Alternative processing during development of a macronuclear chromosome family in *Oxytricha fallax*

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Macronuclear chromosomes in *Oxytricha fallax*, a hypotrichous ciliate, are very short. They often belong to small families of cross-hybridizing chromosomes of two or three different sizes. For example, the 81-MAC family consists of three sizes of macronuclear chromosomes (4.9, 2.9, and 1.6 kbp) (Cartinhour and Herrick 1984). We show that the family actually consists of two closely related sets of three each and that the two sets are independently created by alternative processing of two separate precursor (micronuclear) versions. Chromosomes of a set share a common 1.6-kbp region, which contains a transcribed gene coding for a 25-kD protein. Different-sized macronuclear chromosomes of a set result from alternative choices of positions for telomere formation. All six members of the family are reproducibly generated in each developing macronucleus, and their copy numbers are stably maintained during vegetative replication of the macronucleus (Herrick et al. 1987). Here we argue for the existence of three distinct copy control elements in the 81-MAC family chromosomes. A model is discussed in which, following polytenization of the micronuclear chromosomes, different chromatids are processed differently, and, subsequently, replication-competent macronuclear chromosome products are amplified under the influence of the vegetative copy control elements.

[Key Words: *Oxytricha fallax*; macronuclear chromosomes; copy control elements]

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Irreversible developmental genome alterations affect essentially every locus in the somatic nucleus of the hypotrichous ciliated protozoa (for review, see Klobutcher and Prescott 1986; Ammermann 1985; Steinbrück 1986). Although each organism is only a single cell, it contains two highly different nuclei with very different roles. The macronucleus directs the vegetative processes of clonal growth, whereas the micronucleus is vegetatively silent and its primary role is to carry the germ line through clonal expansion to the next conjugation. During conjugation, gametic nuclei are exchanged and join to form the zygotic nucleus of the genetically new individual. One mitotic daughter of the zygotic nucleus gives rise to the new macronucleus, and the other becomes the micronucleus. The exconjugant, so equipped, resumes vegetative fission and establishes a clone of cells called a karyonide.

The developing macronucleus undergoes three distinct periods of change. During the first, chromosomes are polytenized. In the second, many DNA sequences are destroyed, including spliced-out internal eliminated sequences (IESs), whereas retained segment ends receive telomeres consisting of blocks of dC₄A₄ repeats. The resulting linear macronuclear chromosomes are extremely numerous (~20,000 kinds) and extremely small (heterogeneous, but averaging ~2 kbp). Each chromosome analyzed to date bears only a single gene. In the last period of macronuclear development, the chromosomes repli-

cate repeatedly, amplifying the contents of the macronucleus to a vegetative level characteristic of a given species and commensurate with the cell's size.

In a survey of randomly chosen *Oxytricha fallax* macronuclear chromosomes, cloned whole chromosomes (pMAs) were used to probe blots of native macronuclear DNA (Cartinhour and Herrick 1984). We were struck by the high frequency of what were termed "families" of macronuclear chromosomes, the two or three members of which strongly cross-hybridize but differ in size. Subsequently other such families have been discovered, including the *O. nova* c4 + c5 chromosome family (Klobutcher et al. 1986), *O. fallax* histone H3 and H4 families (K. Williams and G. Herrick, unpubl.), and an *O. nova* H4 family (see Discussion). The 81-MAC family, a representative from our initial survey, was chosen for further study (for an example of its blot hybridization pattern, see Fig. 2A). It consists of three different-sized chromosomes, 4.9, 2.9, and 1.6 kbp long. From restriction site mapping (Cartinhour and Herrick 1984) they appear to share a common region of between 1.3 and 1.6 kbp, with the smallest chromosome (MAC III) consisting primarily or entirely of the common region, plus telomeres. The two larger chromosomes, MAC I and II, bear unique 3.3–3.6 and 1.2–1.5 kbp "arms" appended to the right or left, respectively (Fig. 1).

We suggested that the 81-MAC family of chromosomes is generated by alternative DNA processing

during macronuclear development. However, an unambiguous demonstration of alternative processing was complicated by the existence of more than one sequence version of the family sequences. Cloning of micronuclear and macronuclear DNA's and extensive DNA sequence determinations have led us to conclude that there are three versions of the 81-MAC family sequences in the micronucleus, vA, vB, and vC (Herrick et al. 1987). Each version is defined by a set of micronuclear and/or macronuclear DNA clones showing perfect sequence overlaps. The three versions differ from each other primarily by 2.1–4.6% base substitutions. The genetic relationship between the three versions is unclear;

they could be related loci, or, at another extreme, they could be allelic (discussed in Herrick et al. 1987). Whereas all three versions must be represented in the germ line (micronucleus), we have no evidence for vC DNA in the macronucleus. However, as we show here, vA and vB chromosomes are present in the macronucleus. Version A chromosomes bear an *SphI* site in their common regions, whereas vB chromosomes lack it (*SphI*⁺ vs. *SphI*⁻, Fig. 1). Maps of cloned vA and vB macronuclear chromosomes (pMAs) relevant to alternative processing are shown in Figure 1 with solid lines; maps of macronuclear chromosomes we demonstrate to exist here are shown with faint lines. Whereas a cloned mi-

Version A (*SphI*⁺)

Version B (*SphI*⁻)

