Evolution of nuclear dualism in ciliates: a reanalysis in light of recent molecular data

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Ciliates are defined by the presence of dimorphic nuclei. Following conjugation, both the functional macronucleus (MAC) and the transcriptionally inactive germline micronucleus (MIC) develop from a zygotic nucleus. As the MAC develops, germline chromosomes are processed by excision of internal sequences, fragmentation and amplification of the remaining chromosomes. The extent of processing varies among lineages and, in all but one class of ciliates, the resulting MACs divide by an unusual process termed 'amitosis'. Research on these chromosomal rearrangements, largely from studies of only a handful of taxa from two of the nine classes of ciliates, has failed to find evidence of homologous processing among ciliate lineages. This observation, coupled with the structural diversity of MAC genomes among ciliates, led to the hypothesis of multiple origins of at least two MAC properties: (1) the ability to divide and (2) the mechanisms underlying chromosomal processing. Applying this logic to a more inclusive analysis of ciliate lineages, where an even greater diversity of MAC structure is observed, increases the potential number of origins of these MAC characteristics. Here, it is proposed that a single origin of a relatively plastic mechanism underlying MAC development better explains the observed diversity in MAC structure and processing among ciliates. Such a mechanism is suggested by the demonstration of epigenetic effects during MAC development in *Paramecium* and *Tetrahymena*.

Keywords: Ciliophora, genome evolution, chromosomal rearrangements, origin of macronucleus, epigenetic effects

INTRODUCTION

Ciliates are a diverse, monophyletic assemblage of microbial eukaryotes defined by the presence of cilia in at least one phase of their life cycle and by the division of nuclear function into two types of nuclei – the 'germline' micronucleus (MIC) and the 'somatic' macronucleus (MAC) (Hausmann & Bradbury, 1996; Prescott, 1994; Raikov, 1996). During conjugation, meiotic products of the MIC are exchanged between cells and fuse to form a zygotic nucleus (Fig. 1). This zygotic nucleus then divides mitotically and daughter nuclei either differentiate into MACs or remain unprocessed as MICs. During cell division, which occurs asexually in ciliates, MICs divide by mitosis while the MACs of all but one class of ciliates divide by an unusual form of nuclear division termed 'amitosis'.

Neither condensed chromosomes nor mitotic spindles are seen during MAC division. Instead, chromatin moves in an atypical manner along microtubules located either within or outside the MAC (Raikov, 1996). MAC division is absent from the class Karyorelictea, where a new MAC must differentiate from the MIC following every cell division (Raikov, 1996).

The development of the MAC occurs through chromosomal rearrangements, including fragmentation, elimination of internal sequences and amplification of the processed chromosomes. To date, most studies of the molecular basis of nuclear dimorphism have focused on five genera representing two of the nine ciliate classes: *Tetrahymena* and *Paramecium* (class Oligohymenophorea) and *Oxytricha*, *Euplotes* and *Stylonychia* (class Spirotrichea). Relatively limited chromosomal processing marks the first two genera, while the genomes of spirotrichs are extensively fragmented to create MACs with hundreds to thousands of copies of 'gene-sized chromosomes' (defined here as

Abbreviations: IES, internally excised sequences; MAC, macronucleus; MIC, micronucleus; Div⁺ MAC, macronucleus capable of dividing.

Fig. 1. Generalized ciliate life cycle. (1) A ciliate with its MIC (black circle) and MAC (black oval). (2) Two ciliates of different mating types preparing for conjugation – the MICs undergo meiosis. (3) Conjugation: haploid MICs are exchanged generating (4) ciliates with genetically novel zygotic nuclei (grey circles). (5) The zygotic nucleus divides and one daughter nucleus begins to differentiate into a MAC; the old MAC begins to fragment. (6) A new MAC has differentiated and the old MAC degrades. (7, 8) The ciliate divides asexually (e.g. Coyne *et al.*, 1996; Prescott, 1994).

Fig. 2. The hypothesis of Orias (1991a, b) redrawn from Herrick (1994). Ciliates are shown in bold. The dashed line leading to foraminifera indicates the speculative nature of this portion of the topology. Black circles, MAC differentiation; black triangles, gene-size MAC 'chromosomes'; black rectangles, origin of Div⁺ MAC.

DNA molecules averaging 2–4 kb in length with telomeres on either end; Prescott, 1994; Coyne *et al.*, 1996).

While common features of chromosomal rearrangements do exist in all ciliates, differences in MAC development and MAC structures among a limited sample of taxa led to the speculation that components of this system have evolved independently multiple times (Fig. 2; Herrick, 1994; Orias, 1991a). However, recent data indicating an even greater diversity among MACs, reviewed below, challenge the validity of this

model. Combining these data with observations on a potential epigenetic effect during MAC development provides a new model that requires only a single origin of a relatively plastic mechanism of chromosomal rearrangements to explain the variation in MAC structure observed among ciliates.

