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Non-Mendelian Inheritance and Homology-Dependent Effects in Ciliates

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ABSTRACT

Ciliates are single-celled eukaryotes that harbor two kinds of nuclei. The germline micronuclei function only to perpetuate the genome during sexual reproduction; the macronuclei are polyploid, somatic nuclei that differentiate from the micronuclear lineage at each sexual generation. Macronuclear development involves extensive and reproducible rearrangements of the genome, including chromosome fragmentation and precise excision of numerous internal sequence elements. In *Paramecium* and *Tetrahymena*, homology-dependent maternal effects have been evidenced by transformation of the vegetative macronucleus with germline sequences containing internal eliminated sequences (short single-copy elements), which can result in a specific inhibition of the excision of the homologous elements during development of a new macronucleus in the sexual progeny of transformed clones. Furthermore, transformation of the *Paramecium* maternal macronucleus with cloned macronuclear sequences can specifically induce new fragmentation patterns or internal deletions in the zygotic macronucleus. These experiments show that the processing of many germline sequences in the developing macronucleus is sensitive to the presence and copy number of homologous sequences in the maternal macronucleus. The generality and sequence specificity of this *trans*-nuclear, epigenetic regulation of rearrangements suggest that it is mediated by pairing interactions between zygotic sequences and sequences originating from the maternal macronucleus, presumably RNA molecules. Alternative macronuclear versions of the genome can be maternally inherited across sexual generations, suggesting a molecular model for some of the long-known cases of non-Mendelian inheritance, and in particular for the developmental determination and maternal inheritance of mating types in *Paramecium tetraurelia*.

I. INTRODUCTION

A. Non-Mendelian inheritance in ciliates

Long before the advent of molecular genetics, ciliates enjoyed a period of success during which they were strongly associated with non-Mendelian phenomena, and in particular with the question of cytoplasmic inheritance (Nanney, 1985; Preer, 1993). They had become the favorite models of a few biologists, such

as T. M. Sonneborn, who believed that Morgan's chromosome theory fell short of explaining all heredity and could not by itself—with no role credited to the cytoplasm—account for complex phenomena such as development and evolution (Harwood, 1985). The early discovery of mating types (Sonneborn, 1937) had made *Paramecium* one of the first single-celled organisms in which genetic analyses could be conducted. Although many characters were found to follow Mendelian inheritance and were ascribed to nuclear genes, it was observed from the very beginning that a number of hereditary characters did not behave in a Mendelian way (Preer, 1993, 2000). One of these non-Mendelian characters, which could hardly have been ignored by the *Paramecium* geneticist, was the mating type itself. Opposite mating types were shown to develop from identical genotypes, and in some species mating types even proved to be cytoplasmically transmitted to sexual progeny. Other well-studied cases of cytoplasmic inheritance included the "killer" trait (production of a toxin that killed other cells) and the serotypes (antigenic variants of the cell surface).

To explain these observations, Sonneborn and others proposed around 1945 that such characters were determined by "plasmagenes," cytoplasmic particles endowed with the genetic properties of reproduction and mutability (Sonneborn, 1948, 1949). Not all plasmagenes, however, were held to be completely independent from nuclear genes; indeed, Mendelian mutations could abolish their maintenance in the cytoplasm, and some plasmagenes even appeared to *originate* from nuclear genes. In its most general form, the plasmagene theory assumed that phenotypic characters can be determined by both nuclear genes and plasmagenes, and that their inheritance patterns in crosses depend on which component differs in the strains analysed (Sonneborn, 1950). Accumulating evidence, however, soon revealed that different mechanisms were at work in different cases, and the search for a unifying theory was abandoned. The killer trait was found to be determined by the presence of an endosymbiotic bacterium multiplying in the cytoplasm, and serotypes were rationalized as the stable inheritance of alternative patterns of expression for a set of nuclear genes encoding variant surface proteins. Unresolved cases of cytoplasmic inheritance, such as that governing *Paramecium* mating types, were confined to the periphery of mainstream research by the strong nuclear hegemony that had built on the remarkable success of Mendelian genetics. As a result of this initial emphasis on cytoplasmic inheritance, ciliates were increasingly perceived as being "anomalous" organisms, and despite Sonneborn's advocacy of the view that the peculiarities of any organism could only help define the universal principles of life (Schloegel, 1999), *Paramecium* genetics largely fell into oblivion (Preer, 1997).

But ciliate research survived, as these organisms continued to prove useful models in other domains, such as cellular morphogenesis or membrane excitability. In the late 1970s, molecular biology began to revive some interest in the genetics of ciliates through a variety of fundamental discoveries, including telomere structure, genome-wide DNA rearrangements, self-splicing introns,

deviant genetic codes, telomerase, histone acetyltransferase, and novel histone modifications. Interestingly, some of the new molecular findings again revealed puzzling non-Mendelian phenomena, which have now been characterized as homology-dependent effects. As the Mendelian era is culminating with the first complete sequences of eukaryotic genomes, it is no longer possible to ignore the fact that dividing cells must inherit, in addition to the genome, essential regulatory information that is not to be found in the genome sequence itself, and much excitement is being redirected at some poorly understood epigenetic mechanisms of gene regulation. Homology-dependent effects, in particular, cannot be accounted for by classical paradigms of molecular genetics; yet there are now indications that their widespread occurrence in eukaryotes reflects the evolutionary conservation of a sophisticated machinery. It is the purpose of this review to present available information on homology-dependent effects in ciliates, and their relevance to known cases of non-Mendelian inheritance. The diversity and novel aspects of ciliate effects have the potential of exerting a significant impact in this domain. As in many eukaryotes, ciliate homology effects can lead to the specific silencing of almost any gene during vegetative growth, but in addition they have been shown to participate in the programming of genome rearrangements that occur during development. More generally, the evidence for maternal effects directing an epigenetic programming of the zygotic genome through homology-dependent mechanisms may turn out to be of interest in other systems. For a proper understanding of these maternal effects, some introduction to ciliate biology is necessary.

B. The ciliate life cycle

Ciliates are a monophyletic group of unicellular organisms belonging to the Alveolates, one of the major phyla that emerged at about the same time as plants and the metazoan/fungi clade during the "Big Bang" of eukaryotic evolution (Philippe *et al.*, 2000). One of their most distinctive features is a unique system of separation of germline and somatic functions, which takes the form of two different nuclear lineages coexisting in the cytoplasm of each cell. The germline micronucleus is a diploid nucleus, which divides by mitosis and is transcriptionally silent during vegetative growth. Its only genetic function is to undergo meiosis and produce gametic nuclei during sexual events. In contrast, the somatic macronucleus is a large, highly polyploid nucleus which divides by a nonmitotic process and is responsible for vegetative transcription. It governs the cell phenotype but is lost during sexual events; its genetic material is not transmitted to sexual progeny. Although the numbers of nuclei of each type vary in different species, all nuclei in one cell always originate from a single, diploid zygotic nucleus.

Sexual processes are usually induced by starvation. The sequence of nuclear reorganization is similar in all species with minor variations, and is described in Figure 10.1 for the *P. aurelia* species (see Sonneborn [1974] for full details). An

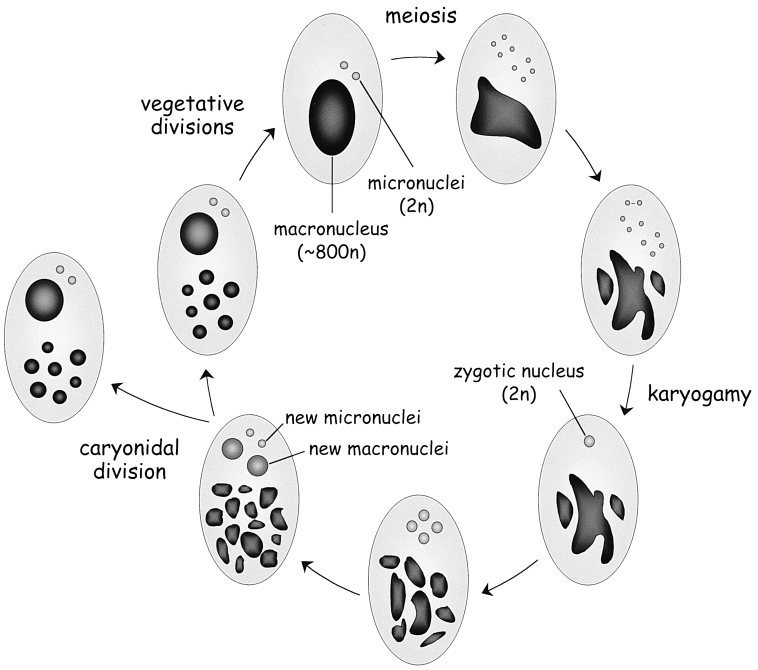

Figure 10.1. The sexual cycle of *Paramecium aurelia* species. Vegetative cells have two micronuclei and one macronucleus. After meiosis of the two micronuclei, seven of the eight haploid products (small circles) degenerate and the surviving one divides once more, yielding two genetically identical gametic nuclei (two small circles with a line between them). In conjugation, one of the gametic nuclei is exchanged with the partner cell before caryogamy; in autogamy, the two gametic nuclei fuse together. In both cases, the diploid zygotic nucleus then undergoes two rounds of mitotic divisions. Of the four products, two become the new micronuclei, while the other two begin to enlarge and develop into new macronuclei. Rearrangements begin after a few replication cycles and are completed by the first cell fission (caryonidal division). During the caryonidal division, the new micronuclei divide by mitosis while the new macronuclei segregate without division to the two daughter cells, called caryonides, and start dividing only at the second cell fission. Each meiotic cell thus gives rise to two caryonidal clones, which derive from distinct events of macronuclear development. Starting shortly after meiosis of the micronuclei, the parental macronucleus is progressively fragmented into about 30 pieces, which segregate randomly to daughter cells during the first vegetative fissions and then disintegrate.