Figure 1. Maps of the 81-MAC family chromosomes. Intervals are as deduced by restriction analyses of cloned and uncloned DNAs (Cartinhour and Herrick 1984; Herrick et al. 1985) and adjusted on the basis of sequence information. Maps for DNAs representing versions A (central *SphI* site present) and B (central *SphI* site absent) are segregated. For each version the micronuclear map is shown (MIC), followed by the maps of the three macronuclear chromosomes derived from it (MAC I–III). Map regions shown as solid lines have been cloned; the clone names are given at the left. Underlined portions have been sequenced; numbered regions are referred to in the text and in Fig. 3. Macronuclear map regions indicated by broken lines (e.g., version A, MAC II) are deduced from results of blotting experiments (see text). Micronuclear map regions indicated by featureless dotted lines are deduced to exist from the existence of corresponding macronuclear DNAs (see text). Boxes at the ends of macronuclear chromosome maps indicate blocks of telomeric dC₄A₄ repeats (see Fig. 3). Two bubbles in the micronuclear version A map indicate the positions of IESs not present in macronuclear DNA; the leftward portion of this DNA is not shown (full map in Herrick et al. 1985). Restriction site symbols: (S), *SphI*; (S)⁻, “*SphI*” site referred to in the text; (K), *KpnI*; (O), *XhoI*; (B), *BamHI*; (G), *BglII*; (H), *HindIII*; (R), *EcoRI*.

macronuclear vA DNA has been obtained (λ MI 21, Fig. 1), no micronuclear vB clones are available, and we only deduce the vB micronuclear DNA must exist (in some undetermined form) because of the existence of corresponding cloned vB macronuclear DNAs (discussed in Herrick et al. 1987). It is important to realize that although there are three sequence versions and there are three sizes of macronuclear chromosomes, the two numbers "three" are not related. Specifically, we provide evidence that there are two complete sets of three macronuclear chromosomes each (vA MAC I, MAC II, and MAC III; and vB MAC I, MAC II, and MAC III) and that each set of three chromosomes is generated independently from its corresponding vA or vB micronuclear precursor.

Most steps of macronuclear development presumably are invariant. However, some do vary from one developing macronucleus to the next, or even within a given developing macronucleus, as shown in a variety of ciliates both genetically (reviewed by Preer 1969; Bruns 1986) and biochemically (reviewed by Karrer 1986; Austerberry and Yao 1987). For instance, variation in the position of break-telomere addition sites recently has been shown to result in the production of variably sized macronuclear chromosomes (Yao, cited in Yao 1986; Baroin et al. 1987; Forney and Blackburn 1988; Klobutcher et al., in prep.). Such variability in principle can provide somatic flexibility to the species. A special case of variation is that in which the same set of different products of a given germ line sequence is generated *reproducibly in each developing macronucleus*. Such a situation is analogous to many instances of alternative RNA processing in metazoans wherein the development of the soma leads to a controlled generation of differently processed forms of the same primary transcript in different tissues or times in development (for review, see Breitbart et al. 1987). We adopt the term "alternative processing" to describe the generation of the 81-MAC family of chromosomes because we show that different products of a given micronuclear sequence were generated in a single developing macronucleus, and, furthermore, that the same set of different products is generated each time a new macronucleus develops. To our knowledge this is the first demonstration of alternative processing in any somatic genome alteration system.

Results

Different macronuclear chromosomes from the same micronuclear sequence

In contrast to the numerous differences between versions, scattered every ~20–50 bp, the sequenced regions of pMA81 and pMA83s share 704 bp and 1296 bp of perfect homology with λ MI 21, respectively, and 866 bp of perfect homology with each other. Thus, we consider these three DNAs to represent a single version (vA). Because pMA81 and pMA83s are independently obtained clones, each of which carries an intact, telomere-to-telomere macronuclear chromosome—a MAC I and MAC III, respectively (Fig. 1)—and both of which are the same version (vA), it is firmly established that vA micronu-

clear DNA was processed to give rise to a MAC I chromosome on one hand and a MAC III chromosome on the other. The possibility that any vA MAC II chromosome also was created is left open. Similarly, pMAs 82d and 83, which are *SphI*⁻ (Fig. 1) and differ in sequence considerably from vA and vC, are perfectly matched in the 221 bp of sequenced overlap. These clones show that the putative vB micronuclear sequence was processed to generate a vB MAC II and a MAC III chromosome. This one-at-a-time examination of individual, cloned chromosomes is not suited for studying the full composition of the family in total macronuclear DNA, so a different approach was taken.

Version-specific blot hybridization probes were designed and synthesized, based on our knowledge of sequence differences between the versions. Figure 2 compares the results of hybridization of identical, native macronuclear DNA blots with one of two probes, a vB-specific 16-mer (Fig. 2B), and an 18-mer with a sequence shared by all three versions (Fig. 2A). Note first that hybridization and wash conditions were set so that vA and vC control DNAs did not hybridize to the vB probe (lane B2 vs. A2). The vB 81-MAC family has all three sizes of chromosomes, and their relative numbers are in the same proportion as in the family as a whole: MAC III chromosomes predominate, and MAC I and II chromosomes are in roughly equal numbers. With a vA-specific oligomer probe, we have demonstrated a full set of vA MAC I, II, and III chromosomes, present in the same relative proportions (III>I $\approx$ II), but rarer than the vB set (not shown).

These blot hybridizations thus confirm what was suggested from the analyses of the cloned DNAs; that is, the 81-MAC family contains two subfamilies, vA I+II+III and vB I+II+III, each produced independently by processing of distinct but highly similar vA and vB precursor sequences.