CURRENT HYPOTHESIS AND RECENT DATA

Macronuclear division

The evolution of MACs capable of dividing (Div⁺ MACs) remains unclear as there are two equally parsimonious ways to reconstruct the history of this characteristic of ciliates. As described above, Div⁺ MACs are found in all classes of ciliates except the Karyorelictea. Where Div⁺ MACs occur, the unconventional process of amitosis is required to divide the polyploid, processed chromosomes of MACs. While mitosis in the micronucleus, the more 'typical' eukaryotic nucleus, occurs through intranuclear orthomitosis (spindle fibres arise at opposite poles within MICs; Raikov, 1982), Div⁺ MACs divide by amitosis with microtubules either outside the MAC (in the class Heterotrichea) or inside the MAC (in all other ciliates, subphylum Intramacronucleata; Lynn, 1996).

According to one school of thought, Div[−] MACs represent the ancestral state for ciliates. Raikov (1976, 1982) argued that the nearly diploid, Div[−] MACs of karyorelictids represent an ancestral state that developed early in the evolution of nuclear dimorphism of ciliates. Similarly, Orias reasoned that (a) nuclear dimorphism in ciliates is homologous to that of the heterokaryotic foraminifera, and (b) the inability of the somatic nucleus of both the foraminifera and the karyorelictids to divide is a shared ancestral character (Orias, 1991a, b). Orias (1991a, b) and later Herrick (1994; Fig. 2) further argued that the variability in

Fig. 3. Evolution of nuclear dualism in ciliates. Ciliates are shown in bold. Phylogeny based on bootstrap analysis of small-subunit rRNA sequences in PAUP* 4.0b4a (Swofford, 1999) with likelihood parameters estimated by the hierarchical ratio tests in MODELTEST v. 3.0 (Posada & Crandall, 1998). Sequences were aligned in CLUSTAL W (DNASTAR, Madison, WI, USA) with a gap penalty of 30 and a gap length penalty of 5. Gaps and ambiguously aligned regions were removed for the analysis. The bootstrap values above the lines are based on 1000 replicates performed using a heuristic search. Sequences of *Chilodonella uncinata* and *Heliophrya erhardi*, *Euplotes crassus*, *Metopus palaeformis* and *Nyctotherus ovalis* represent a consensus of four to five clones. Outgroups are the dinoflagellate *Gymnodinium mikimotoi* and apicomplexan *Cryptosporidium parvum*. Black circles, parentally mediated MAC differentiation; black triangles, gene-size MAC 'chromosomes'; black rectangles, origin of Div⁺ MAC; white rectangles, loss of Div⁺ MAC; H₁, hypothesis 1: single origin followed by loss; H₂, hypothesis 2: two origins.

MAC structure indicates at least three independent origins of Div⁺ MACs in ciliates: one for the relatively unprocessed oligohymenophoran MAC, one for the dramatically processed spirotrich MAC and a third for the heterotrichs.

Since the models put forth by Orias (1991a, b) and elaborated by Herrick (1994), our understanding of the origin of dimorphic nuclei in ciliates has been altered by the taxonomic placement of foraminifera with respect to ciliates. Both recent analyses of ultrastructure (Taylor, 1999) and molecular data (Darling *et al.*, 1996; Pawlowski, 1996) do not support the sister status of ciliates and foraminifera; instead, these analyses place foraminifera towards the base of the eukaryotic tree. Hence, the origin of heterokaryotic nuclei in ciliates appears to be independent of that in foraminifera.

Based on analyses of small-subunit rRNA genealogies, Hammerschmidt *et al.* (1996) discussed the two equally parsimonious mappings for Div⁺ MACs: (1) an early

origin of MAC Div⁺ followed by a loss in the Karyorelictea or (2) two independent origins of Div⁺ MACs, one in the heterotrichs and one in all of the other ciliates (see Fig. 3). The authors favour the former hypothesis and argue that the extranuclear microtubules involved in amitosis in the Heterotrichea represents a derived state (Hammerschmidt *et al.*, 1996). Implicit in this argument is the assumption that having intranuclear microtubules is the ancestral state of all nuclear division in ciliates as this placement of microtubules is present in ciliate MICs and the MACs of ciliates in the subphylum Intramacronucleata. However, it is still possible that Div⁺ MACs evolved from Div⁻ MACs twice, once with intranuclear microtubules and once with extranuclear microtubules.

