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The DNA of Ciliated Protozoa

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INTRODUCTION

Ciliates emerged as an evolutionary group more than 10^9 years ago, separating from the eukaryotic line that led to plants and animals before the fungi appeared (87, 101, 194, 198) (Fig. 1). By that time meiosis and fertilization had been established in eukaryotes, and modern ciliates share these functions with other contemporary eukaryotes. In the 10^9 plus years since the evolutionary establishment of the first progenitor in the group, the ciliates have diverged into a rich assortment of subgroups containing many thousands of species. A sense of the evolutionary diversity among the ciliates themselves and between the ciliates and other eukaryotes can be gained from Fig. 1, redrawn from reference 216. For example, the genetic distance between the two ciliates *Euplotes* and *Tetrahymena* is about the same as between corn and the rat. Despite their great genetic diversity, the ciliates remain united by two characteristics: the possession of complexes of cilia, used for swimming or crawling and for phagocytic food capture, and the presence of nuclear dimorphism or duality. Each ciliate contains a germ line nucleus (micronucleus) used for sexual exchange of DNA and a somatic nucleus (macronucleus) for production of RNA to support vegetative cell growth and cell proliferation. When two cells mate, they exchange haploid micronuclei and develop a new macronucleus from a micronucleus. This review is about some structural and functional properties of the micro- and macronuclear genomes and about the remarkable developmental processing of DNA that has evolved with nuclear duality in ciliates.

The molecular genetics of only a few among the thousands of species of ciliates have been studied so far. These include *Tetrahymena* and *Paramecium* species (in the order Hymenostomatida) and *Oxytricha*, *Stylonychia*, and *Euplotes* species (in the order Hypotrichida), and this review is primarily about these organisms. Few investigations of the molecular genetics of the thousands of organisms belonging to the other 50 to 60 orders and suborders of ciliates have yet been made. Much more detailed, earlier information about many of the issues discussed in this review may be found in *The Molecular Biology of Ciliated Protozoa* (106), in *The Protozoan Nucleus, Morphology and Evolution* (289), in *Molecular Approaches to the Study of Protozoan Cells* (159), and in reviews by Yao (354) and Nomoto et al. (249).

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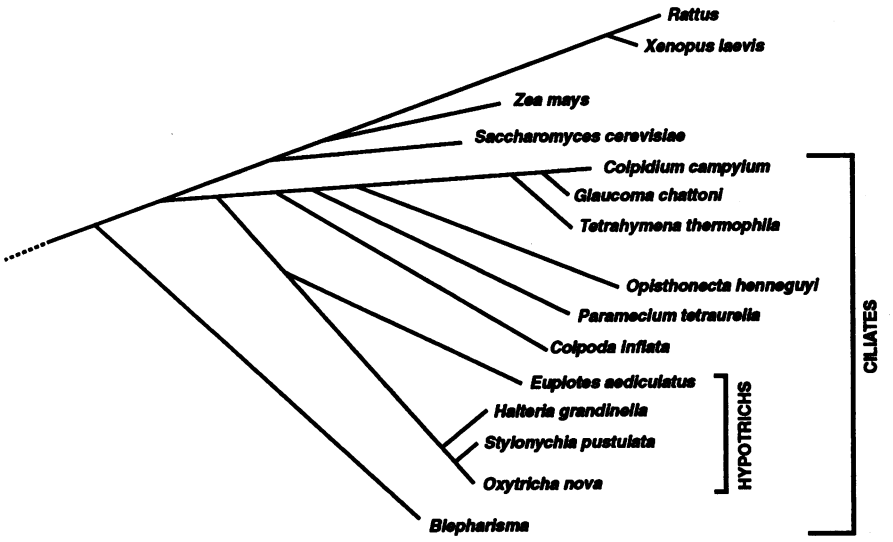

FIG. 1. Phylogenetic tree of eukaryotes including 11 genera of ciliates, derived from nucleotide sequence homologies. The addition of *Blepharisma* (not a hypotrich) is based on the phylogenetic tree of Fleury et al. (101). The evolutionary distance between branch points is proportional to the horizontal distance between them. Redrawn from reference 216.