Processing mechanism

Figure 3 compares portions of the DNA sequences of the vA MAC I and III chromosomes carried in pMA81 and pMA83s (see maps, Fig. 1). The right subtelomeric sequence of the MAC III chromosome ("1" in Fig. 1) is colinear with the sequence at the right end of the common region of the MAC I chromosome ("2" in Fig. 1; Fig. 3B) and bears no resemblance to the right MAC I subtelomeric region ("4" in Fig. 1; Fig. 3A). Despite the closely adjoining IES (Fig. 3B), the micronuclear sequence at the latent telomere position ("3" in Fig. 1) is colinear with the macronuclear sequences, and these results eliminate direct involvement of the IES in the telomere formation mechanism. This situation is in contrast to a more complex one in *Paramecium*, where variations in IES processing appear to be involved in variations in the site of telomere formation (Forney and Blackburn 1988). Note also that the left ends of these vA MAC I and III chromosomes are colinear with each other and with the vA micronuclear DNA ("6," "5," and "7" in Fig. 1). The simplest interpretation of these results is that these chromosomes were created by excision of different segments

Figure 2. Composition of the 81-MAC family. Native macronuclear DNA and a mixture of control plasmids representing each of the three versions were subjected in duplicate to electrophoresis through a 0.7% agarose gel and blotted to nitrocellulose. Part A of the blot was hybridized with an oligomer probe with a sequence conserved in all three versions. Part B of the blot was hybridized with an oligomer that perfectly matches vB, but which mismatches with the two other versions. Both oligomers hybridize to areas near the right end of the common region. Lanes A1 and B1 each carry 4 μ g of macronuclear DNA. Lanes A2 and B2 received equal aliquots of a mix of restriction-cut plasmids bearing each of the three versions of the right end of the common region on different-sized restriction fragments: vA, 0.48 ng of *Kpn*I-digested pMA83s, 5863 bp; vC, 0.35 ng of *Kpn*I-digested 22p206 [carries the 0.40-kbp *Kpn*I–*Hind*III fragment of vC micronuclear DNA in pEMBL8+), 4.40 kbp; vB, *Pst*I-digested pMA83, 1.6 kbp. The vA and vC plasmid amounts were chosen to represent the signal expected in the macronuclear DNA sample if each is present at average copy number (1000/macronucleus); the vB plasmid DNA was inadvertently underrepresented in the mixture.

from different polytene chromatids and that telomeric repeats were then added. We have not studied the areas involved in the generation of the extreme left and right ends of the family (left MAC II, right MAC I), but we assume they are similar to those at the two ends of the common region. If so, the family would appear to be generated by chromosome breakage variously at four sites, with attendant telomere addition. In contrast to this “excision” model, it is equally possible that the en-

tire region is excised by breaks at only two invariant, flanking sites, followed by exonuclease removal of alternative lengths of material before termination with the addition of telomeres. However, for ease of discussion, we will ignore this possibility in favor of simple breakage and telomere addition.

Like Klobutcher et al. (1984) we find no micronuclear dC₄A₄ repeats near the positions of latent telomeres (at “7” and “3” in Fig. 1; Fig. 3B, 3D), bolstering the conclusion (Dawson and Herrick 1984; Klobutcher et al. 1984) that all macronuclear telomeres must receive dC₄A₄ de novo, either in concert with or after removal of distal sequences.

The right ends of the common regions of the chromosomes cloned in pMA83 and μ 1A1 are not exactly the same but differ by 18 bp in the position of the break–telomere addition events that made them, even though they both are derived from vB sequences (“8” vs. “9”, Fig. 1; Fig. 3C). [The right end of the vA chromosome of pMA83s is another 5 bp further beyond the μ 1A1 end (“1” vs. “9” Fig. 1; Fig. 3B,C), but this might be attributable to some genetic difference between vA and vB]. Similarly, the two vA left common region ends of pMA83s and 81 chromosomes were created 33 bp apart (“5” vs. “6” Fig. 1; Fig. 3D). These results argue against the simple expectation that single, specific sites at each end of the common region are responsible for the generation of the common region telomeres. Instead they suggest that the target of the process is an area with some diffuse property, as opposed to being a specific short sequence such as recently described in *Tetrahymena* (Yao et al. 1987). Consistent with this notion, we have examined both the areas surrounding the two common region ends and the latent c2 chromosome reported for *O. nova* by Klobutcher et al. (1984) and have found no consistent homology with the 15-bp *Tetrahymena* “chromosome break sequence,” nor have we been able to identify any conserved sequence or potential secondary structure shared by the four *Oxytricha* sites.

Transcription from the 81-MAC family

A polyadenylated transcript has been detected on RNA blots probed with various portions of the common region (Fig. 4). Its apparent size is 1.33×10^3 nucleotides on both formaldehyde and methylmercury denaturing gels. It is 10–50 times less abundant than the actin transcript we detect with the pOFACT (1.6) probe of Kaine and Spear (1982), and its abundance is not noticeably affected by our usual 2- to 7-day preharvest 4°C incubation of the cells (not shown). Use of complementary single-stranded probes demonstrates that it is transcribed from right to left (not shown). Using a variety of nick-translated subclones as probes (not shown), we have neither detected homology of this transcript with regions outside the common region, nor have we detected any other transcripts from the arms of either MAC I or MAC II, either in total RNA or in polyadenylated RNA preparations. (Since we have no intact MAC II clone, instead we used the λ MI 21 *Bgl*II–*Eco*RI fragment that spans the left end of the common region; see Fig. 1.)

A.
 TCTAAGATTGTTAAGATTATGGATAATTCATTTTGTAGAAATATTTAAATTCAAATTTATACTACTATACTACACATGGGGTTTTGGGGTTTTGGGGTTTTGGGG
 TTGATTGCTTATTTAAATAATTTAATAACTTTATATTAATTATGTTTATTACAAATTTATTCATTAAGTTCATTCAAAAAGGGGTTTTGGGGTTTTGGGGTTTTGGGG

B.
 IES
 \/
 AATTTAATAACTTTATATTAATTATGTTTATTACAAATTATTTCAATTAAGTTCATTCAAAAATAAATCTCTGCCACCTTTAAATCTATATTTAGGTTAACTCAAGTCTTCAAACAA
 AATTTAATAACTTTATATTAATTATGTTTATTACAAATTATTTCAATTAAGTTCATTCAAAAATAAATCTCTGCCACCTTTAAATCTATATTTAGGTTAACTCAAGTCTTCAAACAA
 AATTTAATAACTTTATATTAATTATGTTTATTACAAATTATTTCAATTAAGTTCATTCAAAAAGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG

C.
 AATTaTAATAACTTTATATTAATTATGTTTATTACAAATTATTTCAATTAAGcTCATTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG
 AATTaTAATAACTTTATATTAATTATGTTTATTACAAATTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG

D.
 GATTTAGAATGATATATGATATAATGCTTTATAATGTACTTTTCAAAAGTAAGTACTAGGACTGTGATATTAATATCAACTAAGAGTATATATAAATTTAATATTTTAAATAGGTTTAA
 CCCCBAACCCCAACCCCAACCCCAACCCCAAGTAAGTACTAGGACTGTGATATTAATATCAACTAAGAGTATATATAAATTTAATATTTTAAATAGGTTTAA
 CCCCBAACCCCAACCCCAACCCCAACCCCAAGAGTATATATAAATTTAATATTTTAAATAGGTTTAA

Figure 3. DNA sequences of chromosome ends and common region ends. Sequences are presented 5' to 3' as read left to right in the orientation shown in Fig. 1. Telomeric dC₄A₄ and dG₄T₄ repeats are underlined. (A) vA MAC I and III right ends: pMA81 vs. pMA83s; see positions "4" and "1" of Fig. 1. (B) vA common region right ends: λMI21, pMA81, pMA83s; see positions "3," "2," and "1" of Fig. 1. An IES in λMI21 has been removed where indicated. (C) vB common region right ends: pMAμ1A1 vs. pMA83; see positions "9" and "8" of Fig. 1. Nucleotides that differ from vA are lower case. (D) vA common region left ends: λMI21, pMA83s, pMA81; see positions "7," "5," and "6" of Fig. 1.

A protein-coding gene in the common region

The vA MAC II chromosome of pMA83s was sequenced and was searched for open reading frames, with the assumption that the genetic code used only TGA as "stop," as appears to be the case for ciliates in general (reviewed by Preer 1986). Three long open reading

frames were found, all reading from right to left. Their positions are indicated at the bottom of Figure 5. Of these three only the longest appears to be protein coding, as judged by several criteria. First, its codons are minimally diverged from RnY (purine, any base, pyrimidine) over nearly its entire length (Fig. 5, center), a property of genes from a variety of sources (Shepherd 1981), including *Paramecium* surface antigen genes (Preer 1986). Second, it resides in a relatively GC-rich area (Fig. 5, top), as is generally true for protein-coding genes in GC-poor genomes (ciliate data reviewed by Karrer 1986). Third, its coding information is almost completely conserved among the three versions, despite numerous nucleotide differences (Fig. 6): that of vB differs at 14 bp from vA but shows no coding differences from vA; that of vC differs at 18 bp and shows a single conservative valine-alanine difference. By contrast, the other two vA open reading frames noted are very poorly conserved from version to version (not shown). Fourth, codon usage in the conserved open reading frame is typical of other ciliate genes (see Horowitz et al. 1987). In general, GC-rich codons are avoided: of nine Arg codons, all are AGA and none is AGG or CGN; of 14 Leu codons, none is CTG. In contrast, 11 of 20 Ala codons are GCT, and 10

Figure 4. Northern blot of polyadenylated RNA hybridized with a common region probe. The RNA lane received 0.54 μg of polyadenylated RNA isolated from cold-shifted cells. The DNA lane received 25 ng of macronuclear DNA. Electrophoresis was through a 1% agarose, 2.2 M formaldehyde gel. The probe was prepared from a subcloned *Hind*III–*Bgl*II fragment from the common region (see Fig. 1); the labeled single strand was not purified for this experiment. For further details, see Experimental procedures. Denatured DNA size standards were run in an adjacent lane, and the apparent size of the RNA was calculated by reference to a linear regression plot of the mobilities of the size standards, the nearest being 1126, 1307, and 1444 nucleotides long.

Figure 5. Sequence features of the vA MAC III chromosome from pMA83s. (Top line) Restriction map with carets indicating positions of internal eliminated sequences present only in the micronuclear DNA. Base composition (% G + C): each point represents the average base composition of a window of 40 bp, with the window moved 20 bp for each calculation. RnY analysis (Shepherd 1981): percent mutation away from poly(RnY) is plotted for each of the three frames, using a 90-bp window and 21-bp step. (●) Frame 1; (+) frame 2; (○) frame 3. The top panel labeled "frame" scores the frame with the lowest deviation from poly(RnY) for each interval. The bottom panel shows the positions of the three largest open reading frames; solid arrows indicate ATG-to-TGA intervals, whereas the broken line preceding the ATG in frame 1 is free of TGA stops.

of 18 Gly codons are GGT. Two of nine Gln codons are nonstandard, one TAA and one TAG.

This conserved open reading frame would code for a slightly basic ($B/A = 23/19$) 25,407-dalton primary translation product. A search of the National Biomedical Research Foundation (NBRF) protein sequence data base revealed significant homologies with four major, highly conserved regions of metazoan and plant ADP/ATP carrier proteins (Aquila et al. 1982; Arends and Sebald 1984; Baker and Leaver 1985; Adrian et al. 1986). The significance of this finding (not shown) in the context of alternative DNA processing is as yet unclear.

The IES near the left end of the common region (76 bp; Herrick et al. 1987) interrupts the micronuclear counterpart of the gene in such a way that translation would emerge from the IES out of frame and terminate just beyond (Fig. 6). Thus, its precise excision is necessary for production of the functional macronuclear gene, as also is true in a case reported by Klobutcher et al. (1984).

Finally, the 78 codons upstream of the first ATG/Met codon are open. Like the downstream open reading

frame, this section is relatively GC-rich and is minimally different from poly(RnY) (Fig. 5). Its 78-residue peptide sequence is completely conserved among the three versions despite several nucleotide differences (Fig. 6). Conceivably, it might be translated in frame with the downstream region if its transcript were spliced to an upstream or a *trans* exon.