important point is that meiosis of the micronuclei eventually results in two genetically identical gametic nuclei in each cell, as only one haploid product survives and this undergoes an additional division. If the cell is engaged in conjugation with a partner of compatible mating type, karyogamy occurs after the reciprocal exchange of one of the two gametic nuclei, resulting in identical zygotic nuclei

in the two conjugants. In the absence of a partner, some species can undergo autogamy, a self-fertilization process by which the two identical gametic nuclei fuse together. In both cases new micronuclei and new macronuclei develop from mitotic products of the diploid zygotic nucleus. The parental macronucleus is not immediately degraded: although DNA replication is rapidly inhibited, transcription continues actively throughout the development of the new macronuclei. The parental genome thus contributes ~80% of the RNA synthesized during the first two cell cycles in *Paramecium* (Berger, 1973).

The genetic consequences of conjugation and autogamy are illustrated in Figure 10.2A by a cross between two homozygous *Paramecium* cells differing by a pair of alleles. Because the exchange of gametic nuclei always makes the two conjugating cells become identical in genotype (see the heterozygous F1

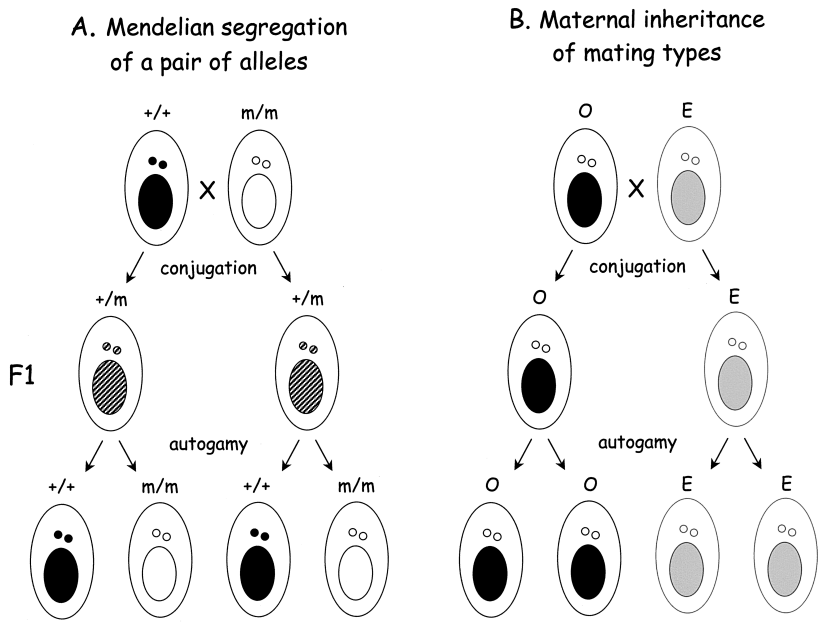

Figure 10.2. Inheritance patterns in *Paramecium aurelia*. (A) Mendelian segregation of a pair of alleles in a cross between homozygous mutant (*m*/*m*) and wild-type (+/+) cells. Conjugation results in identical zygotic nuclei in the two F1 exconjugants (genotype *m*/+). Autogamy of the F1 clones yields entirely homozygous F2 progeny that have a 50% chance of receiving each of the parental alleles. (B) Maternal inheritance of mating types in *P. tetraurelia*. Mating types O and *E* follow a cytoplasmic pattern of inheritance, but are determined by the differentiated macronuclei. Maternal inheritance results from the recurrent programming of the zygotic macronucleus by the maternal macronucleus during development. Adapted with permission from Figure 2 in E. Meyer and S. Duharcourt (1996), *J. Eukaryot. Microbiol.*, **43**(6):453–461.

exconjugants in Figure 10.2A), any phenotypic difference between F1 clones is indicative of non-Mendelian effects. In *Paramecium*, there is almost no exchange of cytoplasm between conjugating cells under normal conditions. This makes the system ideally suited for the detection of cytoplasmically inherited characters, such as those encoded in mitochondria. However, a cytoplasmic pattern of inheritance can also reflect maternal effects on zygotic development, exerted through the cytoplasm by the maternal macronucleus (see Figure 10.2B and Section I.D). Note that ciliates are hermaphrodites; "maternal" refers here to the cytoplasmic parent of each progeny cell. Finally, genetic analyses are greatly facilitated in *Paramecium* by the occurrence of autogamy, as this process generates an entirely homozygous genotype in just one sexual generation. F2 individuals obtained by autogamy of a heterozygous F1 clone have a 50% chance of receiving each parental allele, which translates into a characteristic 1:1 segregation of characters in the F2 population (Figure 10.2A).

C. Developmental genome rearrangements

Developmentally regulated rearrangements occur in a variety of other eukaryotes, but rarely on a scale as massive as that seen in ciliates (for reviews, see Coyne *et al.*, 1996; Klobutcher and Herrick, 1997; Prescott, 1994; Yao *et al.*, 2002). During the development of a new macronucleus, the genome is amplified to a high ploidy level (~50*n* in *Tetrahymena*, ~1000*n* in *P. tetraurelia*), and rearranged at a large number of sites ranging from 6000 in *Tetrahymena* to perhaps as many as 100,000 in hypotrichous ciliates. The process results in the elimination of a sizable fraction of the genome, which varies from 10–15% to 95% in different species. Virtually all transposable elements and repeated sequences belong to the eliminated fraction. A recent pilot survey of the *P. tetraurelia* macronuclear genome ($\sim 10^8$ bp) revealed a very high gene density (Dessen *et al.*, 2001), leading to an estimate of at least 20,000 genes and possibly many more. As a rule, rearrangements are highly reproducible from one sexual generation to the next. They occur at specific time periods, after some initial amplification of the diploid genome but before the final ploidy level is reached. Although little is known about the molecular mechanisms involved, a link between heterochromatin formation and DNA elimination is suggested by the identification of two chromodomain proteins that are required for rearrangements in *Tetrahymena*. There appears to be significant differences in mechanistic details among ciliates; here we shall limit ourselves to a brief overview of the two main types of rearrangements that are observed in all species.

The first type is the precise excision of internal eliminated sequences (IESs). Both coding and noncoding regions of the micronuclear genome contain various sequence elements that reside between two short direct repeats and are deleted during macronuclear development. Flanking sequences are rejoined

with high precision, leaving one of the repeats in the macronuclear sequence. The excision of each IES usually results in a unique junction sequence in the mature macronucleus, although a reproducible set of alternative junctions have been described in *Tetrahymena* and for a few *Paramecium* IESs. IESs can be either relatively large transposable elements, mostly Tc1/mariner family members with copy numbers ranging from dozens to tens of thousands per haploid genome, or short, single-copy, noncoding elements that are also very numerous (about 50,000 different elements in the *Paramecium* haploid genome). One class of single-copy IESs, called TA IESs because they are invariably bounded by two 5′-TA-3′ repeats, is present in widely divergent ciliates. Although they contain no other invariant sequence motif, a short, loosely conserved consensus is present at their ends, adjacent to the TA boundaries. Point mutations at some consensus positions, or in the TA repeats, were shown to abolish excision. The resemblance of this consensus to the extremities of inverted repeats of Tc1/mariner transposons has led to the hypothesis that single-copy IESs represent ancient, degenerate copies of such transposons that are flanked by the duplication of a TA integration site. Developmental excision of both transposon-like and TA IESs was shown to generate covalently closed circular molecules with a species-specific junction structure.

The second type of rearrangement observed in all species is the fragmentation of germline chromosomes into smaller molecules, which are healed by *de novo* telomere addition. The average size of these "macronuclear chromosomes" varies considerably among species, from ~600 kb in *Tetrahymena thermophila* to about 2 kb in hypotrichous ciliates such as *Euplotes crassus*, in which most macronuclear chromosomes contain a single gene. Mechanisms for fragmentation also appear to be quite diverse. In both *Tetrahymena* and *Euplotes*, the process is directed by short *cis*-acting sequences, but only in the latter are telomeric repeats added at a fixed number of nucleotides from the *cis*-acting sequence element. In contrast, no putative *cis*-acting consensus sequence has been identified in *Paramecium aurelia* species. In these species, the process appears to be associated with the elimination of significant lengths of germline-limited sequences around fragmentation sites. Macronuclear telomeres are formed by addition of telomeric repeats at multiple random positions within regions that are 0.8–2 kb in length. Although these telomere addition regions do not contain any conserved sequences, they are reproducibly used each time a new macronucleus develops. Additional heterogeneity is seen for some macronuclear chromosome ends, which have a choice of several alternative telomere addition regions, spaced 2–20 kb apart. All of this heterogeneity is observed within each developing macronucleus. Furthermore, some fragmentation events are facultative: in these cases the deletion of germline sequences can lead either to chromosome fragmentation or to the joining of the sequences flanking the eliminated segment. These internal deletions differ from IES excision by their larger size and the variability of breakpoints (A. Le Mouël *et al.*, unpublished). In two cases where they have been examined,

the germline sequences that were eliminated at fragmentation sites were found to contain different Tc1/mariner transposons and, for one of them, a 69-bp minisatellite (A. Le Mouël, unpublished; O. Garnier, unpublished). As both transposable elements and satellites are commonly associated with heterochromatin in eukaryotic genomes, this finding suggests that chromosome fragmentation in *P. aurelia* may result from the elimination of heterochromatin through a developmentally regulated, non-sequence-specific mechanism.

