Autonomously Replicating Macronuclear DNA Pieces Are the Physical Basis of Genetic Coassortment Groups in *Tetrahymena thermophila*

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ABSTRACT

The macronucleus of the ciliate *Tetrahymena thermophila* contains a fragmented somatic genome consisting of several hundred identifiable chromosome pieces. These pieces are generated by site-specific fragmentation of the germline chromosomes and most of them are represented at an average of 45 copies per macronucleus. In the course of successive divisions of an initially heterozygous macronucleus, the random distribution of alleles of loci carried on these copies eventually generates macronuclei that are pure for one allele or the other. This phenomenon is called phenotypic assortment. We have previously reported the existence of loci that assort together (coassort) and hypothesized that these loci reside on the same macronuclear piece. The work reported here provides new, rigorous genetic support for the hypothesis that macronuclear autonomously replicating chromosome pieces are the physical basis of coassortment groups. Thus, coassortment allows the mapping of the somatic genome by purely genetic means. The data also strongly suggest that the random distribution of alleles in the *Tetrahymena* macronucleus is due to the random distribution of the MAC chromosome pieces that carry them.

Heritably reconfigured somatic genomes are rare but have wide phylogenetic distribution among eukaryotes. Such rearrangements include chromosome fragmentation (breakage of a chromosome into multiple pieces), chromosome elimination (loss of entire germline chromosomes), and chromatin diminution (loss of DNA through internal deletion; Tobler 1986). Somatic chromosome fragmentation in the parasitic nematode Ascaris (Muller et al. 1996), discovered by Boveri in the last century, provided graphic evidence for Weissmann’s germ-line theory. Fragmentation of germline-derived chromosomes and chromatin diminution occur in every ciliated protozoan so far investigated (Prescott 1994).

Ciliated protozoa are unicellular eukaryotes that maintain two related and differentiated genomes within a common cytoplasm: the micronucleus (MIC) and the macronucleus (MAC). The micronucleus is diploid, lacks gene expression, and is the germline of the cell. The macronucleus contains the somatic genome, which is fragmented, polyploid, and actively expressed. Site-specific chromosome fragmentation and amplification occur during conjugation, when a mitotic sister of the micronucleus differentiates into a new macronucleus (Coyne et al. 1996). In the ciliate *Tetrahymena thermophila*, the MAC consists of 200–300 identifiable autonomously replicating pieces (ARPs, also known as MAC chromosomes; Altschuler and Yao 1985; Conover and Brunk 1986), derived from the five pairs of germline-derived chromosomes. With the exception of the 9000-copy rDNA macronuclear piece, the ploidy of the bulk MAC DNA is estimated at an average of 45 copies of each ARP per G1 MAC. This estimate is based on the kinetics of phenotypic assortment (Allen and Nanney 1958; Orias and Flacks 1975; Doerder et al. 1992 and see below) and is supported by measurements of amount and kinetic complexity of MIC and MAC DNA (Woodard et al. 1972; Yao and Gorovsky 1974).

Attempts to show any cellular apparatus capable of ensuring the regular distribution of daughter ARP copies, e.g., kinetochores, have failed (Davidson and LaFountain 1975). Moreover, early genetic work in Tetrahymena showed that when cells with a MAC heterozygous at a given locus undergo vegetative multiplication, subclones that irreversibly express the phenotype associated with homozygotes for either of the alternative alleles arise. This phenomenon, called phenotypic assortment, is well explained by a model of random distribution of allele copies (Allen and Nanney 1958; Orias...
We have recently identified an extensive collection of DNA polymorphisms between inbred strains B and C3 of *T. thermophila* using the randomly amplified polymorphic DNA (RAPD) method (Williams et al. 1990). The mapping of these RAPDs and the identification of meiotic linkage groups in the MIC (Orias 1997) allowed us to study the assortment behavior of many loci in the MAC of double heterozygotes. This led to the discovery of pairs of loci, closely linked in the micronucleus, that show a highly correlated pattern of macronuclear assortment. In other words, assortants with parental allele combinations at both loci are much more frequent than those of recombinant type (Longcor et al. 1996). This phenomenon was called coassortment, and a set of loci that coassort with one another in all combinations defines a coassortment group. We next sought to determine the physical basis for a coassortment group. Labeled DNA probes from two loci (1PM 8 and 1KF 2) in the PM 8 coassortment group were shown to hybridize to MAC ARPs of identical size in inbred strain B cells. With both probes, a strong hybridization signal was observed at 1.2 Mb and a fainter signal at 1.0Mb. In contrast, DNA probe from a neighboring locus (1KN3), which assorted independently of the other two, hybridized to an ARP with a different size (1.0 Mb) and restriction pattern. These observations led us to the hypothesis that MAC ARPs are the physical basis of coassortment groups.