Discussion

The 81-MAC family chromosomes are generated by alternative processing

The macronuclear DNA analyzed here contains a complete set of three vA and three vB chromosomes. Because *O. fallax* is capable of intraclonal conjugation (although not autogamy) at some stages of clonal life, one might argue that these various 81-MAC chromosomes may not coexist in the same macronucleus. If so, they are not the result of alternative processing within the same macronuclear precursor nucleus, but instead could result from processing variations from one developing macronu-

cleus to the next (see introduction). However, all macronuclear DNA used in these experiments was obtained from subkaryonide 3.5, a clone we established from a single vegetative cell. Both the parent culture and subkaryonide 3.5 have been senescent and sterile ever

since we obtained them. Therefore, we are certain that the various 81-MAC family chromosomes all represent only one developing macronucleus.

Are these various chromosomes generated *reproducibly* each time a new macronucleus develops? Elsewhere

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CCCCAAAACCCAAAACCCAAAACCCAAAACCC
CTTTGAATGAACCTAATTGAAATAATTTGTAATAAACATAATTAATATAAAGTTATTAAATTTTAAATA
AGCAATCAAGAAAGATGCCTACTTAATGGAAGGTAAATTTATCAATAAAATATCGAATCATCGATTATAAAAT
TTATTTTGTAGTTGAGTACTGGAGAAGAAGGTACCAACGCATGAAGTATGAAAGATTGCTGCTGTAATGTTATT
GCTCTCATAACTCATGCCGCTGTAAGTTAATACCCTAAAGCACAATATACTATGCTTTGTGATATTTAATCATAT
TCTTAATGTAATATAGACTTAACCACTCGATATGGTTAGAAATCAGATCTCAATGCTGTAAGAGGGCAAGACCTT
CTCAGGCCCTTGGTTATCAAAAAGGTTGGTATCCATTCTAAATTATGGAAGAGATTATGCAGCAGCGGTGGACT
CAGAAAAATCTATTCTGCGTAAGTGTATAACAAAATTTGGGTTATCCAAAACATCTCTCAATTTTATAATTGA

GGA ATT GCA ATA AAT TTA GCT AAT TTA TAT TGG TAT AGT TTC GAC ACA TTC TTC   54
Gly Ile Ala Ile Asn Leu Ala Asn Leu Tyr Trp Tyr Ser Phe Asp Thr Phe Phe

TTC AGA ACA GTT TGT TAC ACT ACT GCT AGA GTA ACA GCC TTC GGT TAT TTC TAC   108
Phe Arg Thr Val Cys Tyr Thr Thr Ala Arg Val Thr Ala Phe Gly Tyr Phe Tyr

GAT AAG GTA AAT AAA GAT CCG AGA AGA GTT GCT CGT CCA GAC TTC TTG GTA GCA   162
Asp Lys Val Asn Lys Asp Pro Arg Arg Val Ala Arg Pro Asp Phe Leu Val Ala

GCA GGA GTA CTC GGA GGA TTT ATT GCT GGT GTA GTG ACA AAT CCG ATC GAT ATC   216
Ala Gly Val Leu Gly Gly Phe Ile Ala Gly Val Val Thr Asn Pro Ile Asp Ile

GTC TAT AAT AGA ATG CAG GTT GAT GAG CTT TAT CCA CAA GCT GCC AGA AGA AAC   270
Val Tyr Asn Arg MET Gln Val Asp Glu Leu Tyr Pro Gln Ala Ala Arg Arg Asn

TAC TCA AAT ACA ATT TAA GGA CTT GCG AAA GTT GCT GAA GAA GGT GCT CTC TTC   324
Tyr Ser Asn Thr Ile gln Gly Leu Ala Lys Val Ala Glu Glu Gly Ala Leu Phe

AGA GGT GCT GGT GCC AAC GGT TTT AAG CTT GCA GCT ATT TGT TCA TCA ATG ACA   378
Arg Gly Ala Gly Ala Asn Gly Phe Lys Leu Ala Ala Ile Cys Ser Ser Met Thr

AAC ATC TAC GAT TGG TGC AAG GAG AAC TCC TAC TTC TTC TTT GGT CCT CAT TGG   432
Asn Ile Tyr Asp Trp Cys Lys Glu Asn Ser Tyr Phe Phe Phe Gly Pro His Trp

ATT AAT AGA TTG TGG GGT ACT GCC GTA GCT GTT GCT ATT GGA ACT GGT GTA AGC   486
Ile Asn Arg Leu Trp Gly Thr Ala Val Ala Val Ala Ile Gly Thr Val Val Ser
Val/Ala

ATG CCT TTT GAT ATG ATC AGA ACA AGA CTA CAC ACC ATG AGA CCA CTA CCA AAC   540
Met Pro Phe Asp Met Ile Arg Thr Arg Leu His Thr Met Arg Pro Leu Pro Asn

GGT CAA ATG CCA TAT AAC GGC ATG ATT GAT TGC TTC AAC AAG ATC ATA AAG TAC   594
Gly Gln Met Pro Tyr Asn Gly Met Ile Asp Cys Phe Asn Lys Ile Ile Lys Tyr

GAG TGC AAC TCA AAA TGG ATG TCT AAC TTT GGA TCA TTC TAT GCA GGA GGT GAA   648
Glu Cys Asn Ser Lys Trp Met Ser Asn Phe Gly Ser Phe Tyr Ala Gly Gly Glu

GCC TAC TTC CTC AGA TTG TTC TTG ATT TGT TAT CTT TCT CAA TTC TTG GTC GAT   702
Ala Tyr Phe Leu Arg Leu Phe Leu Ile Cys Tyr Leu Ser Gln Phe Leu Val Asp