NUCLEAR DUALITY

Different ciliate species contain different numbers of micronuclei (Fig. 2). *Tetrahymena* species have one micronucleus, *Paramecium* species have two to many depending on the species, and *Oxytricha* species usually have two to four. *Stylonychia* species have 2 micronuclei, *Euplotes* species have 1, and, at an extreme, *Urostyla grandis* (a hypotrich) has 5 to 20. The multiple micronuclei in a single organism are all genetically identical; they all are derived by mitosis from one original micronucleus formed by fertilization at cell mating. Micronu-

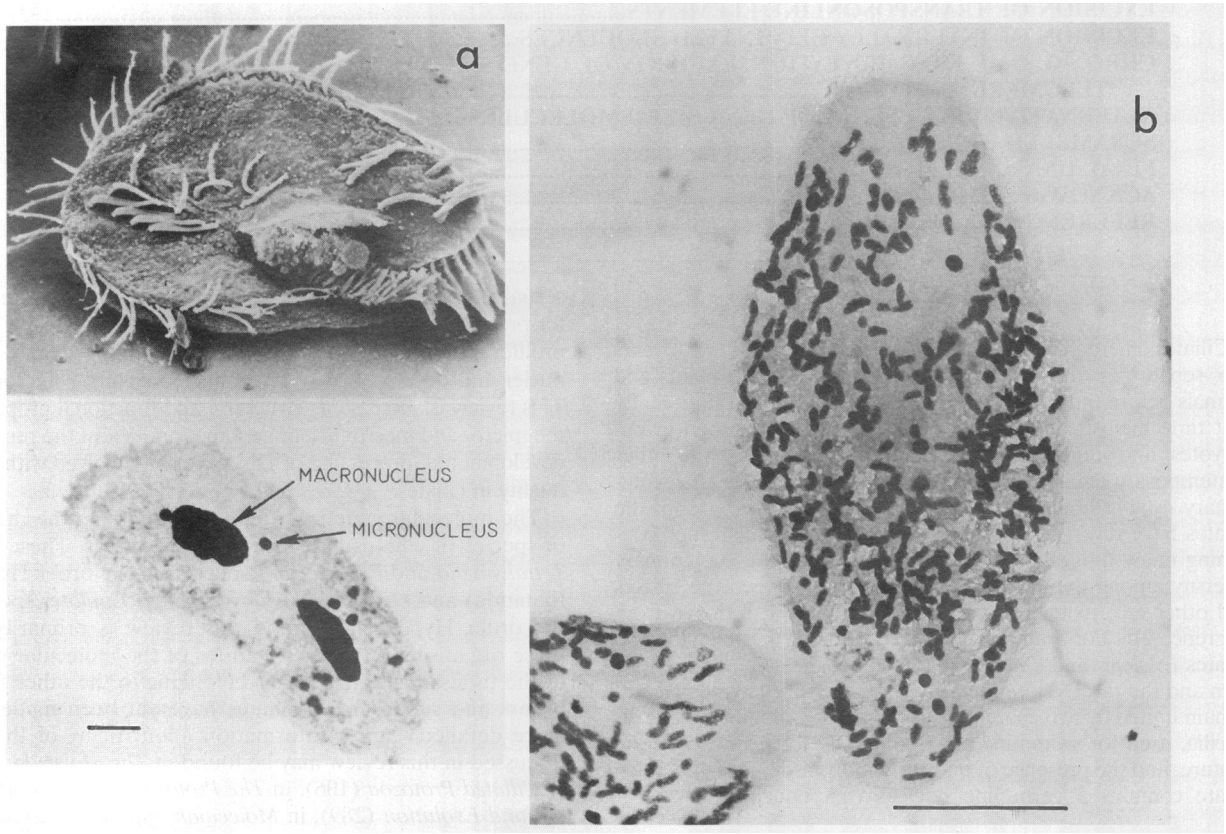

FIG. 2. (a) Scanning electron micrograph (top) and Feulgen-stained micrograph (bottom) of *O. nova*. Bar, 10 μm. (b) Feulgen-stained micrograph of the hundreds of macronuclei of *U. grandis*. The several micronuclei are spherical. Bar, 50 μm.