Chromosomal rearrangements

As with MAC division, the apparent lack of conservation of sequences involved in chromosomal rearrangements within and between the oligohymeno-

Table 1. Summary of MAC processing in four genera of ciliates

IES excision, fidelity of removal of IES (internally excised sequences); IES structure, nature of repeats at ends of IES; CB fidelity, location of CB (chromosome breakage) points; CB 'signal', nature of sequence at CB. Based on information given by Coyne *et al.* (1996) and Klobutcher & Herrick (1997).

Genus	IES excision	IES structure	CB fidelity	CB 'signal'
<i>Tetrahymena</i>	Imprecise	Variable	Precise	Conserved sequence
<i>Paramecium</i>	Precise	TAs	Variable	Variable
<i>Oxytricha</i>	Precise	Variable	Variable	Variable
<i>Euplotes</i>	Precise	TAs and others	Precise	Conserved sequence

phorans and spirotrichs studied to date has led to the hypothesis of multiple independent origins of chromosomal rearrangements within ciliates (Table 1; Coyne *et al.*, 1996; Klobutcher & Herrick, 1997). As fragmentation is coupled to *de novo* synthesis of telomeres on MAC chromosomes (Coyne *et al.*, 1996; Prescott, 1994), comparisons of the location of MAC telomeres with the corresponding sequences in the MIC allows identification of fragmentation sites (chromosomal breakage points) that are the site of telomere addition in MAC chromosomes. A conserved, polypurine rich chromosomal breakage sequence (cbs) has been identified in *Tetrahymena thermophila* (class Oligohymenophorea) and its close relatives (Coyne & Yao, 1996; Yao *et al.*, 1987). However, no evidence of a conserved sequence has been found in *Paramecium* (class Oligohymenophorea), where telomere addition can occur over a 200–800 bp region in developing MACs (Steele *et al.*, 1994). Within the class Spirotrichea there is also variation in chromosomal breakage points. As with *Paramecium*, there is no evidence of a conserved sequence at chromosomal breakage points in *Oxytricha* (class Spirotrichea) where again there is some heterogeneity in the breakage sites (Herrick *et al.*, 1987). In contrast, breakage is very precise in *Euplotes crassus* (class Spirotrichea) where a conserved 14 bp sequence is associated with the telomere addition sites (Baird & Klobutcher, 1989; Klobutcher *et al.*, 1998). However, this sequence is not similar to the conserved cbs of *Tetrahymena*.

As with chromosomal breakage, analysis of the taxa sampled to date indicates that a diversity of mechanisms may be involved in the excision of internally excised sequences (IESs) within and between classes of ciliates. IESs are sequences of variable length always bounded by repeats that are removed during MAC development (Coyne *et al.*, 1996; Klobutcher & Herrick, 1997). Based on sequence comparisons, IESs can be classified either as transposable elements or as 'short' IESs (reviewed by Klobutcher & Herrick, 1997). Several classes of transposable elements that are removed during macronuclear development have been found in the spirotrichs *Oxytricha* and *Euplotes* (Klobutcher & Herrick, 1997). Transposable elements have not been found in either *Paramecium* or *Tetrahymena* (Klobutcher & Herrick, 1997).

A second group of IESs, short non-transposable elements, has been found in all taxa examined to date, but vary in length and sequence both within and between classes (Klobutcher & Herrick, 1997). 'Short' IESs are found within coding regions in both spirotrichs and *Paramecium* and are excised precisely. In *Euplotes* and *Paramecium*, 'short' IESs are flanked by 'TA' direct repeats and contain a conserved, eight nucleotide inverted repeat sequence (Klobutcher & Herrick, 1995). (This same sequence is also found in the Tec transposons of *E. crassus*, leading to the hypothesis that these, and perhaps all, IESs are excised by a transposon-like mechanism; Klobutcher & Herrick, 1997.) In contrast, the direct repeats flanking 'short' IESs in *Oxytricha* are variable between elements and no evidence of conserved sequences have been found to date (Ribas-Aparicio *et al.*, 1987). The 'short' IESs in *Tetrahymena* are quite different as no IESs have been found interrupting coding sequences of *T. thermophila* and excision in this genus is imprecise (reviewed by Coyne *et al.*, 1996). Also, although conserved sequences have been identified that are essential for excision of individual elements in *Tetrahymena* (Godiska & Yao, 1990), no conserved features have been identified between IESs. The current interpretation for the lack of conserved features among ciliates is that the mechanisms underlying MAC differentiation, like the ability of MACs to divide, has arisen independently multiple times (Klobutcher & Herrick, 1997).