TAC TAT AAC GAA AAC TAC TAT GAT CAA GAA TTC TGG CAA CCA TAG AGA TTC CAC   756
Tyr Tyr Asn Glu Asn Tyr Tyr Asp Asp Gln Glu Phe Trp Gln Pro gln Arg Phe His

TAT CAA AGT GGT ATC GAT TAC GAT ATT CAT GAT CCA TAC ACA GAT GCC TTC AAT   810
Tyr Gln Ser Gly Ile Asp Tyr Asp Ile His Asp Pro Tyr Thr Asp Ala Phe Asn

AAA AAA TTA GTT GCT ACT TAT ACT ACA GCT ACT GGA GGT ATG GGA GCT GCT CAC   864
Lys Lys Leu Val Ala Thr Tyr Thr Thr Ala Thr Gly Gly Met Gly Ala Ala His

CCT TCA GGA AAA GAA AAT CTC GCA ATT ATC   894
Pro Ser Gly Lys Glu Asn Leu Ala Ile Ile

TGAGAAATCAAAAAATAAATTGTCTCTAACATCGAGCATTATCACATGCTTTTTTGTAAAGTAGAGATTATTT
GTTATTTAAACCTATTTTAAATATTAATTTATATACTCTTAGTTGATATTAATATCACAGTCCTAAGTTAC
TTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG

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Figure 6. Amino acid conservation of the longest open reading frame of the common region. The entire sequence of the pMA83s insert is shown, with the open reading frame translated (represented in Fig. 5 by the entire arrow in frame 1). The first in-frame ATG is at nucleotide 229, and the first in-frame TGA is at 895; corresponding amino acids are shown below each codon; "gln" is assigned to the single TAA and TAG codons at 286 and 745. The A version is shown. Each underlined, boldface base is different in one or both of the other two versions. In order encountered, the base at the position for each version is listed the vA/vB/vC: A/T/T, T/T/C, C/T/C, T/A/T, C/C/A, T/C/C, G/A/G, C/T/C, G/C/C, A/C/A, G/G/A, T/A/T, T/T/A, T/T/C, C/T/T, T/T/C, A/A/C, A/A/T, C/T/T, A/G/G, C/A/A, T/C/C, T/T/C, C/C/T. One amino acid change results, as indicated below the line, in the order vB/vC. The overlined ATTCA (781–785) represents the sequence, present twice in micronuclear DNA, bounding the IES near the left end of the common region (Fig. 1). Note immediately after the ATTCA an out-of-frame TGA stop codon (underlined) at which translation would stop were it to read through the IES (see text).

we show that the macronuclear DNAs of the young and sexually immature karyonides 9D1 and 9D2 have native and *SphI*-digested macronuclear DNA blot patterns indistinguishable from each other and from that of subkaryonide 3.5 macronuclear DNA in a side-by-side test (Herrick and Cartinhour 1984; Herrick et al. 1987). Although we have not performed DNA sequencing or version-specific hybridizations with 9D1 and 9D2 DNAs, these results, in conjunction with our thorough analysis of subkaryonide 3.5 DNA, very strongly imply that the complete set of three vA and three vB chromosomes is reproducibly generated in each developing macronucleus. Finally, the typical pattern of 81-MAC I, II, and III chromosomes has been seen in at least 39 independently isolated, wild karyonides of related *Oxytricha* species (R.L. Hammersmith, pers. comm.; R.L. Hammersmith and G. Herrick, unpubl.). We thus feel justified to call the 81-MAC family an alternative processing family, because we are confident that the full set of chromosomes is reproducibly generated within each developing macronucleus. Furthermore, each vegetative individual maintains the full set at its disposal (see below). Whether mechanisms have evolved to take advantage of the alternative forms of the 81-MAC family is not known. It is suggestive that even though homology has diverged sufficiently to require lowered stringency of hybridization to detect the family in the wild *Oxytricha* isolates, nonetheless the alternative processing feature has been fully maintained, since not only are all three chromosome sizes seen, but they are in the same relative stoichiometry we observe in *O. fallax*. In any case, given the apparent ubiquity of such alternative processing in the formation of the macronucleus, it seems likely that alternative processing does play a role in gene control in at least some alternative processing chromosome families.

Whether the pattern of microheterogeneity of common region ends seen in subkaryonide 3.5 DNA (Fig. 3) is reproduced in each developing macronucleus is unknown; thus, it may not represent alternative processing, but simply high-resolution variability.

Copy control in the vegetative macronucleus

An important feature of the 81-MAC family is the unequal and strictly maintained copy numbers of its six members during extended karyonidal expansion (Herrick et al. 1987). It is now clear that a specific, active mechanism regulates the number of copies of the *Tetrahymena* rDNA macronuclear chromosome. One component of the mechanism has been demonstrated genetically; it resides on the chromosome and participates in the partitioning of a fixed, total rDNA chromosome number between competing allelic forms (Larson et al. 1986; Tondravi and Yao 1986). Whether such active mechanisms exist for other macronuclear chromosomes is unknown, and various models of active and passive copy control have been proposed and discussed (see Preer and Preer 1979). However, it seems reasonable provisionally to take the rDNA case as the general para-

digm, in the absence of evidence to the contrary. Given this assumption, given our demonstration of strict maintenance of copy number in the 81-MAC family (Herrick et al. 1987), and now given our knowledge of the anatomy of the chromosomes, we can consider the nature and location of distinct sequence elements that presumably participate in the maintenance of the composition of the family.