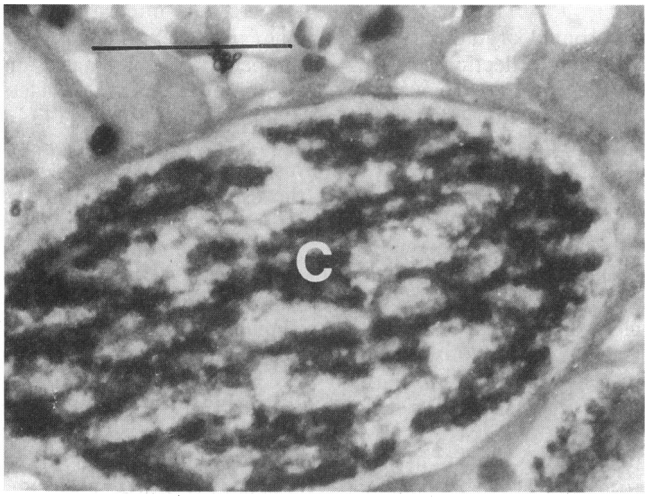

FIG. 3. Late-prophase micronucleus in *Onychodromus quadricornutus*. No chromosomes are distinguishable, only parallel strands of chromatin. Bar, 2 μm. From reference 207.

clei divide mitotically during vegetative growth, but the form of mitosis is different from that seen in plant and animal cells. Mitosis occurs intranuclearly, i.e., without breakdown of the nuclear envelope, and individual chromosomes are not distinguishable. Rather, the mitotic micronucleus contains long strands of chromatin (Fig. 3) that distribute to produce two genetically equivalent daughter micronuclei. The details of the process are poorly understood.

Tetrahymena and *Paramecium* species generally have a single ovoid macronucleus. *Oxytricha* and *Stylonychia* species have two, and all *Euplotes* species contain a single, highly elongated macronucleus (Fig. 2). *U. grandis* has several hundred macronuclei, and the number is variable. These are small, about the size of micronuclei, and are dispersed throughout the cell; in this case the term "macronucleus" is a misnomer. In most species the multiple macronuclei fuse into a single macronucleus just before nuclear division. This is a spectacular event in *U. grandis*, with hundreds of macronuclei fusing into one gigantic macronucleus in a matter of minutes (Fig. 4). As cytokinesis begins, the composite macronucleus in various species quickly undergoes one or more rapid, successive amitotic divisions to produce the appropriate number of daughter macronuclei in each daughter cell. Nothing is known about the triggering of macronuclear fusion at the beginning of cell division or its molecular mechanism or what controls and accomplishes amiotic division at the end of cell division. Perhaps the cytoskeleton mediates these events.

Chromosomes are not visible in dividing macronuclei; the DNA is packaged in long, parallel bundles of chromatin strands during amitosis (Fig. 3). Amitosis is an imprecise division mechanism; the DNA is unequally distributed to daughter macronuclei (37, 62, 81, 174, 193, 205, 226, 320, 344), one macronucleus sometimes receiving as much as 10 or 15% more DNA than the other.