We have more recently discovered a size polymorphism between inbred strains B and C3 in the PM 8 ARP, reported in this article, and we (Wickert et al. 2000) have identified two more loci, 1EO3R and 1J09, that coassort with 1PM 8 and 1KF 2 (Figure 1). These advances have allowed us to investigate the relationship between the assortment of the ARP size polymorphism and the assortment of the loci believed to lie on it. In this article, we provide experimental evidence for the random distribution of ARP copies during vegetative multiplication and decisive genetic evidence that the 1.2 Mb ARP is the physical correlate of the PM 8 coassortment group. Our results strongly support the hypothesis that MAC ARPs are the physical basis of coassortment groups in Tetrahymena. These findings provide a firmer foundation for mapping the fragmented somatic genome of Tetrahymena by purely genetic means. The coassortment map of the left arm of chromosome 1 (Wickert et al. 2000) strongly supports the idea that coassortment groups indeed provide a fundamental genetic view of the Tetrahymena MAC genome.

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inbred strain background</th>
<th>MIC genotype</th>
<th>MAC phenotype</th>
<th>MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB210</td>
<td>B</td>
<td>gal1-1/gal1-1</td>
<td>2dg-s</td>
<td>VI</td>
</tr>
<tr>
<td>SB1969</td>
<td>B</td>
<td>chx1-1/chx1-1</td>
<td>cy-s</td>
<td>II</td>
</tr>
<tr>
<td>SB3546</td>
<td>C3</td>
<td>pm1-1/pm1-1</td>
<td>pm-s</td>
<td>VI</td>
</tr>
<tr>
<td>SB3539</td>
<td>C3</td>
<td>chx1-1/chx1-1</td>
<td>cy-s</td>
<td>I</td>
</tr>
</tbody>
</table>

s, sensitivity; MT, mating type. CH X1, GAL1, and PM R1, previously known as ChxA, galA, and Pmr (Allen et al. 1998), respectively, are loci defined by mutations that confer resistance to cycloheximide (cy), 2-deoxy-d-galactose (2dg), and paromomycin (pm), respectively (Bruns and Cassidy-Hanley 1993). The wild-type alleles confer sensitivity. All of these strains are heterokaryons; i.e., their MIC and MAC differ genetically.

### Materials and Methods

**Strains, culture, and crosses:** Routine methods used in our lab for *Tetrahymena* storage, culture, and genetic crosses have been described (Orias and Bruns 1975; Hamilton and Orias 1999; Orias et al. 1999). *Tetrahymena* strains used in crosses first reported in this article are shown in Table 1. All the DNA polymorphisms used here are RAPDs (Williams et al. 1990) between inbred strains B and C3 (Brickner et al. 1996). Genetic methods for working with RAPD polymorphisms in *Tetrahymena* have been described (Lynch et al. 1995; Brickner et al. 1996; Longcor et al. 1996). Tetrahymena RAPD loci have a leading “1” to indicate their identification in this laboratory. The names of RAPDs for which the polymorphic band is templated by B DNA end in R, while absence of a final R indicates one for which the polymorphic band is templated by B DNA (Allen et al. 1998). ARPs and coassortment groups (PM 8 in this work) are named after one RAPD in the group, omitting its leading 1.

Two panels of B/C3 terminal assortants were used. One panel (cell lines SB1805–1840) consists of 36 terminal assortants described in Longcor et al. (1996), passed further so as to reach at least 500 fissions. The other panel (cell lines SB4210–4221) consists of 12 additional assortants obtained by crossing strains SB1969 and SB3546 (see Table 1) and passing progeny lines for at least 350 fissions. Given the age of these lines, there is at least 95% probability that assortment will be complete at any given locus (Deerdore et al. 1975). “Young” progeny are mixtures of progeny derived from more than 1000 conjugating pairs and maintained in mass culture for roughly 200 fissions after conjugation. Young B homokaryotes were obtained by crossing strains SB1969 and SB210. Strains SB3539 and SB3546 were crossed to obtain the young C3 homokaryotes, and SB1969 and SB3546 were crossed to make the young B/C3 heterokaryotypes.