First, within a version subset MAC IIIs predominate, and the numbers of MAC Is and of IIs are about the same (Fig. 2B shows the vB subset; see also Herrick et al. 1987). We have estimated the relative copy numbers to be ~1 : 1 : 10 (MAC I : II : III), although exact values have not been measured. MAC III has only two sequences not found on either MAC I or II. These span the junctions between the subtelomeric regions and the telomeric repeats (Fig. 3B,D); it is possible that these junction sequences are responsible in a positive manner for maintaining the elevated numbers of IIIs, although if so, the picture is complicated by the subtelomeric microheterogeneity. It is simpler to see how the I and II arms might have *negative* copy control elements. These elements simply might be inert, extra DNA, serving, for instance, to separate the telomere by a critical distance from a second kind of element (replication origin?) in the common region (see below). Alternatively, they might be more conventional, compact units placed at discrete places in each arm.

Second, the vB subset is an estimated 10-fold more numerous than the vA subset (Herrick et al. 1987). Since all members of a version subfamily share only their common regions, the version difference must—within the rDNA paradigm—be caused by some version-specific element *within* the common region. In summary, in light of the rDNA work, our results suggest the existence of both common-region and arm elements, which would necessarily interact to control chromosome copy number during each vegetative cell cycle.

Generation of the family in the developing macronucleus

Chromosome breakage and telomere addition take place immediately after chromosome polytenization (see introductory section). We suggest that different chromatids give rise to alternative products produced by different combinations of breaks (Fig. 7). Note that, even with long autoradiography exposures and excellent probes, we have never seen even hints of chromosomes consisting of solely a left or a right arm, or of a common region with both arms (not shown). One explanation could be that chromatid breakage is highly coordinated and only certain combinations of breaks are made.

A simpler model takes into account the idea that control mechanisms exist to adjust the copy numbers of at least the I+II+III subset of the products one expects from a random “partial digest” of chromatids. If we assume that only the common region has an origin of replication, then arm-alone fragments would be lost rapidly by dilution (in contrast to active destruction, which is

Figure 7. Model for alternative processing of polytene chromatids. See text for explanation. Chromatids are marked where breaks will be made and where telomeres will be added in the second step. (–) The positions of negative copy control elements are represented on the arms. (+) An orDNA replication is represented in the common region.

not addressed in this model). Similarly, the absence of chromosomes with a common region and both arms could be a consequence of their bearing a negatively acting copy control element in each arm, leading to a sufficient deficit in replication capacity to cause such chromosomes to be lost by dilution within a few generations, as is the *Tetrahymena* 11-kbp rDNA chromosome (Pan and Blackburn 1981). Thus, the standard stoichiometry of the three competent chromosomes would become stably established long before a clone has expanded sufficiently to allow biochemical examination of the composition of its family. Presumably, such adjustments would be made already in the several rounds of macronuclear DNA replication that precede emergence of the exconjugant into the vegetative phase (Fig. 7).

An attractive feature of this view is that it provides a possible explanation for the existence of two quite different modes of DNA amplification during macronuclear development. By this view, the polytenization step provides multiple substrates for production of various products, and, if sufficient numbers of chromatids are made, then it becomes statistically unlikely that any of the various products will be omitted from the emerging macronucleus. Having evolved to this state (alternative vs. variable processing), the system is free to evolve

mechanisms of differential expression from the alternative chromosomal forms upon which it can depend. The purpose of the second amplification step would be not only to make more copies of the products of the rearrangement step, but also to provide a means for sorting out alternative products and equipping the exconjugant with a mature, copy-adjusted macronucleus competent to drive vegetative processes.

Gene expression from the 81-MAC family chromosomes

To learn what purpose alternative processing might serve, a search of 81-MAC DNA for transcription units and genes was begun. With the possible exception of the long, conserved pre-ATG portion of the conserved open reading frame, our results are consistent with the notion that there is a single gene, solely within the common region, and that the arms are devoid of genes. Of course, our failure to detect other transcripts besides the polyadenylated ~1300-nucleotide species merely could reflect insensitivity of our techniques; for example, a small or rarely used alternative exon or a tRNA gene could easily have escaped detection. Furthermore, genes expressed only in special conditions would have been missed. However, it is not necessarily unreasonable to consider the idea that the arms are nongenic. Both we (K. Williams and G. Herrick, unpubl., in *O. fallax*) and R. Stears and C. Jahn (in *O. nova*; pers. comm.) have detected histone H4 genes on three sizes of macronuclear chromosomes, reminiscent of the 81-MAC family. The middle-sized 1.7-kbp chromosome of *O. nova* has been cloned and sequenced. The 0.3-kbp H4 gene is quite close to one end; the bulk of the chromosome is devoid of long open reading frames and GC-poor. Whether the H4 chromosome family is produced by alternative processing is not known, but one might expect the 81-MAC arms not to have genes. This, of course, need not mean that the arms have no function.

Two kinds of known subtelomeric genes have unusual control features. The case of "expression-linked copies" of trypanosome surface antigen genes is widely recognized (Boothroyd 1985). The surface antigen genes of *Paramecium* are environmentally regulated and subtelomeric (Preer 1986). The history of the *P. tetraurelia* mutant d16 is intriguing (Epstein and Forney 1984). It was selected twice for inability to express its A surface antigen gene, but then reverted to an apparently full wild-type phenotype. Nonetheless, when its DNA was analyzed, the A gene showed an aberrantly processed subtelomeric region, such that the telomere was misplaced immediately 3' of the gene. This suggests that telomere placement has some effect on the expression of the neighboring gene. Nearly all genes in the macronucleus of a hypotrich might be considered subtelomeric. It seems possible that there is but one gene in the 81-MAC family, located completely within the common region, and that the two subtelomeric arms are involved in some way with differential control of the copies of that gene on the respective chromosomes. We currently are devising experiments to determine from which chromosome(s) the ~1300-nucleotide RNA is transcribed.