The micronucleus serves as a germ line nucleus, undergoing meiosis followed by exchange of haploid micronuclei between two mating cells (discussed below). It is genetically silent or nearly so during vegetative growth. When a *Paramecium* cell is experimentally constructed to contain alleles that differ in the micronucleus and macronucleus, only the macronuclear alleles govern the cell phenotype (306). In *Tetrahymena* species, no genes in the micronucleus that influence the phenotype of a

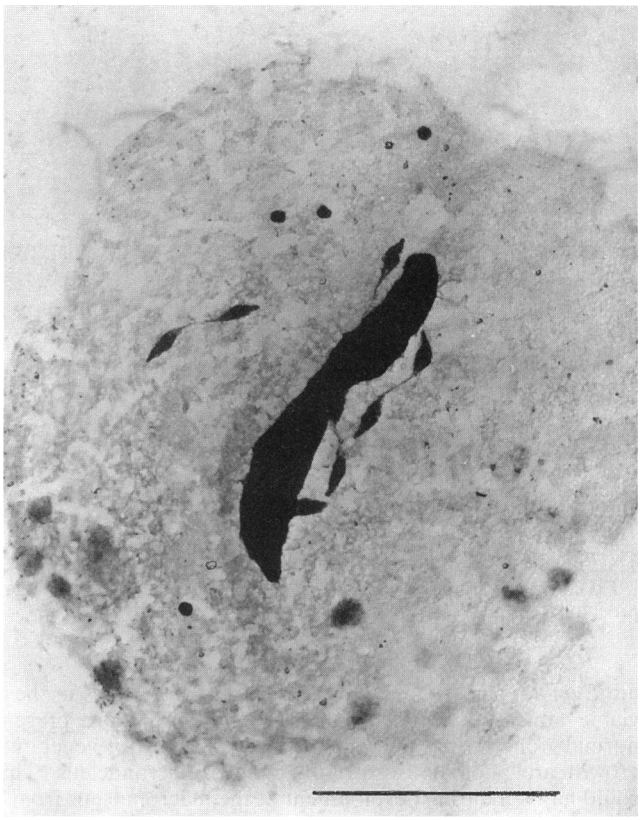

FIG. 4. *U. grandis* beginning amitosis. The hundreds of macronuclei have fused into one composite nucleus. The spindle-shaped structures are micronuclei in metaphase. Several micronuclei are still in interphase. Bar, 50 μm.

vegetative cell have been identified (51, 225). This does not preclude the possibility that some micronuclear genes, perhaps only a few particular genes, are expressed during vegetative growth. A small amount of RNA synthesis has been detected autoradiographically in *Stylonychia* (15), *Tetrahymena pyriformis* (241), and *Paramecium caudatum* (258, 291) micronuclei. In *Paramecium* and *Tetrahymena* species, RNA labeling was detectable only during the rather short period of micronuclear DNA synthesis. In an extensive study Gorovsky and Woodard (117) found no autoradiographic evidence of RNA synthesis in the *Tetrahymena* micronucleus. The issue is worth further investigation. Total genetic silence of the micronucleus seems puzzling because experimental removal of the micronucleus(ei) from various species of ciliates usually results immediately in a lower reproductive rate or renders a cell incapable of vegetative growth, with consequent death (5, 234, 244, 333, 336). This clearly says that the micronucleus does make a contribution, perhaps expressing a few micronucleus-specific key genes that are absent from the macronucleus and are essential for vegetative growth (8, 164).

This idea seems to be contradicted by the fact that amicronucleated ciliates created by mutagenesis (164), by removal of the micronucleus with a micropipette (233), or by elimination of the micronucleus by UV or X irradiation (15) sometimes survive and proliferate at a normal rate. Moreover, amicronucleated ciliates are often encountered in the wild, and these proliferate vigorously in laboratory culture. These include *Tetrahymena* (86), *Stylonychia* (20), *Oxytricha* (Fig. 5) (76, 273), and *Paraurostyla* (273) species, among others, but amicronucle-