**RAPD polymerase chain reactions:** The conditions used for RAPD PCR were as in Williams et al. (1990). Reactions (25 μl) were set up in siliconized, 600-μl-capacity microfuge tubes with standard wall thickness. The primers were 10-mers available from Operon Technologies, Inc. Reaction mixtures for RAPD PCR amplification were as described in Lynch et al. (1995), except that the reactions for 1PM 8 contained 5 mm (instead of 2.5 mm) MgCl2 to maximize contrast in the RAPD polymorphic band. Primers and polymorphic band sizes (kb) for the RAPDs used in this work are: 1PM 8, B17 and B20, 0.5;
An ARP size polymorphism between inbred strains B and C3 undergoes phenotypic assortment: Four loci (1J09, 1EO3R, 1KF2, and 1PM8) that map to MIC chromosome 1L co assort with one another in the MAC and define the PM8 co assortment group (Longcor et al. 1996; Wickert et al. 2000). The relevant segment of MIC chromosome 1L and the data that establish the MAC co assortment of these four loci are shown in Figure 1. We now report the discovery of an ARP size polymorphism. It was detected after probing a pulsed-field blot containing DNA from young homoyzogotes of inbred strains B and C3, using labeled DNA from the 1PM8 locus. An autoradiogram is shown in Figure 2A. The probe hybridized to a 1.2-Mb band in the B DNA lane and a 0.9-Mb band in the C3 DNA lane. The B vs. C3 difference defines an ARP size polymorphism between these two inbred strains. Young B/C3 heteroyzogotes showed a mixture of bands of both sizes, whether tested in mixed population (Figure 2A) or tested as single-cell lines subcloned by 20 fissions of age (not shown).

Figure 1.—The PM8 co assortment group. Data for this figure are taken from Wickert et al. (2000). (A) Segment of the map of MIC chromosome 1L containing the four loci that comprise the PM8 co assortment group. (B) Data demonstrating coassortment of the four loci with one another. Names of terminal assortant strains are written vertically; the SB prefix has been omitted. 1 and 0 represent presence and absence of B-derived polymorphic band for 1PM8, 1KF2, and 1J09 and absence or presence of the C3-derived band for 1EO3R, respectively. Data for terminal assortants of recombinant type are shaded. LOD values against independent assortment range from 4.4 (1PM8 vs. 1J09 or 1EO3R) to 14.5 (1J09 vs. 1EO3R).

Figure 2.—The four loci in coassortment group PM8 are carried in the same MAC ARP. All parts represent autoradiograms of Southern blots of pulsed-field gels of intact whole cell DNAs. (A) A PM8 ARP size polymorphism. DNAs are from C3 homozygotes, B homozygotes, and B/C3 heterozygotes, probed with labeled cloned 1PM8 genomic DNA. All DNAs were prepared from mass cultures less than 20 fissions old (see materials and methods). (B–E) Cloned DNAs from the four loci of the 1PM8 coassortment group hybridize to ARP homologues of identical size. Lanes 1–6: Blotted DNA from terminal assortant cell lines 5B-4212–5B-4217, respectively. Each cell line was initiated with a different B/C3 heterozygote and subcloned after asexual propagation for roughly 350 fissions. The blot was successively stripped and probed with labeled cloned DNA of the four RAPDs indicated.
Figure 3.—Assortment patterns of loci in the PM8 coassortment group and of sizes of PM8 ARP polymorphs in panels of B/C3 heterozygotes. Names of terminal assortant strains are written vertically; the SB prefix has been omitted. Key to RAPD symbols: For 1PM8, 1KF2, and 1JO9, (B) B-allele-determined band present, (C) B-allele-determined band absent; for 1EO3R, (B) C3-allele-determined band absent, (C) C3-allele-determined band present. Key to ARP size symbols: Presence of ARP of corresponding size is shown with a B or a C, depending on the known or suspected B or C3 origin (see discussion). No ARP size data are available for SB1819. Recomb., simplest events generating terminal assortants of apparent recombinant type. (D) Deletion of B alleles (see discussion section); (X) normal crossing over; (C) gene conversion. LOD against independent assortment of PM8 ARP homologues (assuming that 1.0 and 1.4 bands are B derived) for the 1JO9/1EO3R RAPD alleles is 12.1.