D. Mating type determination in *P. tetraurelia:* maternal inheritance of developmental alternatives

More than 60 years after the discovery of mating types in *P. aurelia*, the molecular basis for the expression of alternative types is still unknown. Nevertheless, a considerable sum of experimental observations has led to a detailed description of the developmental process of mating type determination. The summary given below shows that the maternal macronucleus can direct differentiation of the zygotic macronucleus for stable alternative characters, providing an interesting precedent for the maternal homology-dependent effects presented in Section II (for reviews, see Sonneborn, 1974, 1977).

Each species of the *P. aurelia* group has two complementary mating types, *O* and *E*. Both types can be produced in entirely homozygous wild-type strains, indicating that they are not determined by genotypic differences. Rather, they correspond to alternative differentiated states of the macronucleus. Mating type is determined in an irreversible manner during a critical period of macronuclear development and remains unchanged throughout vegetative growth, until the macronucleus is lost at the following conjugation or autogamy and replaced by a new one.

Of special interest here is the cytoplasmic pattern of inheritance of mating types at sexual reproduction, a feature that is observed in roughly half of the *P. aurelia* species, including *P. tetraurelia* (see Figure 10.2B). After conjugation of an *O* cell with an *E* cell, the two new macronuclei developing in the cytoplasm of the *O* parent are almost always determined for *O*, while those developing in the *E* parent cell are almost always determined for *E* (Nanney, 1957). Likewise, autogamy produces a zygotic macronucleus that is determined for the same mating type as the maternal macronucleus. Numerous experiments, including the transfer of cytoplasm between cells at the appropriate stage of development, have shown that the *E* maternal macronucleus produces a cytoplasmic factor that causes the zygotic macronucleus to be determined for *E*, hence the capacity to produce, in turn, the same factor at the following sexual event. This *E*-determining factor was shown to act only at a specific stage of macronuclear development and to have no effect on mature macronuclei. As no evidence for an *O*-determining cytoplasmic factor was obtained, mating type *O* appears to be the default developmental

alternative. Although recurrent determination of the zygotic macronucleus by the maternal macronucleus gives a cytoplasmic pattern of inheritance, this is more properly called maternal inheritance, to distinguish it from true cytoplasmic inheritance.

The timing and irreversibility of mating type determination suggest that it could be achieved during macronuclear development by an alternative rearrangement of the genome. Some support for this possibility came from studies of the only Mendelian mutation known to affect mating type determination in *P. tetraurelia*, the *mtF*^{E} mutation. All other mating type mutations restrict homozygotes to the *expression* of type O during sexual reactivity, but do not affect *determination* (Brygoo, 1977; Byrne, 1973). In contrast, *mtF*^{E} is a pleiotropic mutation which affects the process of macronuclear development, making determination for *E* constitutive in homozygotes, regardless of the maternal mating type (Brygoo and Keller, 1981a), and also causing stable differentiation for several unrelated mutant characters (Brygoo and Keller, 1981b). Among *mtF*^{E}-induced developmental abnormalities, one was shown to be the failure to excise an IES located in the G gene, which encodes a nonessential surface antigen, resulting in a nonfunctional form of the gene in the macronucleus (Meyer and Keller, 1996). This suggests that the *mtF* gene product is involved in the excision of a subset of germline IESs, and that the *mtF*^{E} pleiotropy reflects rearrangement defects at a number of unrelated loci. Constitutive determination for mating type *E* may thus result from the inability to rearrange a putative gene controlling mating type.

If type O is indeed a default state determined by the excision of an IES from a mating type gene, could the maternal *E*-determining cytoplasmic factor specifically block excision of this IES in wild-type cells? Again, indirect evidence was obtained through the study of the *mtF*^{E} pleiotropy. Indeed, like mating type *E*, some (possibly all) of the *mtF*^{E}-induced characters can be maternally transmitted to sexual progeny, even when the wild-type *mtF* allele is reintroduced in the cytoplasmic lineage by conjugation and made homozygous by autogamy (Brygoo and Keller, 1981b). These mutant characters are further inherited in subsequent generations but can revert with relatively high frequencies at each autogamy and revert independently from each other. Thus, they appear to be determined by epigenetic alterations arising during macronuclear development, each of which is self-reproduced from maternal to zygotic macronucleus. A striking example, which can be described in molecular terms, is the retention of the G gene IES in the macronuclear genome. Once induced by the *mtF*^{E} mutation, the character is stably maintained through an indefinite number of autogamies in genetically wild-type cells (Meyer and Keller, 1996). Like mating types, it is also maternally inherited at conjugation (Duharcourt *et al.*, 1995). The next section shows that this results from a homology-dependent control of IES excision that is exerted by the maternal macronucleus.

II. REGULATION OF IES EXCISION BY HOMOLOGY-DEPENDENT MATERNAL EFFECTS

A. An IES inhibiting its own excision in *P. tetraurelia*

The genetic analysis presented above showed that wild-type *P. tetraurelia* cell lines can be induced to retain a particular IES located in the G gene during macronuclear development. The IES^+ (retention) and IES^- (excision) states of the macronucleus are alternative characters that show maternal inheritance in breeding analyses, confirming that they are not attributable to any genotypic difference. If developmental excision of the IES in the zygotic macronucleus is controlled by the maternal macronucleus, it could either be induced by maternal copies of the correctly rearranged G gene, or be inhibited by maternal copies of the IES-retaining G gene. To answer this question, the macronucleus of IES^+ or IES^- cells was transformed by direct microinjection of plasmids containing a fragment of the G coding sequence in either its micronuclear (IES^+) or macronuclear (IES^-) versions (Duharcourt *et al.*, 1995). After being introduced into the *Paramecium* macronucleus, any DNA molecule above ~4 kb can be stably maintained at a wide range of copy numbers during vegetative growth, replicating autonomously as extrachromosomal monomers and multimers after *de novo* addition of telomeres. Transformed clones were grown and allowed to undergo autogamy, which leads to the loss of the transformed maternal macronucleus, and excision of the IES was examined in the new macronucleus of sexual progeny. While the IES^- plasmid proved unable to induce excision in the progeny of transformed IES^+ cells, the IES^+ plasmid resulted in the retention of the IES in the progeny of transformed IES^- cells (Figure 10.3).

The effect of the IES^+ plasmid was clearly dependent on its copy number in the maternal macronucleus: low copy numbers resulted in a partial effect, i.e., the retention of the IES on only a fraction of the ~1000 copies of the genome in the postautogamous macronucleus. The IES sequence itself is responsible for the phenomenon, since the IES^- plasmid, which contained the same length of flanking sequences from the G gene, had no detectable effect, even at high copy numbers. Furthermore, a plasmid containing only the IES, but no flanking sequences, also caused IES retention in sexual progeny from transformed cells, although with a reduced efficiency (see Section II.B). In contrast, an internal deletion removing two-thirds of the 222-bp IES sequence completely abolished the effect.

The IES was still retained after additional autogamies of first-generation IES^+ cells, showing that injection of the IES is sufficient to turn the wild-type cell line into a permanent IES^+ cell line. Inspection of a few other IESs in the same gene or in other genes showed them to be correctly excised. Furthermore, IES^+ cells have no obvious phenotype, indicating that the vast majority of germline

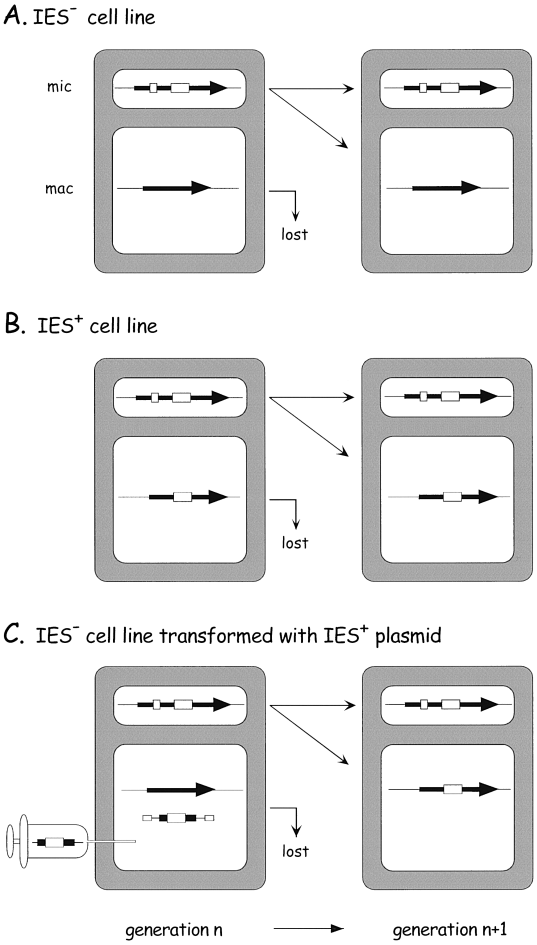

Figure 10.3. Maternal inhibition of IES excision. (A) In the IES$^-$ (wild-type) cell line, all IESs (open boxes) are removed from the G gene (arrow) during macronuclear differentiation. (B) In the IES$^+$ cell line, developmental excision of the 222-bp IES is specifically inhibited, although the germline genome is entirely wild type. (C) Transformation of the IES$^-$ macronucleus with a plasmid containing the 222-bp IES specifically inhibits excision of this IES after autogamy. Adapted from Figure 2 in *Cell* **87,** Meyer, E., and Duharcourt, S., Epigenetic programming of developmental genome rearrangements in ciliates, pp. 9–12, Copyright © 1996, with permission from Elsevier Science.