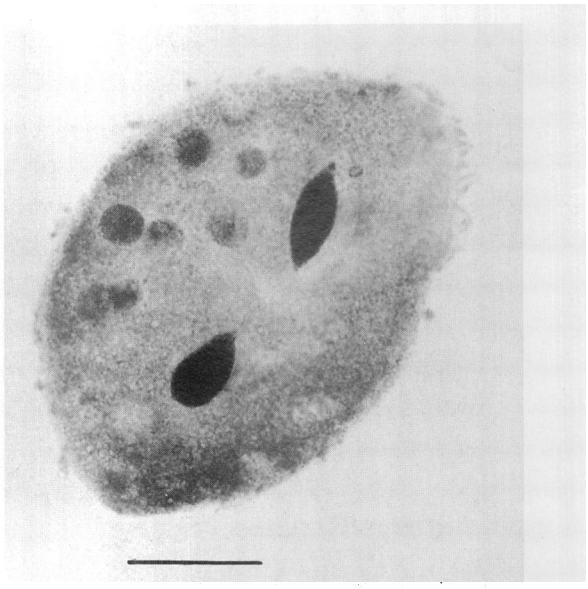

FIG. 5. Amicronucleated *O. nova* cell stained by the Feulgen procedure. Bar, 25 μm.

ate *Euplotes* strains have not been reported. Perhaps in these viable amicronucleated cells, a few genes that are present normally only in the micronucleus and needed for vegetative growth are anomalously present in the macronucleus. This could happen during development of the macronucleus from a micronucleus after cell mating; the few genes normally restricted to the micronuclear genome because they are eliminated from the developing macronucleus (anlage) may escape elimination and be included in the new macronucleus (164). (The developing macronucleus is called the anlage in this review.) The proposition of failed elimination is consistent with the observation that the macronucleus of a viable amicronucleated strain of *T. thermophila* contains a few DNA sequences that are normally restricted to the micronuclear genome (167, 169) and that may represent a particular gene or genes whose products have a special role in vegetative cell growth. Study of such normally micronucleus-specific sequences might not only define the vegetative contribution of the micronucleus but also provide information about the mechanism that selectively eliminates particular DNA sequences during macronuclear development. The normal retention in the micronucleus and elimination from the micronucleus of one or a few active genes essential for vegetative growth would, by making the micronucleus essential for vegetative growth, eliminate from a population organisms that had lost the capability of sexual reproduction.

MICRONUCLEAR AND MACRONUCLEAR STRUCTURE

The DNA in the vegetative micronucleus occurs in uniformly and densely packed chromatin and is otherwise featureless when observed by light or electron microscopy. It resembles the chromatin in a sperm cell or in a nucleated erythrocyte. There are no nucleoli. The micronuclear envelope has typical pores. The DNA in the macronucleus occurs in many chromatin bodies dispersed in the nucleoplasm (see Fig. 12). Autoradiography has shown a high rate of RNA synthesis associated with these bodies. Multiple nucleoli are present, and the nuclear envelope has numerous pores. The DNA in both macronuclei and micronuclei is organized into nucleosomes (52, 116, 200, 212). The DNA repeat unit is longer in macronuclear nucleosomes than in micronuclear nucleosomes, probably reflecting the greater compaction of chromatin and the transcriptional inactivity of the micronucleus. The nucleosomal core unit of macronuclear DNA is the usual ~143 bp (111, 200, 212, 329).

The *Oxytricha* (52, 54), *Stylonychia* (211), and *Tetrahymena* (128, 161) macronuclei contain the usual five major histones. Histone H1 is absent from the micronucleus of *T. thermophila*; three major peptides appear to be associated with the linker region of DNA between nucleosomes in place of H1 (7). How the histone differences between micronucleus and macronucleus are related to the transcriptional differences is unclear.

The amino acid sequences of the histones, most notably of histone H4, differ substantially from those of other eukaryotes (32, 112, 115). Among the 102 amino acid residues of H4, pea plant H4 differs from cow H4 in two positions and yeast H4 differs from cow H4 in eight positions. *Tetrahymena* H4 differs from cow H4 in 20 positions. The H4s of *Oxytricha* (130) and *Stylonychia* (331) species, two closely related hypotrichs that have the same H4 amino acid sequence, differ from human H4 in 18 amino acids. *Oxytricha* and *Stylonychia* H4s differ from *Tetrahymena* H4 in 20 positions. These differences reflect not only the ancient split of the ciliates from the main eukaryotic line but also the enormous genetic distances within the ciliate group.