Experimental procedures

DNA and RNA isolations

All materials were derived from *Oxytricha fallax* subclone 3.5. Macronuclear DNA was isolated as described previously (Cartinhour and Herrick 1984). All cloned DNAs were isolated previously (Cartinhour and Herrick 1984; Herrick et al. 1985, 1987); each cloned macronuclear DNA (pMA) discussed was isolated independently and shown subsequently to be distinct from each of the others by restriction mapping and/or sequence determination. RNA was isolated by modifications of the procedure of Klobutcher et al. (1984): lysis was in 2% SDS, 100 mM Tris-HCl (pH 8.1), 10 mM EDTA, 50 µg/ml potassium polyvinylsulfate, and bentonite was omitted; DNase I (Cooper, DPFF grade) was pretreated with iodoacetate (Zimmermann and Sandeen 1966). Polyadenylated RNA was isolated by chromatography on oligo(dT)-cellulose (Collaborative Research); following denaturation in 15 mM methylmercury hydroxide, total RNA was diluted 50-fold into 0.5 M NaCl and applied to the column at room temperature (Richards and Ehrenfeld 1980). Normally cells were prepared for harvest by a final feeding of autoclaved bacteria, followed by a period of 2–7 days at 4°C to prevent encystment while allowing previous food vacuoles derived from *Chlamydomonas* to be chased out (Dawson and Herrick 1982). In some cases, RNA was prepared from room temperature-grown cells immediately after they had cleared the final feeding of *Chlamydomonas*; no significant differences in total or polyadenylated RNA yields were observed: per 10⁶ cells, 0.64 mg total RNA, and 5 µg polyadenylated RNA (0.8% of total).

DNA sequencing

Regions to be sequenced were subcloned into one of the pEMBL plasmid vectors, single-strand templates were prepared, and sequences were determined by the dideoxy chain-termination method (Dente et al. 1983; Herrick et al. 1985). The macronuclear chromosome of pMA83s was subcloned in alternative orientations into pEMBL plasmids, and complementary deletion sets were prepared and sequenced by the method of Henikoff (1984). This provided a common region fully sequenced on both strands. Other regions were sometimes sequenced on only one strand when they could be compared to corresponding regions that had been sequenced on both strands. The DNA sequences generated can be obtained from the authors or from GenBank. The DNA sequences corresponding to the marked regions of the clones shown in Figure 1 have the following names and GenBank accession number: SRRHGKH-a, #13033; mic-KH-a, #13042; Gp-a, #13034; pSRRHG-b, #13031; Kp-b, #13032. The name indicates the single-letter codes for the landmark restriction sites (see key to Fig. 1) encountered from left to right in the sequence of a version, with the version indicated by the suffix. The sequences are exclusively macronuclear, except for mic-KH-a, which is the vA micronuclear *KpnI*–*HindIII* interval carrying the rightward IES. See accession numbers 13029–13042 for the full set of sequences relating to the 81-MAC family and Herrick et al. (1985, 1987) for descriptions of sequences not described here.

DNA sequences were assembled and manipulated by a variety of computer programs, including RnY analysis (Shepherd 1981) and homology search programs provided by J. Shepherd and run for us by G. Lark, NucFreak (written by E. Kofoid), PC/Gene, and GEL and SEQ at Intelligenetics (NIH 1 U41:RR-01685-05). The Intelligenetics IFIND program was used to search the NBRF protein sequence data base [Release 10].

Gel electrophoresis, blotting, and hybridization

Southern blotting from agarose gels was as described previously (Cartinhour and Herrick 1984). Northern blots were made from formaldehyde (Goldberg 1980) or methylmercury hydroxide gels (Bailey and Davidson 1976) by capillary transfer onto Gene-Screen nylon (DuPont), followed by UV cross-linking (Church and Gilbert 1984).

Southern blot probes were DNA oligomers synthesized as described previously (Herrick et al. 1985). The vB-specific 16-mer 5'-GCAATTTTATAAATCG corresponds to nucleotides 26–41 to the right of the common region *KpnI* site (see Fig. 1). It mismatches with the corresponding regions of vA (TAAATTTTATAAATCG) and vC (GAAATTTTAGAAATCA). The control 18-mer 5'-GGCATCTTTCTTGATTGC exactly matches nucleotides 85–102 to the right of the common region *KpnI* site in all three versions. Oligomers were 5' ³²P-labeled by kination (Zoller and Smith 1984) and used directly as probes. With the 16-mer, prehybridization (2 hr) and hybridization (20 hr) were performed at 37°C, or 3°C below the calculated *T_d* (Suggs et al. 1981), by the protocol of Wood et al. (1985), with 8.4 pmoles of probe. The blot was washed in 6× SSC at 4°C prior to stringent washes of 1 min in 6× SSC at 41°C and 47°C (*T_d*+4°C and *T_d*+12°C, respectively). With the 18-mer, prehybridization (2 hr) and hybridization (22 hr) were performed at 46°C, by the protocol of Church and Gilbert (1984), with 12.2 pmoles of probe. The blot was washed in 6× SSC at 22°C prior to a 2.5-min stringent wash in 6× SSC at 52°C (*T_d*+6°C).

Northern blot probes were prepared by a modification of the procedure of Miller (1984) in which 3000 Ci/mM ³²P were incorporated by DNA synthesis across the insert of a single-stranded pEMBL8 subclone, primed by a standard DNA sequencing primer. This product was usually isopropanol-precipitated, denatured, and used directly as a probe. For polarity determinations, the labeled single strand was isolated by restriction cutting at the far side of the insert, electrophoresis in formaldehyde-agarose, and excision of the gel fragment, which was melted and used as a probe. In some cases, nick-translated (Maniatis et al. 1975) plasmids were used as probes. Hybridization was performed at 65°C by the procedure of Church and Gilbert (1984), followed by stringent washes in 0.5× SSC, 0.1% SDS at 65°C.

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Alternative processing during development of a macronuclear chromosome family in *Oxytricha fallax*.

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