IESs are not affected. Thus, when present in the maternal macronucleus, this IES specifically causes the retention of the homologous germline IES during development of a new macronucleus.

B. A homology-dependent maternal effect

Do other IESs show similar maternal effects? Another study addressed this question by transforming the macronucleus of wild-type cells with large segments of micronuclear DNA containing the G gene or another nonessential surface antigen gene, *A*, belonging to the same multigene family (Duharcourt *et al.*, 1998). The injected segments contained 6 and 9 IESs, respectively. Thirteen of these 15 IESs were examined in the postautogamous progeny of transformed cells, and five were found to be retained on part or all of the copies of the new macronuclear genomes. Specificity between the two genes was complete: each micronuclear gene induced the retention of some of the IESs located in its own homologous copy (two for *G*, three for *A*), but did not affect any of the IESs in the other gene. For three of these five IESs, the test of plasmids containing only one IES showed that each of them acts only on itself and does not affect other IESs in the same gene. A control DNA fragment containing most of the macronuclear G gene had no effect on any of the IESs tested. The sequence of IESs retained in the macronuclear genome was found to be identical to the germline sequence, although two of the IESs contain smaller, internal IESs that were fully excised from the new macronuclear copies. These small internal IESs are also excised when excision of the larger IESs is blocked by point mutations at their boundaries, rather than by the maternal epigenetic effect (Mayer and Forney, 1999; Mayer *et al.*, 1998).

Quantitative analyses showed that the fraction of copies of the new macronuclear genome that retain an IES increases with the copy number of the IES-containing DNA fragment in the maternal macronucleus. However, different IES sequences were clearly retained with different efficiencies. In the most efficient case, 50% retention was obtained with a maternal copy number of ~0.4 copies per haploid genome (cphg), i.e., ~400 copies per macronucleus, and 100% retention with ~1 cphg; in the least efficient case, 50% retention was not reached with ~6 cphg. For a given IES, the efficiency was further shown to depend on the length of flanking sequences linked to the IES sequence carried by the DNA fragment in the maternal macronucleus. While 50% retention, in the most efficient case, required ~40 cphg of a plasmid containing only the IES sequence, this level was obtained with ~3 cphg of a plasmid that also contained some 600 bp of flanking sequences, and with only ~0.4 cphg of the 12-kb DNA fragment containing the entire micronuclear gene. The latter value is close to the maximum efficiency (~0.3 cphg in this case), which was observed for IES-retaining endogenous chromosomes in the maternal macronucleus.

These quantitative analyses were performed after mass cultivation of pools of 40 different caryonidal clones (clones deriving from single events of macronuclear development) picked from the postautogamous progeny of each transformed clone. Although the *average* fraction of new macronuclear copies retaining the IES is a fairly regular function of the maternal IES copy number, a great variability was observed among individual caryonidal clones deriving from the same transformed clone. Caryonidal clones showing 0% or 100% retention were common in mass populations giving an average figure of 50%, although individual clones showing partial retention were also found. Thus IES retention in each developing macronucleus appears to be a stochastic event, the probability of which is determined by the copy number of the IES in the maternal macronucleus.

C. Epigenetic inhibition of IES excision in *Tetrahymena thermophila*

A very similar phenomenon has been described in *Tetrahymena thermophila* (Chalker and Yao, 1996). Transformation of the vegetative macronucleus with either one of two IESs that are closely linked in the germline genome, the M and R elements, resulted in the retention of the homologous IES in the macronuclear genome of sexual progeny after conjugation of transformed clones. Here again the effect was quite specific, although parental copies of the M element were also found to interfere weakly with the excision of R. In addition, the presence of IES-bearing constructs in the parental macronucleus appeared to increase the frequency of aberrant excision events (i.e., deletions using novel endpoints), which are rarely seen in the wild type. As was observed in *Paramecium,* IES sequences alone were sufficient for the effect, and flanking macronuclear sequences had no effect at all. Conjugation of IES-retaining F1 progeny yielded F2 clones that still retained the IES in their macronuclear genome, showing that IES retention had become a non-Mendelian, heritable trait.

In this study, the fraction of IES-retaining copies in the new macronucleus did not appear to correlate with the copy number of IES-bearing constructs in the parental macronucleus. As the *Tetrahymena* macronucleus does not have the capacity to maintain introduced DNA fragments as autonomously replicating molecules, macronuclear transformation relies on an rDNA-based vector which allows the replacement of the endogenous rDNA minichromosome by a modified version carrying the sequence of interest. Introduced IESs, therefore, had the same high copy number as the rDNA, which is overamplified during macronuclear development and then maintained at ~180 cphg, or ~9000 copies per macronucleus. Such a high copy number did not result in complete IES retention in the developing macronucleus. As in the *Paramecium* effect, the level of IES retention was very variable, even among different F1 progeny of the same transformed clones, but most of these progeny contained a substantial fraction of correctly rearranged copies. Although IES-retaining F1 clones had at most 50 copies of the IES

in their macronucleus (the normal macronuclear ploidy), the same variability and incomplete retention was also observed in the F2 generation. No correlation was apparent between IES copy numbers in the various F1 and derived F2 macronuclei. However, an average correlation could have been masked by the large stochastic component of the effect, as only a few F2 progeny were analysed for each F1. Since the efficiencies of the various *Paramecium* IESs to cause sequence retention were shown to depend on their sequence context, the efficiency of rDNA-linked IES copies in *Tetrahymena* may also be inherently smaller than the efficiency of chromosomal copies of the same elements.

One interesting difference between the *Tetrahymena* and *Paramecium* effects is that conjugation of IES-transformed cells with normal cells results in IES retention in the progeny of *both* mates in *Tetrahymena*, whereas the IES^+ and IES^- characters show maternal inheritance in *Paramecium*. This may be related to the fact that conjugating cells form a cytoplasmic bridge large enough for complete cytoplasmic mixing to occur before the two conjugants separate in *Tetrahymena*, but not in *Paramecium*. The cytoplasmic "dominance" observed in *Tetrahymena* therefore confirms that the effect of the IES-containing parental macronucleus on the developing zygotic macronucleus is mediated through the cytoplasm.

D. Models

Although they both belong to the class Oligohymenophorea, *Paramecium* and *Tetrahymena* are fairly distant evolutionarily (Tourancheau *et al.*, 1998). Their IESs show a number of differences: *Tetrahymena* IESs are bounded by short direct repeats that are variable in sequence, as compared to the invariant *Paramecium* TA, and their excision appears frequently to use a discrete set of alternative boundaries; they have never been found within coding sequences; they are fewer and longer than *Paramecium* IESs. Nevertheless, the homology-dependent maternal effects leading to the retention of specific zygotic IESs in these two species are remarkably similar, suggesting the conservation of an ancient mechanism, which may also exist in other ciliates.

Among the 13 *Paramecium* IESs tested, there is no obvious difference in size, base composition, or position within the genes between the five that show the maternal effect and the eight that do not. Four of the five IESs showing the effect begin with the sequence 5′-TATT . . . -3′ at both ends, where the T at the 4th position is a rare variant in the general consensus 5′-TAYAGYNR . . . -3′ (Duharcourt *et al.*, 1998). However the significance of this deviation is unclear, as the 5th one has very standard ends. The phenomenon is not limited to IESs located in surface antigen genes, as one of those showing the effect is inserted upstream of the gene's promoter. Furthermore, two of two *Tetrahymena* IESs tested showed the effect, suggesting it may be observed for a large number of other IESs in both genomes.

The most puzzling aspect of this *trans*-nuclear effect is its sequence specificity. The limits of this specificity have not been fully explored, but minor sequence differences, such as a few mutations present in allelic sequences, or the deletion of 28-bp and 29-bp internal IESs in two 370-bp IESs, do not hinder recognition of homologous target sequences (Duharcourt *et al.*, 1998). How can a sequence introduced in one nucleus specifically affect the excision of the homologous sequence in another nucleus? Two different types of models have been proposed. In the first type, IES copies in the maternal macronucleus act by sequestering sequence-specific protein factors that are required for excision in the developing macronucleus. Because it is highly unlikely that each IES requires a different factor, it was proposed, on the basis of the *Tetrahymena* study, that different IESs use different subsets of a smaller number of factors, which could explain the weak effect of the M element on excision of R. However, this model still requires an unreasonably large number of different factors if, as suggested by the *Paramecium* study, a significant fraction of the ~50,000 IESs in the genome can inhibit their own excision with high specificity. Furthermore, such factors would have to bind the inner portion of IES sequences, since constructs carrying only the consensus-bearing extremities, or flanking macronuclear sequences, have no effect by themselves. This is difficult to reconcile with the higher efficiency of molecules containing longer flanking sequences, and with the rapid, apparently unconstrained, evolution of the inner part of IES sequences (Scott *et al.*, 1994a).

