Although 12 of the 13 IESs tested belong to the *A* and *G* genes, one of those showing inhibition (51A-712) is located ~700 bp upstream of the *A*-gene transcription start site. This region is apparently noncoding and is clearly upstream of the *A*-gene promoter, since normal transcription regulation does not require more than ~270 bp of upstream sequences (21,

23). Thus, the effect is not limited to IESs located within surface antigen genes and may concern a sizeable fraction of the estimated ~50,000 different IESs in the genome. The case of paralogous IESs 51G4404 and 51A4404 is interesting because only the former could be inhibited. IESs that have a common ancestor are unlikely to use radically different excision mechanisms, suggesting that the same mechanism can be differently affected depending on the particular sequence. Paralogous IESs often show limited sequence conservation at their very ends (22, 38); the fact that one of the TATT ends of 51G4404 is changed to TATG in 51A4404 can be viewed as supporting the significance of the deviation from the consensus noted above. However, this single criterion is not sufficient to explain the different behaviors of all tested IESs.

The search for possible mechanisms should take into account all basic features of this transnuclear epigenetic phenomenon. The first and most important is sequence specificity. The micronuclear *A* gene can inhibit the excision of *A*-gene IESs in the developing macronucleus but has no effect on *G*-gene IESs and vice versa. Maternal inhibition is caused by the IESs themselves, as shown by macronuclear controls and the effect of 51G4404 without flanking sequences (8). Complete IES specificity was here demonstrated for the effects of 51A-712, 29A6649, and 51G4404; in an independent study, transformation with 51A2591 alone was shown to block the homologous IES (24). Taken together, these results strongly suggest that each IES inhibits the excision of its germ line copy independently from the others. The homology requirement can nevertheless tolerate small differences between the sequence present in the maternal macronucleus and the germ line sequence, such as the introduction of a restriction site within 51G4404 (8), or the allelic differences between *A*⁵⁷ and *A*²⁹ (one substitution and a 12-bp insertion at the most, in 29A2591). This allowed us to show that macronuclear maintained IESs originate from the germ line genome and are not copied from maternal sequences. Short internal deletions in the maintained versions of IESs 51A2591 and 51A6649 (28 and 29 bp of 370) do not seem to impair the effect either, as these shortened copies were themselves able to inhibit excision of the germ line copies in the following sexual generation. In contrast, removing a 147-bp internal segment from the 222-bp IES 51G4404 has been shown to abolish the effect (8).

A second important feature is copy number dependence. Because of the large variability observed among individual karyonidal clones derived from a single transformed clone, the quantitative relationship is most clearly evidenced by the analysis of mass-autogamy samples, which measures the average effect in ~40 different karyonidal clones for each point. Karyonidal variability could in principle reflect the unequal distribution of IES copies during amitotic divisions of the transformed macronucleus, leading to variable copy numbers at the time of induction of autogamy. However, this is unlikely to account for the extreme variability observed in some cases. For instance, the absence of any effect on 51G2832 in some karyonidal clones from the G4 population cannot be explained by the absence of the λ51Gmic insert in the maternal macronuclei of these particular cells during autogamy, because 51G4404 was fully maintained in these clones. Similarly, one karyonide derived from a clone transformed with λ51Amic was found to retain 51A6649 but not 51A2591, although the latter is retained on a higher fraction of copies in the mass-autogamy sample (data not shown). Thus, excision inhibition appears to be a stochastic process in single developing macronuclei; the mass-autogamy correlation indicates that its probability is determined by the copy number of the IES in the maternal macronucleus.