A variant of histone H2A called hv1 is absent from the transcriptionally inactive vegetative micronuclei in Tetrahymena but is present in the macronuclei (308a). The hv1 variant appears transiently in micronuclei during the brief period when they become transcriptionally active in conjugation (336a), suggesting that the absence of hv1 in micronuclei may be causally related to the transcriptional silence of micronuclei at all other times (308a).

MICRONUCLEAR AND MACRONUCLEAR DNAs OF *TETRAHYMENA* AND *PARAMECIUM* SPECIES

The structure and function of the micronucleus and macronucleus are very different, and the differences are reflected in their genomic DNAs. Micronuclei are generally diploid. Genetic studies of *Tetrahymena* (51), *Paramecium* (see, e.g., reference 266), and *Euplotes* (see, e.g., reference 28) species show that genes are present in single copies per haploid micronuclear genome. Even the gene encoding rRNA in *Tetrahymena* species, unlike other eukaryotes, is present as a single copy in the micronucleus (356, 363). The macronuclei of ciliates are usually described as highly polyploid. Sensu strictu, the term "polyploid" is inappropriate because the macronuclear genome is not simply a multiplied version of the total micronuclear genome but, rather, of some fraction of the micronuclear genome (discussed below). The DNA content of the *Tetrahymena* macronucleus (10.2×10^9 bp) is on average about 46 times greater than the micronuclear haploid value (2.2×10^8 bp) (82, 109, 152, 300, 303, 346) (Table 1), but it can be misleading to refer to the macronucleus as 46C, as is often done. The letter C stands for the amount of DNA in a haploid genome, usually measured in germ cells of various eukaryotes; in ciliates this is the micronucleus after meiosis. The term 46C implies the presence of 46 haploid genomes. The macronuclear-to-micronuclear ratio of 46 represents an average and is not firmly fixed. The macronuclear DNA content can vary over an approximately twofold range (7.5×10^9 to 13.2×10^9 bp) in stable *Tetrahymena* cultures and even more widely when nutrient or temperature conditions are changed (301).

The macronuclear DNA content of *P. primaurelia* and *P.*

TABLE 1. Micronuclear and macronuclear DNA content and sequence complexity for five ciliates[a]

Organism	Micronuclear content (bp)	Micronuclear complexity (bp)	Total macronuclear content (bp)	Macronuclear complexity (bp)
Tetrahymena sp.	2.2×10^8	ND[b]	10.2×10^9	2.2×10^8
Paramecium sp.	2.9×10^8	ND	2.42×10^{11}	ND
Oxytricha sp.	1.2×10^9	$(0.3–2.3) \times 10^9$	1.05×10^{11}	5.5×10^7
S. lemnae	1.5×10^{10}	2.2×10^9	7.19×10^{11}	4.7×10^7
E. aediculatus	1.8×10^9	ND	3.47×10^{11}	4.2×10^7

[a] See the text for references for these values.
[b] ND, not done.

tetraurelia is $\sim 2.42 \times 10^{11}$ bp, and the haploid micronuclear content is $\sim 2.9 \times 10^8$ bp (109), giving a macronuclear-to-haploid micronuclear DNA ratio of ~800 (36, 305, 345, 347; see reference 289 for a list of macronuclear DNA contents in various species).