In the second type of model, sequence specificity is achieved by pairing interactions between homologous nucleic acids. However, the parental macronucleus, or its fragments, have never been observed to fuse with the developing macronucleus, and there is ample evidence that its DNA never gets incorporated into the new macronucleus. Indeed, the well-documented Mendelian segregation of alleles during autogamy of heterozygotes shows that only one of the two maternal alleles is present in the zygotic macronucleus; similarly, injected exogenous sequences, such as plasmid vectors, are maintained in the maternal macronucleus but cannot be recovered from sexual progeny. Thus, one has to assume that IES copies, presumably RNA molecules, are exported from the maternal macronucleus to the developing macronucleus, where they could pair with homologous sequences of the zygotic genome. The stronger effect of longer IES flanking sequences in the constructs may then be explained by differences in pairing efficiency. In support of this model, copies of the M and R elements present in the maternal macronuclear chromosomes were recently shown to be transcribed during conjugation in *Tetrahymena* (Chalker and Yao, 2001). This nongenic transcription appears to be determined by internal promoter sequences, since maternal macronuclear flanking sequences are not detectably transcribed when IESs are excised from the chromosomes. IES-driven transcription of chromosomal flanking

sequences, however, might somehow be responsible for the weak effect of M on the excision of R, as these elements are separated only by 2.7 kb. In *Paramecium,* transcription of maternal IESs has not been tested, but the maternal macronucleus is known to be transcriptionally active throughout the development of the new macronucleus. Maternal transcription level and RNA stability might certainly be some of the factors that determine the characteristic inhibition efficiency of each IES.

One version of the pairing model postulated that IESs are in fact constitutively excised from zygotic chromosomes during macronuclear development, leaving a gap that is repaired by polymerization with a homologous template originating from the maternal macronucleus. Since IES excision involves DNA cleavage at the ends of the element in *Tetrahymena* (Saveliev and Cox, 1995) as well as in *Paramecium* (M. Bétermier and A. Gratias, personal communication), IESs could be resynthesized in the new macronucleus by copying maternal IES-containing templates. It should be noted that such a mechanism is not likely to account for the formation of correct macronuclear junctions after IES excision, as these junctions were observed to form spontaneously after autogamy of cells that were completely devoid of junction templates: Indeed, IES^+ *Paramecium* cell lines can be made to return to normal IES excision when the copy number of the IES-containing chromosomal region is first reduced in the maternal macronucleus, through the experimental induction of macronuclear deletions (see Section III.C), to the point where it is no longer sufficient to cause IES retention on all copies of the zygotic macronucleus (Duharcourt *et al.,* 1995). Template-independent formation of macronuclear junctions is also consistent with the reported lack of maternal transcription of these junctions in *Tetrahymena* (Chalker and Yao, 2001). The remaining possibility, that templated repair is involved in cases of IES *retention,* was disproved by injecting IES sequences that were marked by the introduction of a restriction site or by allelic mutations: IESs retained in the new macronuclear genome were copied from the germline genome and not from maternal copies (Duharcourt *et al.,* 1995, 1998).

Thus, the hypothesized pairing interactions apparently cause IES retention by inhibiting endonucleolytic cleavage or recognition of IES sequences. Direct protection of zygotic IESs from the excision machinery by the pairing of maternal transcripts is unlikely, as one would then expect small internal IESs to be retained concomitantly with the larger ones in which they are located. Maternal transcripts may instead induce some epigenetic modification of germline IESs, which in turn would block excision. The modification of specific nucleotides or dinucleotides could affect the excision of each IES to a different extent, depending on the precise location of modified sites relative to IES boundaries. Such a highly localized modification could allow small internal IESs to be excised even when excision of the larger ones in which they reside is inhibited. N6-methyladenine is

known to occur in *Tetrahymena* and *Paramecium* (Cummings *et al.*, 1974; Gorovsky *et al.*, 1973; Karrer and VanNuland, 1998), but no role for this modification in the developmental regulation of genome rearrangements has yet been demonstrated.

Other possible mechanisms are suggested by the recent finding that the zygotic M and R IESs, as well as other *Tetrahymena* germline-limited elements, are transcribed at an early stage of macronuclear development, well before they are excised (Chalker and Yao, 2001). Transcription of the M element was shown to occur from both strands, giving rise to nonpolyadenylated transcripts that are heterogeneous in size, at least a fraction of which originate or terminate in flanking sequences. Brief treatment of cells with actinomycin D during early macronuclear development resulted in impaired IES excision, leading the authors to propose that transcription of zygotic IESs is necessary for their excision, either because it establishes a chromatin structure that allows access to the excision machinery, or because the transcripts themselves are used to guide the excision machinery to the IESs. In both cases, the presence of IES copies in the parental macronucleus could inhibit excision by mechanisms akin to homology-dependent gene silencing. Parental IES transcripts (which appear to be qualitatively different from zygotic transcripts, although they have not been fully characterized) could be responsible for the transcriptional silencing of zygotic IESs, which would reduce their accessibility; alternatively, parental transcripts could cause the degradation of zygotic transcripts by posttranscriptional mechanisms, which would affect the recognition of IESs to be excised.

Whatever the mechanism may be, the homology-dependent effect through which a maternal IES can inhibit its own excision in the zygotic macronucleus provides cells with a simple way to transmit a variety of alternative macronuclear versions of the genome to sexual progeny. For one of the *Paramecium* IESs studied (51G4404), the efficiency of the maternal effect is such that the presence of the IES on a given fraction of copies of the macronuclear genome will result in a *greater* fraction of IES-retaining copies in the macronucleus of the following sexual generation (Duharcourt *et al.*, 1995). The dynamics of the system thus imply that, over the course of many sexual generations in a cytoplasmic lineage, a stable equilibrium can be reached only for two alternative states, IES^- (100% excision) and IES^+ (0% excision). This is reminiscent of the stable maternal inheritance of mating types *O* and *E* in *P. tetraurelia*. The same fundamental asymetry is seen in both systems: IES excision and determination for *O* both appear to be default developmental pathways, while IES retention and determination for *E* both require a specific cytoplasmic signal produced by the maternal macronucleus. The hypothesis that mating type is determined by the regulated excision of an IES is further supported by the pleiotropic effects of the Mendelian mutation mtF^E, which impairs excision of IES 51G4404 regardless of the IES^- or IES^+ state of the maternal macronucleus, and similarly makes determination for *E* constitutive.

III. REGULATION OF ZYGOTIC GENOME AMPLIFICATION AND CHROMOSOME FRAGMENTATION BY HOMOLOGY-DEPENDENT MATERNAL EFFECTS

A. Maternal inheritance of alternative chromosome fragmentation patterns

The first evidence for an epigenetic regulation of the other type of developmental genome rearrangement, the fragmentation of germline chromosomes, came from the study of d48, a variant cell line of *P. tetraurelia*. Originally isolated in a screen for mutants unable to express the A surface antigen, the d48 cell line indeed showed this defect, but genetic analyses surprisingly indicated that it did not carry any germline mutation. Although the A expression defect was found to be due to the complete absence of the *A* gene in the macronucleus, the defect followed a cytoplasmic, rather than Mendelian, pattern of inheritance in crosses with the wild type (Epstein and Forney, 1984). Nuclear transplantation experiments directly confirmed that the d48 micronuclear genome is entirely wild type, and that vegetative micronuclei are not determined in any way for that character: the replacement of the d48 vegetative micronucleus with a wild-type micronucleus did not prevent maternal transmission of the *A* gene deletion to sexual progeny, and the d48 micronucleus, when transplanted into a wild-type cell, gave rise after autogamy to a new macronucleus which contained the *A* gene (Harumoto, 1986; Kobayashi and Koizumi, 1990).

In contrast, injection of wild-type vegetative macronucleoplasm into the d48 vegetative macronucleus was shown to rescue the *A* gene defect in the injected clone, as well as in its postautogamous progeny, thus causing a permanent reversion of d48 to wild type (Harumoto, 1986). The cytoplasm from wild-type cells, when transferred to d48 cells at an early stage of macronuclear development, also proved able to prevent deletion of the *A* gene, but only when it was taken from donor cells that were themselves undergoing autogamy (Koizumi and Kobayashi, 1989). In the absence of any reported evidence for a d48-determining cytoplasmic factor, these experiments collectively suggest that the *A* gene is deleted during development of the d48 macronucleus as a consequence of the lack of a cytoplasmic factor transiently produced by the wild-type, *A*-containing maternal macronucleus, which determines the maintenance of the gene in the new macronucleus.