If the size polymorphs are derived from homologous MIC genetic segments in inbred strains B and C3 and if their copies are randomly distributed during MAC division, they should assort from one another in the course of asexual cell multiplication. To test this prediction, the DNA of nearly 50 terminal assortant lines of B/C3 heterozygotes was subjected to pulsed-field electrophoresis and Southern blots were probed with cloned 1PM8 DNA. Results are illustrated in Figure 2B. We can summarize the results as follows (Figure 3):

Half of the assortants (23) showed exclusively the 0.9-Mb band characteristic of inbred strain C3.

The other half (24) showed one or more bands characteristic of inbred strain B: (a) the 1.2-Mb band previously described (Longcor et al. 1996 and Figure 2A of this article); (b) the 1.0-Mb band previously observed in inbred strain B lines maintained as stock cultures in our laboratory (Longcor et al. 1996); and (c) a novel 1.4-Mb band, seen in one heterozygous cell line (1807 in Figure 3), likely also to be of B origin (see below and discussion section).

Since the C3-derived polymorph (0.9 Mb) completely assorted from the group of putatively B-derived polymorphs (1.2, 1.0, and 1.4 Mb), we conclude that the ARP size polymorphism observed here between inbred strains B and C3 is genetically determined.

Identical hybridization patterns were observed when the blot shown in Figure 2B was successively stripped and reprobed with labeled 1KF2, 1EO3R, or 1JO9 DNA (Figure 2, C–E). The identity and specificity of the hybridization pattern, involving three different band sizes in different assortants, is compelling physical evidence that all four RAPD loci are carried on the same MAC ARP.

The PM8 ARP size polymorphism genetically coassorts with the RAPDs of the PM8 coassortment group: If the ARP is the physical basis of the coassortment group, the assortment pattern of the ARP size polymorphic forms should be correlated with that of the B and C3 alleles of all RAPDs carried on the ARP. The results are summarized in Figure 3 and examples are shown in Figure 4. Among the 24 terminal assortants showing ARP bands putatively derived from inbred strain B, all carried the B allele at the 1JO9 and 1EO3R loci. Conversely, all but one of 23 assortants having the 0.9-Mb ARP form carried the C3 allele at the 1JO9 and 1EO3R loci. (The sole exception, line SB1818, carried the B allele at both loci, presumably as a result of macronuclear crossing over between B and C3 homologous copies of the PM8 ARP.) Thus alleles at these two loci strongly coassort with the B- and C3-derived polymorphs of the PM8 ARP (LOD against independent assortment = 12.1), supporting strongly as well the putative B origin of the 1.0- and 1.4-Mb polymorphs. Highly statistically significant coassortment is also observed with the other two RAPDs in the PM8 coassortment group: 1KF2 (LOD = 5.6) and 1PM8 (LOD = 4.8). The unambiguous coassortment of the ARP polymorphs with the alleles of the four loci provides conclusive molecular genetic evidence that the PM8 ARP is the physical basis of the PM8 coassortment group. Furthermore, the parallel assortment behavior of the ARP size polymorphs and alleles at genetic loci (whose random distribution in Tetrahymena has been well characterized experimentally) provides strong experimental evidence that the ARP copies themselves are randomly distributed at MAC division.

DISCUSSION

Two rigorous lines of evidence lead us to conclude that the PM8 ARP is the physical basis of the PM8 coas-
1. Four distinct RAPD loci that coassort with one another were found to reside on macronuclear ARPs of identical size. Moreover, this correspondence was observed among terminal assortants showing bands of four sizes due to the existence and assortment of heritably distinct ARP size polymorphs.

2. ARP size polymorphs coassort with the B and C3 alleles of each RAPD whose DNA hybridizes to the ARP. Thus we conclude that the coassortment of two loci reflects their MAC synteny.