T ₁₀₀	A ₁₀₀	Y ₉₅	A ₇₀	G ₇₈	Y ₈₃	N	R ₇₈	General consensus	
T	A	T	A	G	T	T	A	51A-10L	Ends of IESs not showing inhibition
T	A	T	A	G	T	T	A	51A-10R	
T	A	C	A	G	T	T	A	51A1416L	
T	A	T	A	G	T	C	G	51A1416R	
T	A	T	A	G	A	T	G	51A1835L	
T	A	T	A	G	A	T	A	51A1835R	
T	A	T	T	G	T	G	G	51A4404L	
T	A	T	G	G	T	A	G	51A4404R	
T	A	C	A	G	C	T	A	51A4578L	
T	A	C	A	G	T	C	T	51A4578R	
T	A	T	C	G	A	T	A	51A6435L	
T	A	T	A	A	T	A	T	51A6435R	
T	A	T	A	C	T	T	A	51G1413L	
T	A	T	T	G	T	T	A	51G1413R	
T	A	T	A	A	T	T	A	51G1832L	
T	A	T	A	A	A	T	A	51G1832R	
T	A	T	A	G	T	G	A	51A28L*	
T	A	T	G	A	A	T	C	51A28R*	
T	A	T	C	G	T	T	G	51A29L*	
T	A	T	A	G	C	A	T	51A29R*	
100	100	100	70	75	75	-	80	% match with consensus	
T	A	T	T	G	C	T	A	51A-712L	Ends of IESs showing inhibition
T	A	T	T	A	T	C	G	51A-712R	
T	A	T	T	C	T	A	A	51A2591L	
T	A	T	T	G	A	G	A	51A2591R	
T	A	C	A	G	C	T	G	51A6649L	
T	A	C	A	G	G	T	T	51A6649R	
T	A	T	T	G	T	A	A	51G2832L	
T	A	T	T	G	A	A	G	51G2832R	
T	A	T	T	A	T	A	A	51G4404L	
T	A	T	T	A	T	T	A	51G4404R	
100	100	100	20	60	70	-	90	% match with consensus	

FIG. 8. Sequences of the ends of tested IESs. The left (L) and right (R) end sequences of each IES are aligned with the degenerate consensus established from the general IES sample (18, 19). All sequences are written 5' to 3'. Subscripts in the general consensus indicate the fraction of sequences in the general sample showing the preferred nucleotide at each position. Sequences marked with asterisks are the ends of internal IES-like segments within 51A2591 (51A28) and 51A6649 (51A29).

Thirdly, for a given maternal copy number, the probability of inhibition varies greatly between different IESs. It should be noted that when the effect is weak (low maternal copy numbers or inefficient IESs), karyonidal variability may not be correctly averaged in pools of ~40 karyonidal clones, which adds to measurement errors inherent in the quantification of faint hybridization signals. Despite these technical limitations, the consistency of the mass-autogamy plots shows that each IES is inhibited with a characteristic efficiency, which does not correlate with any obvious feature such as IES size, position within the gene, or end sequence. The efficiency of 51G4404 was previously shown to increase with the length of flanking sequences present on the maternal molecule (8). It was here shown to be eightfold higher in the context of the λ51Gmic insert than on the largest plasmid tested (851 bp) and very close to that of the chromosome-borne IES (50% inhibition with ~0.3 copies phg). This suggests that the efficiencies determined for other IESs, all present on phage inserts of similar sizes (12.3 to 12.4 kb), are also close to their maximum values. Our attempt to determine directly the efficiencies of chromosome-borne IESs after a second autogamy revealed that their effects are more variable than those of phage inserts, and on average only slightly stronger. In spite of this greater variability, the results confirm the relative efficiencies of the different IESs, indicating that they reflect intrinsic properties of the elements. Since the least efficient IES was only partially inhibited at the highest copy number tested, transformation with

higher copy numbers might have resulted in the inhibition of more IESs. Thus, it may be misleading to classify IESs into two categories according to whether inhibition was observed.

Finally, the observation that internal IES-like segments are excised from the macronuclear maintained versions of 51A2591 and 51A6649 reveals that at least parts of the inhibited IESs are still accessible to the excision machinery. Internal excision events are not linked to the process of maternal inhibition, as they do not occur in the A^{29} allele of 51A2591, nor in the other three IESs showing inhibition. Moreover, a point mutation at the left end of 51A2591 has been reported to impair excision, allowing Mayer et al. (24) to show that, in the absence of any maternal inhibition, the internal 28-bp segment is still deleted in the macronuclear version of the IES. Similarly, a point mutation in one of the TA boundaries of 51A6649 reveals that the excision of the internal 29-bp segment occurs independently from the maternal inhibition (25). It has been suggested that the 51A2591 28-bp segment could have originated from the secondary insertion of some mobile element into a preexisting IES or ancestral transposon (24). Our observation that allelic differences between 51A2591 and 29A2591 are all located within this segment does not support a more recent origin. Since the homologous segment from the A^{29} allele does not appear to be capable of independent excision, the data are perhaps more consistent with the idea that functional IES consensus sequences were created by random mutations in the A^{57} allele. However, it remains intriguing that the only allelic difference between 51A6649 and 29A6649 is also located within the 29-bp IES-like segment.