In the wild type, the *A* gene is located near the end of a macronuclear chromosome; developmental chromosome fragmentation results in the addition of telomeres in one of three alternative regions located 8, 13, and 26 kb downstream of the gene, all three forms being represented in each macronucleus. Molecular characterization of the d48 macronuclear deletion revealed that the gene is lost as part of a larger terminal deletion that also removes all downstream sequences, the d48 telomere forming in a single region located at the 5′ end of the gene

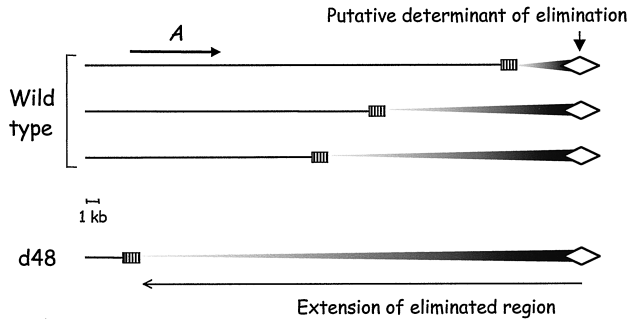

Figure 10.4. The macronuclear A-gene locus in the wild-type and d48 cell lines. The A-gene coding sequence is shown as an arrow. Hatched boxes represent the telomere addition regions. The open lozenge on the right symbolizes a putative germline sequence element determining elimination of this region during macronuclear development.

(Figure 10.4) (Forney and Blackburn, 1988). The d48 cell line thus provides evidence that alternative chromosome fragmentation patterns can be maternally inherited in genetically wild-type cells. However, it should be noted that the alternative pattern is not simply the result of chromosome breakage occurring upstream of the gene instead of at one of the wild-type downstream positions, as one would then expect the A gene to be present on a different macronuclear chromosome. Even if breakage occurred at both upstream and downstream positions, the gene would be present on 13- to 34-kb molecules, which should be long enough to be maintained at normal copy numbers in the macronucleus.

An alternative view is that chromosome fragmentation results from the imprecise elimination of a variable length of downstream sequences, which can be extended to include the A gene in d48. If elimination is determined by a heterochromatin-like structure of germline-limited sequences, the wild-type telomere addition regions could represent alternative boundaries of heterochromatin spreading from downstream sequences toward the A gene. In d48, heterochromatin would spontaneously spread further to include the gene, perhaps because of the attractive presence of four nearly identical 210-bp tandem repeats in the middle of its coding sequence. In support of the gene's intrinsic propensity to be deleted, the use of upstream probes has recently revealed that the wild-type macronucleus does in fact contain, in addition to the forms already described, a limited number of copies of a shorter chromosome form which lacks the gene, ending with telomeres in the d48 region (unpublished results). In this view, the maternal cytoplasmic factor that is necessary for amplification of the gene to wild-type copy numbers would act by *preventing* the spontaneous formation of heterochromatin over the gene.

The d48 alternative fragmentation pattern thus appears to result from a maternal effect that primarily controls the amplification or deletion of the A-gene

genomic region. Can such maternal effects be evidenced in other genomic regions? Scott *et al.* (1994b) have addressed this question by taking advantage of a mutant strain carrying a Mendelian deletion of the *B* gene, another surface antigen gene that is located close to the end of a macronuclear chromosome in the wild type. In this strain the *B* gene is completely absent from the micronuclei, and therefore is also missing in the macronucleus. The mutant micronuclei were replaced with a wild-type micronucleus, resulting in a cell line analogous to d48, which contained the *B* gene in its micronucleus but not in its macronucleus. When transplanted clones were allowed to undergo autogamy, the wild-type micronucleus gave rise to a new macronucleus in which the *B* gene was deleted. Like the d48 deletion, this macronuclear deletion was maternally transmitted to subsequent sexual generations. Because there is no reason to suspect that the macronucleus of the Mendelian mutant lacked anything else than the *B* gene, this experiment suggests that the *B* gene is amplified to a wild-type copy number in the developing macronucleus only if it is already present in the maternal macronucleus.

B. Homology-dependent rescue of maternally inherited macronuclear deletions

If amplification of the *A* gene in the developing macronucleus is determined by its presence in the maternal macronucleus, it can be assumed that the wild-type cytoplasmic factor that allows zygotic amplification is produced by the maternal *A*-gene copies themselves. Indeed, transformation of the d48 vegetative macronucleus with the cloned *A* gene, followed by induction of autogamy, was shown to restore *A*-gene amplification in the developing macronucleus of sexual progeny, thus resulting in a permanent rescue of the d48 defect (Figure 10.5) (Jessop-Murray *et al.,* 1991; Koizumi and Kobayashi, 1989; You *et al.,* 1991). However, the cytoplasmic factor cannot be the protein product of the gene, nor its full-length mRNA, as even truncated copies of the gene were able to rescue the d48 defect when present in the maternal macronucleus. Furthermore, clones transformed with the entire gene often express the A surface antigen throughout vegetative growth, whereas production of the rescuing cytoplasmic factor was shown to be restricted to the period of nuclear reorganization.

Two studies have attempted to identify specific regions of the *A* gene that are responsible for the maternal rescue effect by transforming the d48 macronucleus with different subfragments, and then testing the capacity of postautogamous progeny to express the *A* gene, which requires its presence in the new macronucleus (Kim *et al.,* 1994; You *et al.,* 1994). Rescuing activity appears to be spread over most of the ~8-kb coding sequence, as several nonoverlapping fragments showed the effect. In contrast, the G gene from *P. primaurelia,* which is 78% identical to *A* over the coding sequence, had no rescue activity. Quantitative measures of the rescue efficiency, based on the fraction of progeny cells that

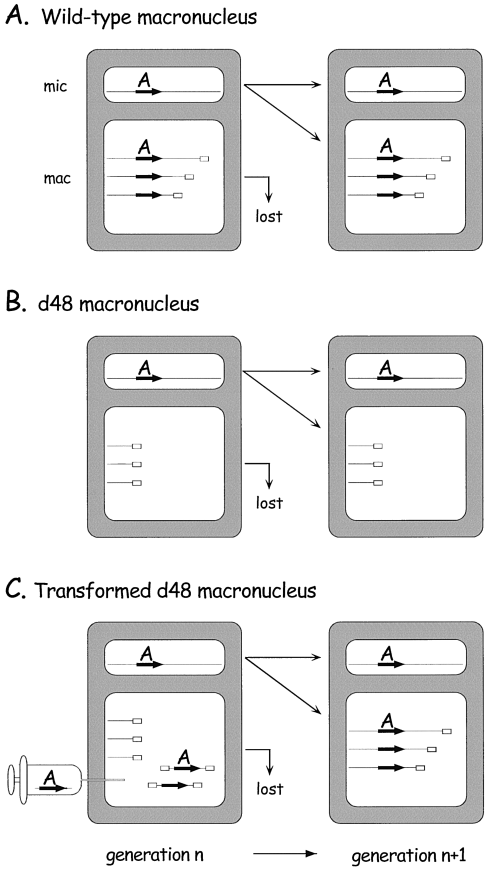

Figure 10.5. Developmental processing of the *A* gene in *P. tetraurelia* during autogamy. (A) Wild-type cell line. Open boxes represent telomere addition regions. (B) d48 cell line. Although the germline genome is wildtype, the macronuclear genome shows a terminal deletion of the *A* gene. (C) Transformation of the d48 macronucleus with an *A*-gene plasmid rescues the macronuclear deletion after autogamy. Adapted from Figure 1 in *Cell* **87,** Meyer, E., and Duharcourt, S., Epigenetic programming of developmental genome rearrangements in ciliates, pp. 9–12, Copyright © 1996, with permission from Elsevier Science.

could be induced to express the *A* gene, indicated that different fragments have different efficiencies, but conflicting results were obtained for some fragments. Interestingly, longer fragments were generally more efficient, and co-transformation with two different fragments resulted in a better rescue than each fragment alone. Constructs that contained the gene's promoter appeared to have a poor rescue efficiency. However, these measures should be taken with caution, as the ability of sexual progeny to express antigen A is not a direct measure of *A*-gene

amplification levels. Furthermore, the copy numbers of the different fragments in the transformed clones were not measured, and this was later shown to be a critical parameter in such maternal effects (see Section III.D).

The sequence specificity of the maternal rescue effect was examined using a genetically wild-type cell line that carried maternally inherited macronuclear deletions of both the *A* and *B* genes (Scott *et al.*, 1994b). These genes are members of the same multigene family and their coding sequences are 74% identical overall, although the central portion is much more divergent than distal portions. Transformation of the maternal macronucleus with the *A* gene restored amplification of the *A* gene in the developing macronucleus, but not that of the *B* gene; similarly, the *B* gene restored only its own amplification. On the other hand, the macronuclear deletion of the *A* gene could be rescued by transformation with a different allele of the *A* gene, showing 97% identity (Forney *et al.*, 1996). Thus, the maternal rescue of macronuclear deletions is a homology-dependent process which does not require any specific sequence within the genes, but requires a minimum level of sequence identity.