This relationship between coassortment groups and ARPs appears to be a general feature of MAC genetics: we have made analogous physical observations with two other groups of coassorting loci (E. P. Hamilton, L. Wong and E. Orias, unpublished observations; S. L. Allen, L. Wong, E. Orias and E. P. Hamilton, unpublished observations). The converse proposition, i.e., that loci in the same ARP should coassort, need not always be true: in theory, MAC recombination (Deak and Doerder 1998) or deletion (of the type observed in the PM8 ARP; see below and Wickert et al. 2000) could occasionally have local intensity high enough to break up coassortment groups. Regardless, we conclude that coassortment provides a general genetic strategy for positively identifying loci that are incorporated into the same MAC DNA piece during MAC differentiation and thus for mapping the MAC by purely genetic means.

The appearance of multiple forms of the inbred strain B ARP homologue was unexpected. Two lines of evidence strongly suggest the B origin of the 1.0- and 1.4-Mb bands observed in our 500-fission-old heterozygotes: (1) We have observed the 1.0 Mb band in old clones of inbred strain B (Longcor et al. 1996) and (2) the 1.0- and 1.4-Mb bands correlate perfectly with the presence of B alleles at the two loci presumed (from the MIC map, Figure 1) to lie in the same half of the PM8 ARP. To explain these and other unpublished observations we propose the following simple model for their appearance. The 1.0-Mb band arises as a consequence of a post-MAC-differentiation deletion of a roughly 200-kb tandem repeat in the B-specified 1.2-Mb ARP. Loss of the repeat is generally accompanied by the loss of the polymorphic copies of both the 1PM8 and 1KF2 RAPD loci. The 1.0- and 1.4-Mb polymorphs could both result from unequal crossing over between two misaligned copies of the 1.2-Mb B ARP homologue. The 1.0-Mb polymorph could equally well (and perhaps more likely) arise from an intramolecular crossover that excises one copy of the tandem repeat. The preceding model accounts for the following observations reported here:

The late generation of both 1.0- and 1.4-Mb bands, neither of which are detectable in young B homozygotes and young B/C3 heterozygotes.

The assortment of the 1.0-Mb band from the 1.2-Mb band, as expected from genetically distinct polymorphs.
The incomplete assortment of the 1.0- or 1.4-Mb bands from the 1.2-Mb band in four heterozygous lines (Figure 3) is consistent with their late (post-MAC-differentiation) generation. This observation stands in contrast with the complete assortment of B vs. C3 polymorphs, which are generated during MAC differentiation, observed in all the heterozygotes examined.

The absence of growth selection against assortants pure for the 1.0-Mb polymorph that might have been expected from the loss of 200 kb of DNA.

Additional work will be needed for a detailed understanding of these deletion events, but the phenomenon has already been useful in providing more discriminating evidence for the correspondence between the genetic coassortment group and a physical ARP.

The overall colinearity of the MIC and MAC maps suggests an uncomplicated model of ARP formation, i.e., exclusively by fragmentation (Wickert et al. 2000). This colinearity, taken together with the ability to identify an ARP restriction pattern (Longcor et al. 1996), suggests an approach to making a physical map of the genome: identifying, for every ARP, which ARPs and in which orientation are derived from the adjacent segments in the MIC. Furthermore, the correspondence between coassortment groups and ARPs described here provides an important way in which to relate the physical map to the genetic map. Efforts to systematically clone at least one RAPD of every coassortment group, to identify the size and restriction pattern of the MAC chromosome piece that corresponds to the coassortment group, and to sequence the RAPD clone are now underway. This information will ultimately be useful in relating the genetic map, the physical map, and the genome sequence.

Mapping a fragmented somatic genome by purely genetic means is so far possible only in Tetrahymena. A maximum-likelihood map detailing the correspondence between the map location of loci on the left arm of MIC chromosome 1L and the coassortment groups formed by these loci in the MAC is shown in Wickert et al. (2000). Analogous coassortment mapping of the other chromosomes is underway. The phenomenon of coassortment generates tools useful for molecular genetics. For example, the ability to genetically map a mutant locus to a coassortment group tagged with a DNA polymorphism and to relate the group to a physical ARP confers the potential to narrow down the location of the locus to an identifiable DNA segment that comprises, on the average, roughly 0.3% of the genome. This should become useful for cloning mutant genes of interest, and more so in the future, as more coassortment groups become identified, more Tetrahymena ARPs become tagged with a DNA polymorphism, and a more complete genome physical map and sequence become available.

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LITERATURE CITED