Two different types of models have been proposed to account for the sequence specificity of maternal inhibition of IES excision in *Paramecium* and *Tetrahymena* spp. (2, 8). In the first type, IES copies in the maternal macronucleus sequester a sequence-specific protein factor that is required for excision in the developing macronucleus. Although formally a possibility, the model would require an unreasonably large number of different factors if, as suggested by the present work, a significant fraction of IESs in the genome can inhibit their own excision with a high specificity. Furthermore, the factor would have to bind the IES itself, since flanking macronuclear sequences have no inhibitory effect; this is difficult to reconcile with the higher efficiency of molecules containing longer flanking sequences.

The second type of model proposes that IES copies are exported from the maternal macronucleus to the developing macronucleus, where they would affect excision by pairing with homologous sequences of the germ line genome. The model easily accounts for the observed sequence specificity; furthermore, the dependence of inhibition efficiency on the length of IES flanking sequences could be explained by differences in pairing efficiency. Although there is no direct evidence, a transfer of nucleic acids between the two nuclei is also likely to be involved in another type of homology-dependent maternal effects on macronuclear development in *Paramecium* spp., affecting the level of amplification of macronucleus-destined sequences (27, 28, 30). RNA would seem to be a better candidate than DNA for a messenger molecule able to leave one nucleus and enter another. That IES-containing plasmids and phage inserts can be transcribed without the need for a polymerase II promoter is suggested by a recent study of homology-dependent gene silencing in vegetative cells, in which aberrantly sized transcripts could be detected following transformation of the macronucleus with plasmids containing promoterless coding sequences (35). Furthermore, the maternal macronucleus is known to remain fully active in transcription throughout the development of the new macronucleus (1).

In the simplest version of this model, pairing of a maternal transcript with the germ line sequence could directly inhibit IES excision by steric hindrance. This is unlikely because excision of the internal IES-like segments in 51A2591 and 51A6649 would then be expected to be inhibited in the same way. Alternatively, a transient pairing interaction could induce some epigenetic modification of the germ line IES, which in turn would block excision. A highly localized modification, such as the methylation or other chemical modification of specific nucleotides or dinucleotides, could affect the excision of each IES to a different extent, depending on the location of modified sites relative to IES boundaries. This could explain the absence of any inhibitory effect for some IESs and for internal IES-like segments. For a given IES, the efficiency of inhibition would also depend on the concentration of effective transcripts, determined by the copy number of the maternal IES. The fact that λ 51Amic can occasionally inhibit 51A6649 without affecting the more sensitive 51A2591 suggests that effective transcripts do not necessarily cover the whole phage insert. Maternal transcription level and RNA stability might thus be additional factors that determine the characteristic inhibition efficiency of each IES.

The targeting of epigenetic modifications to specific genomic sequences through pairing interactions has been implicated in many homology-dependent gene silencing phenomena. These effects have been revealed by transformation in a wide range of eukaryotes (13, 20), including *P. tetraurelia* (35). Both transcriptional and posttranscriptional cases of silencing are frequently associated with epigenetic modifications of the genes; it has recently been proposed that all types of homology-dependent gene silencing in plants are triggered by a common RNA-based mechanism (44). The transnuclear nature of the *Paramecium* maternal effect is not unique. A dominant silencing effect has been observed in multinucleated cells of *Neurospora crassa* (4); in several multicellular organisms, the propagation of homology-dependent silencing signals is thought to involve the transfer of RNA molecules across cellular boundaries (10, 32). The novel aspect of the homology-dependent epigenetic effect described here is that it affects developmental genome rearrangements rather than gene expression.

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