C. Homology-dependent induction of macronuclear deletions

Transformation of the maternal macronucleus can also have opposite consequences on the copy number of zygotic sequences in the new macronucleus. This opposite effect was first observed with the G surface antigen gene, which lies ~5 kb from the end of a macronuclear chromosome, in the sibling species *P. primaurelia*. When the wild-type macronucleus was transformed with a high copy number of the cloned G gene, autogamy of the transformed clones resulted in the complete deletion of the gene in the new macronucleus (Meyer, 1992). Like the d48 deletion, the induced deletion also removed downstream sequences up to the wild-type telomere, the new telomere addition region being located upstream of the gene and overlapping the 5′ end of the injected sequence (Figure 10.6). The induced deletion also showed maternal inheritance in conjugation with the wild type or after a second autogamy, although its heritability was weaker than that of the d48 deletion: many second-generation clones regained variable lengths of G-gene sequences on a fraction of macronuclear copies. Further work showed that similar terminal deletions can be obtained in sexual progeny by transforming the wild-type macronucleus with nonoverlapping subfragments of the G-gene region, indicating that the deletion effect does not depend on a particular sequence in that region (Meyer *et al.*, 1997). However, some differences were noted in the sizes of deletions induced by different fragments (see Figure 10.6).

The maternal deletion effect is not restricted to subtelomeric surface antigen genes, as it could be reproduced at two other macronuclear subtelomeric regions that did not contain such genes (unpublished results). Furthermore, transformation with an arbitrarily chosen fragment internal to a macronuclear

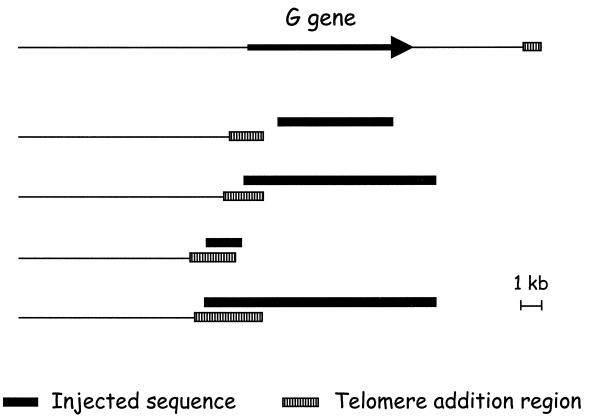

Figure 10.6. Induced terminal deletions in the G-gene region. The top line shows the single telomere addition region (hatched box) downstream of the G gene in the wild-type macronucleus. Black boxes below represent the fragments injected into the maternal macronucleus, and the hatched boxes the telomere addition regions observed in the sexual progeny of transformed clones. Adapted with permission from Figure 6 in E. Meyer and S. Duharcourt (1996), *J. Eukaryot. Microbiol.* **43**(6):453–461.

chromosome, ~80 kb away from the nearest telomere, was shown to induce *internal* deletions of the homologous sequence in sexual progeny, albeit only on a fraction of new macronuclear copies (Meyer *et al.*, 1997). A number of macronuclear molecules carrying these internal deletions were cloned and sequenced to study the deletion endpoints. Alignment with the full-length macronuclear sequence, which is never deleted in the wild type, revealed that deletions occurred between short (3–12 bp) direct repeats, one of which was left in the macronuclear sequence. Strikingly, these repeats were made almost exclusively of alternating Ts and As. Twenty-four sequenced deletion endpoints were clustered in a few hotspots, located on either side of the sequence that had been injected into the maternal macronucleus. These hotspots were the same in several caryonidal clones derived independently from transformed clones, although they were mostly outside the injected sequence and were found to coincide precisely with short segments showing the highest density of 5′-TA-3′ dinucleotides. These TA-rich short segments themselves coincided with intergenic regions, which are very short in the *Paramecium* genome, so that individual genes appear to be the smallest segments that can be deleted. These results show that the maternal deletion effect does not necessarily lead to chromosome fragmentation. However, maternally induced deletions could be facilitated when the injected sequence is close to a wild-type macronuclear telomere, i.e., close to "spontaneously" eliminated germline sequences, in which case the two regions programmed for elimination appear to be bridged, resulting in a single deletion event removing the whole region. It is not known

whether induced internal deletions would show the same maternal inheritance as terminal macronuclear deletions in subsequent sexual generations.

The surface antigen multigene family of *P. tetraurelia* was used to study different parameters that could affect the efficiency of the maternal deletion effect, such as the structure and copy number of the injected sequence and its level of sequence identity with the targeted zygotic sequence (O. Garnier, unpublished results). To this end, a ~10-kb fragment containing the *A* gene was injected at various copy numbers either as a circular or prelinearized plasmid, or as a linear fragment with *Paramecium* macronuclear telomeres at each end. By quantifying both the copy numbers of the injected *A* gene in the maternal macronucleus and the resulting *A* gene copy numbers in the macronucleus of postautogamous progeny, it was concluded that deletion efficiency is not strongly affected by the structure of injected sequences, but is highly dependent on their copy numbers. Under 20 cphg (~20,000 copies per macronucleus) in the maternal macronucleus, the injected sequence induced little or no reduction of the *A*-gene copy number in the zygotic macronucleus; above 40 cphg, all macronuclear copies of the *A* gene were deleted. The newly induced macronuclear deletion of the *A* gene showed the same stable maternal inheritance in further sexual generations as the original d48 deletion.

In the same experiments, zygotic amplification of paralogous genes *G*, *B*, and *C* was also examined in the sexual progeny of *A*-transformed clones. These genes respectively show 78%, 74%, and 55% of overall sequence identity with *A*, although identity levels can be locally higher in any pair. Like *A*, the *G* and *B* genes could be completely deleted with more than 40 cphg of the *A* gene in the maternal macronucleus, but in intermediate cases where the *A* gene was only partially deleted, they were less affected than *A*. Copy numbers of the more divergent *C* gene were not affected, even at much higher maternal *A*-gene copy numbers. Thus, the maternal deletion effect is a homology-dependent effect that is induced only at high copy numbers. The effect of the length of the injected sequence was not fully explored, but comparison with a previous analysis of the effects of short (213–851 bp) *G*-gene fragments (Duharcourt *et al.*, 1995) suggests that deletions are more efficiently induced by large fragments.

An unexpected link with the expression of the injected gene was also uncovered (O. Garnier, unpublished results). The quantitative analysis presented above used an allele of the *A* gene (A^{29}), which is rarely expressed in the transformed clones. Rare cases of A^{29}-transformed clones that did express the A antigen did not show the strong deletion effect that was expected from their A^{29} copy numbers. Similarly, the A^{51} allele, which is ~97% identical to A^{29} but is readily expressed after injection, consistently showed a much lower deletion efficiency. Constructs in which normal expression of the A^{51} allele was made impossible by the introduction of a frameshift in the coding sequence, or by the truncation of its 3′ end, had an increased deletion efficiency, similar to that of the unexpressed

A^{29} allele. Thus, the stable expression of the injected gene appears to be incompatible with the maternal deletion effect. However, inactivating the A^{51} allele by removing its promoter did not significantly increase its deletion efficiency. Intriguingly, another promoterless construct containing an internal fragment of the A^{51} coding sequence also had a reduced deletion efficiency.

D. Resolving the paradox: rescue vs induction of macronuclear deletions

While both the rescue effect and the deletion effect clearly show that maternal macronuclear sequences can influence the copy number of homologous sequences in the developing macronucleus, it is unclear why transformation promotes amplification in some experiments and induces deletion in others. How can these conflicting observations be reconciled? It should first be pointed out that possible outcomes of these experiments are limited by their very design: in the d48 cell line, the only effect of transformation that *can* be observed is the restoration of amplification, but in the wild type it is deletion. Thus, if the same A-gene fragment were capable of inducing both effects, the conclusion drawn would simply depend on which recipient cell line was used. Furthermore, in both sets of experiments some of the tested fragments were found to be more efficient than others, and the most efficient fragments may not be the same for both effects. Although the parameters that determine the efficiencies of both effects are not fully understood, it is interesting to note that promoter-containing fragments of the A^{51} allele generally performed poorly in the d48 rescue, whereas they were the most efficient in inducing deletions in the wild type. Methodological differences also probably play a role. For instance, when the whole A gene was used in rescue experiments, transformants were usually screened on the basis of A gene expression, which involves a convenient immunological test. However, this would screen out any high-copy-number transformant likely to show a strong deletion effect in sexual progeny, as the deletion effect was shown to be incompatible with the expression of the injected gene.

The idea that copy number can influence the type of effect to occur was tested by transforming the macronucleus of a d48-like strain with a wide range of copy numbers of the rarely expressed A^{29} allele (O. Garnier, unpublished results). Very low copy numbers (0.1 cphg) were sufficient to induce a significant rescue of zygotic A gene amplification, and wild-type amplification levels were obtained for maternal copy numbers between 0.6 and 10 cphg. However, further increasing the maternal copy number resulted in a sharp decrease of zygotic copy numbers, and the A gene was again completely deleted in the new macronucleus above 40 cphg in the maternal macronucleus. This experiment showed conclusively that both effects can be induced by injecting the same fragment in the same cell line, and that they are determined primarily by the copy number of the injected sequence.

E. Models

Like the maternal effect leading to the inhibition of the excision of zygotic IESs, both of the homology-dependent maternal effects that modulate the copy number of zygotic sequences are *trans*-nuclear effects. For this reason, attempts to explain the sequence specificity of copy number modulation encounter precisely those problems also encountered by attempts to explain the sequence specificity of the IES effect. Considering the large number of different DNA fragments and subfragments that were shown to induce homology-dependent effects, models involving the production or titration of sequence-specific protein factors by sequences injected in the maternal macronucleus, resulting in a specific effect in the developing macronucleus, are very unlikely. Here again, we are left with the only alternative that sequence information is conveyed by maternal nucleic acids, which, as argued in the case of the IES effect, are presumably RNA molecules. This would imply that almost any DNA fragment can be transcribed, but this is not unreasonable, as a study of the vegetative homology-dependent gene silencing phenomenon (Ruiz *et al.*, 1998) has shown that promoterless coding sequences can indeed be transcribed after injection into the macronucleus.