Macronuclear structure

Another recent development in our understanding of the evolution of chromosomal rearrangements comes from data on the widespread distribution of extensive fragmentation. Extensive fragmentation has been best characterized from members of the classes Spirotrichea (e.g. Coyne *et al.*, 1996; Klobutcher & Herrick, 1997) and two closely related genera in the class Phyllopharyngea (*Trithigmastoma* and *Chilodonella*; Méténier & Hufschmid, 1988). Recent data indicate that additional orders of phyllopharyngeans in the subclass Suctorina, as well as the genera *Metopus* and *Nyctotherus*, of disputed taxonomic position, also extensively fragment their genomes to create gene-sized chromosomes in their MACs (Riley & Katz,

2001). Southern hybridization of total DNA from several genera of phyllopharyngeans and from both *Nyctotherus* and *Metopus* reveal that the α -tubulin gene of these taxa resides on 'chromosomes' less than 2 kb in length. According to the logic presented by Orias (1991a, b) and Herrick (1994), the non-sister status of the phyllopharyngeans, spirotrichs and disputed orders implies additional origins of Div⁺ MACs in ciliates, one for each transition from less fragmented genomes to extensively fragmented genomes.

A NEW MODEL

By combining our knowledge of ciliate systematics with a growing understanding of MAC development, it is possible to construct a new model for the evolution of chromosomal processing in ciliates (Fig. 3). Under this model, a single origin of nuclear dimorphism in ciliates evolved along with a relatively plastic mechanism that has allowed for the evolution of diverse MACs. Until recently, the signals for chromosomal rearrangements were believed to be located predominantly in the zygotic nucleus, while the parental MAC, which eventually fragments and disappears, was thought to play a minimal role. Data on the role of the parental MAC in directing chromosomal rearrangements in the newly developing MACs of *Paramecium* and *Tetrahymena* (Duharcourt *et al.*, 1995, 1998; Meyer *et al.*, 1997; Meyer & Duharcourt, 1996) provide a relatively plastic mechanism of chromosomal rearrangements that may have allowed for the observed diversity in chromosomal processing among ciliate MACs.

A growing number of experiments indicate that a *trans*-acting factor is specifically involved in regulating rearrangements during MAC development (Meyer & Duharcourt, 1996). These experiments involve tracing the effects of microinjection of sequences into the parental MAC on the development of homologous sequences in the new MAC following conjugation. For example, injecting a high-copy number of surface A antigen genes into the parental MAC of *Paramecium tetraurelia* can rescue a mutant phenotype caused by the deletion of a portion of this gene from the parental MAC (Jessop-Murray *et al.*, 1991). Injection of multiple copies of the surface G antigen gene (normally found at the end of a MAC chromosome) into the parental MAC of *Paramecium primaurelia* can cause this segment of DNA to be deleted in the newly developing MAC (Meyer, 1992). Finally, injection of IESs into the parental MACs of both *P. tetraurelia* (Duharcourt *et al.*, 1998) and *T. thermophila* (Chalker & Yao, 1996) specifically prevents the excision of matching sequences from the developing MAC. Although the nature of the mechanisms underlying these epigenetic phenomena is still debated, the data are consistent with a *trans*-acting factor operating between the parental and newly developing MAC.

Under the proposed model, the evolution of nuclear dualism in ciliates was associated with, or evolved

because of, the evolution of a mechanism whereby the parental MAC directly affects the development of a new MAC. Such an epigenetic mechanism may have allowed for the evolution of diverse MAC genome structure, such as the multiple origins of extensive fragmentation, observed in ciliates. Moreover, the presence of an epigenetic mechanism could also allow coevolution between template and target sequences to occur independently in multiple lineages. This might result in the observed variation in *cis*-acting regulatory sequences required for IES excision and chromosomal breakage in different ciliate lineages. The proposed model provides a more plausible explanation for the dramatic variation we observe among ciliate MACs. Instead of having to argue for independent origins of MAC division and extensive fragmentation and mechanisms of chromosomal rearrangements, we can now envision a single origin of a relatively plastic, epigenetic mechanism that allowed for the dramatic variation observed among MACs.

Clearly, more data, particularly on the nature and distribution of an epigenetic mechanism, are needed to test the proposed model. Do *trans*-acting factors from the parental MAC affect the newly developing MACs of all ciliate classes or just of the oligohymenophorans *Paramecium* and *Tetrahymena*? Development of microinjection systems in other ciliates will be necessary to answer this question. Even if the parental MAC turns out to have a regulatory role during MAC development in all ciliates, we need additional comparative data on the mechanisms of chromosomal processing (i.e. IES excision and chromosomal breakage sequences) to determine if the mechanisms are homologous, or have components that are homologous, among ciliate lineages. Finally, the number of origins of Div⁺ MACs remains a mystery as we cannot distinguish between a single origin of Div⁺ MACs in the ancestor of all extant ciliates followed by a loss in the karyorelictids from two independent origins of Div⁺ MACs, one in the heterotrichs and one in the subphylum Intramacronucleata (Fig. 3).

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