In both effects, RNA copies of the sequences injected into the maternal macronucleus could exert their effects by pairing with homologous zygotic sequences in the developing macronucleus. It remains to be explained how these pairing interactions could either promote amplification or induce the deletion of the targeted sequence. In the rescue effect, very low copy numbers of maternal *A* gene sequences are sufficient to allow *A*-gene amplification. It is tempting to believe that RNA copies of the injected sequences are functionally equivalent to the cytoplasmic factor required for zygotic amplification of the *A* gene in wild-type cells, which appears to be produced by maternal *A*-gene copies. The timing of production of the wild-type cytoplasmic factor, which is limited to sexual events, indicates that it is distinct from the normal *A*-gene mRNA, which is known to be continuously produced during vegetative growth in A-expressing cells. A specialized transcription system would also be implied by the fact that normal *A*-gene amplification occurs in wild-type cells expressing a different surface antigen, as the expression of different surface antigen genes is mutually exclusive, and regulated primarily at the transcriptional level. The rescuing activity of nonoverlapping subfragments of the *A* gene indicates that pairing of maternal transcripts over the whole gene is not necessary, although their additive effects suggest that this would further enhance *A*-gene amplification.

We have proposed that, in the absence of maternal copies of the *A* gene, a heterochromatin-like structure determined by downstream germline-limited sequences is able to spread upstream to include the *A* gene and ultimately result in *A*-gene deletion. Preliminary results indicate that in the d48 cell line, the *A* gene is first amplified for at least a few replication cycles during early macronuclear

development before being eliminated by an active mechanism (O. Garnier, unpublished results). The timing of this elimination coincides roughly with that of chromosome fragmentation at another genomic site (A. Le Mouël, personal communication). The pairing of maternal transcripts could block heterochromatin spreading, either directly or because it first induces some epigenetic modification such as DNA methylation. In contrast to the rescue effect, the deletion effect requires a large excess of maternal *A*-gene sequences. Could an excess of the maternal transcripts that would otherwise rescue a deletion result in opposite consequences? Although this cannot be excluded, another possibility is that an excess of maternal *A*-gene sequences leads to the production of qualitatively different RNA molecules, with opposite effects on zygotic sequences. For instance, double-stranded RNA molecules could be produced from head-to-head multimers of the injected sequences, which represent only a fraction of the total copy number. This would be consistent with the higher deletion efficiency of promoter-containing fragments.

The idea that qualitatively different deletion-inducing transcripts could be double-stranded molecules further suggests an alternative mechanism for the deletion effect. Instead of pairing directly with zygotic sequences, double-stranded RNA could result in deletions indirectly, by preventing rescuing transcripts (such as the wild-type cytoplasmic factor) from promoting amplification. In many other eukaryotes, double-stranded RNA has been shown to induce the specific degragation of single-stranded homologous transcripts, providing a simple explanation for the inactivation of rescuing transcripts. This model would explain why an excess of maternal *A*-gene sequences is functionally equivalent to their complete absence. Some support for this hypothesis comes from the study of homology-dependent gene silencing in *Paramecium* (Ruiz *et al.*, 1998). High-copy number transformation of the wild-type macronucleus with promoterless and terminatorless coding sequences indeed results in the specific disappearance of endogenous homologous transcripts during vegetative growth of the transformed clones. Vegetative silencing was further shown to be associated with the production of aberrantly sized transcripts from both strands of the injected sequence. Although the exact role of these aberrant transcripts remains to be determined, in at least one case the silencing mechanism was shown to be posttranscriptional (Galvani and Sperling, 2001), raising the possibility of an RNAi-like mechanism. A connection between the maternal deletion effect and the vegetative silencing phenomenon is suggested by the observation that expression of the injected *A* gene is incompatible with the deletion effect. Most of the deletion-inducing fragments of the *A* gene were indeed shown to silence the expression of the endogenous *A* gene during vegetative growth of the transformed clones, before autogamy is induced (O. Garnier, unpublished results). These observations support the idea that similar transcripts may be responsible for both effects.

IV. CONCLUSIONS

Studies of the developmentally regulated rearrangements of the genome in ciliates have generally been based on the assumption that rearrangement patterns are determined entirely by the sequence of the germline genome. Classical paradigms of molecular biology have inspired the view that the reproducibility of these patterns can be explained by *cis*-acting sequence elements directing the action of *trans*-acting protein factors. While a few such *cis*-acting elements have indeed been identified, the comparison of germline micronuclear sequences with their macronuclear rearranged counterparts has not been very successful in explaining the reproducibility of IES excision in many species, or of chromosome fragmentation in *Paramecium*. We have reviewed the available evidence for an epigenetic programming of rearrangements, which provides an alternative type of explanation for their reproducibility: genetic analyses, as well as macronuclear transformation experiments, reveal that homology-dependent maternal effects play an important role in specifying rearrangement patterns. The mechanistic models discussed for each of these effects are necessarily very speculative, as nothing is known of their biochemistry. The most robust conclusion is probably that the sequence specificity of these *trans*-nuclear effects cannot be explained by the production or titration of sequence-specific protein factors, and therefore implies a direct comparison of homologous nucleic acids through pairing interactions. As in other cases of *trans*-nuclear or *trans*-cellular homology-dependent effects (Grishok *et al.*, 2000; Strauss, 1999; van West *et al.*, 1999; other chapters in this volume), the cross-talk between different genomes is best explained by RNA molecules moving in and out of nuclei to convey sequence information.

For convenience, maternal homology-dependent effects were here classified according to the type of rearrangement they affect, IES excision or chromosome fragmentation. However, in *Paramecium*, chromosome fragmentation appears to result from the elimination of specific germline sequences, in a process that differs from IES excision only by the larger size of the deleted segments, the variability of deletion endpoints, and the frequent lack of rejoining of flanking sequences. In this respect, it is interesting to note that the maternal effect leading to inhibition of IES excision is formally analogous to the maternal effect resulting in the amplification of genomic regions located close to macronuclear telomeres, such as the *A*-gene region. In both effects, the presence of normal copy numbers of these sequences in the maternal macronucleus is sufficient to determine their maintenance in the macronuclear genome of sexual progeny. These effects may therefore draw on similar mechanisms. There is clear evidence that a cytoplasmic factor produced by maternal sequences actively promotes *A*-gene maintenance in the developing macronucleus; such a factor is also likely to mediate IES retention. In both cases, it was hypothesized that maternal transcripts

somehow protect homologous sequences of the zygotic genome from deletion, even though the mechanisms through which these sequences would otherwise be deleted may be different.

Another interesting connection was made between the maternal deletion effect and the phenomenon of homology-dependent gene silencing (Ruiz *et al.,* 1998). Both effects can be induced by transformation of the macronucleus with high copy numbers of a nonfunctional gene, which results in the silencing of homologous endogenous genes during vegetative growth of the transformed clones, and also in the deletion of these genes after autogamy, during the development of a new macronucleus. Both effects can be observed with many different genes, and the requirements of copy number and sequence similarity with the targeted genes appear to be similar. Both effects are likely to be RNA-mediated and may therefore rely on similar mediators. Interestingly, the study of homology-dependent gene silencing in many other systems has led to the idea that these effects evolved as a mechanism of defense against transposable elements (Matzke *et al.,* 2000; Plasterk and Ketting, 2000). Such mechanisms theoretically provide cells with a simple way to silence any transposable element on the sole basis of its copy number in the genome, without prior knowledge of its sequence. Homology-dependent mechanisms indeed are responsible for the silencing of retrotransposons in *Drosophila* (Jensen *et al.,* 1999; Malinsky *et al.,* 2001), and perhaps also in mammals (Whitelaw and Martin, 2001). The transposon connection is certainly relevant in ciliates; it has been proposed that the developmental system of genome-wide rearrangements is the result of a co-evolution process which allows the host genome to be purged of all transposable elements before it is expressed (Klobutcher and Herrick, 1997). If any sequence can be deleted in the developing macronucleus in response to an excess copy number in the maternal macronucleus, the developmental elimination of transposable elements may similarly be determined by homology-dependent effects, on the basis of their high copy numbers in the germline genome itself.

Whatever its mechanisms and evolutionary origins, the developmental remodeling of the genome appears to involve a comparison of the zygotic genome to be rearranged with the maternal macronuclear genome, which allows alternative rearrangement patterns to be transmitted to sexual progeny through the cytoplasm, i.e., independently from the germline genome. This system could account for many of the long-known cases of non-Mendelian inheritance. In particular, it suggests an attractive model of mating-type determination and inheritance in *P. tetraurelia,* which illustrates its potential usefulness in maintaining phenotypic polymorphism within populations of genetically identical cells. Because changes in rearrangement patterns occur much more frequently than Mendelian mutations, the maternal inheritance of alternative macronuclear editions may also help cell populations to adapt to a rapidly changing environment without having to alter their germline genome, which can thus be kept optimized for the long term.

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