The genomes in the two kinds of nuclei are obviously intimately related, because the macronucleus forms from a micronucleus after mating. Therefore, all of the DNA sequences present in the macronucleus are derived from the micronucleus, but the macronuclear sequences are only a subset of the micronuclear sequences. Hybridization between *Tetrahymena* micronuclear and macronuclear DNAs has shown that 10 to 20% of the micronuclear DNA sequence complexity is eliminated in the anlage (150, 152, 357, 358). *Tetrahymena* micronuclear DNA is composed of about 20% moderately repetitious sequences (copy number of ~200 or less [339, 358]) and 80% unique sequences; most of the members of the repetitious families and some unique sequences make up the eliminated DNA (50, 150, 166, 339, 352). The sequence complexity of macronuclear DNA determined by reassociation kinetics is $\sim 2.2 \times 10^8$ bp and presumably a little less than the sequence complexity of micronuclear DNA. Reassociation kinetics usually do not give a precise measure of complexity. The macronuclear-to-micronuclear ratio of 46 means that the macronucleus contains about 57 copies, on average, of the unique sequences in the micronuclear genome. The reassociation kinetics of macronuclear DNA suggest that most sequences are amplified about equally (152, 358), but there are some differential amplifications, notably DNA molecules encoding rRNA (discussed later) and at least a few other molecules (48).

ELIMINATION AND FRAGMENTATION OF DNA IN *TETRAHYMENA* SPECIES

There are ~6,000 cutting-splicing sites at which sequences are deleted from anlage DNA. The deleted sequences range in length from about 100 bp to more than 10 kbp (53, 351, 355, 362) and are distributed throughout the five chromosomes of *Tetrahymena* species (50, 150, 166). Among the eliminated repetitious DNA are interstitial, tandem repeats of the telomere sequence 5′-dC_4dA_2-3′ (61, 366). Some or all of the interstitial 5′-dC_4dA_2-3′ repeats may be arranged in inverted repeat pairs, defining the termini of a transposable element belonging to a family of transposable elements (called the Tel-1 family) that are excised and destroyed in the *Tetrahymena* anlage (61). The function of the various eliminated sequences is not known. Possibilities are contributions to mitosis and/or meiosis; one or more eliminated sequences may be essential for vegetative growth, as discussed above.

In addition to the ~6,000 cutting-splicing events, micronuclear DNA is cut into permanent subchromosomal fragments. Micronuclear DNA has a very high molecular weight; assuming one DNA molecule per chromosome, as in other eukaryotes, the five chromosomes would average ~44,000 kbp each in length (one-fifth of 2.2×10^8 bp). Fragmentation produces up to 200 different subchromosomal DNA molecules ranging in size from less than 100 to over 1,500 kbp, with an average of 800 kbp (10, 66) (an exceptionally small molecule in *Tetrahymena* species is a 21-kbp DNA molecule encoding rRNA [discussed below]). A variable amount of DNA ranging from 54 bp to >4 kbp is eliminated at each breakage site. Fragmentation is precise to the degree that particular macronuclear DNA sequences are reproducibly found in the same-sized fragment. Each fragment is present on average in ~57 copies per macronucleus. Telomeric repeats of 5′-dC_4dA_2-3′ are added to the ends of the fragments in the anlage (360) by telomerase (370) and provide for stability and replication of the fragments in the vegetative macronucleus. Telomere structure and function and telomerase are discussed below.

A particular fragmentation event in the *Tetrahymena* anlage excises the single copy of the rRNA gene (356) from its micronuclear chromosomal location (90, 105, 359). During or after excision, the gene is duplicated into a palindrome to create a 21-kbp linear DNA molecule containing two head-to-

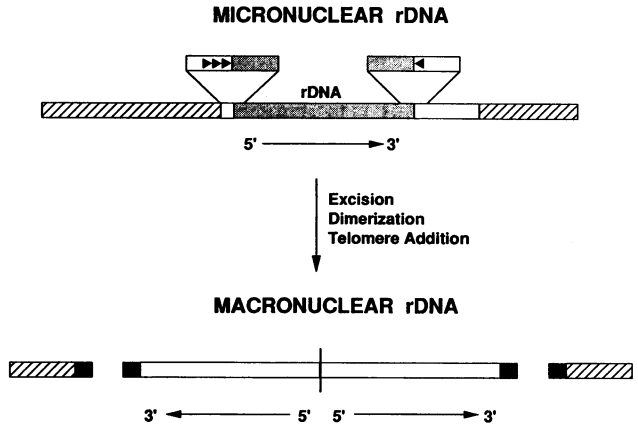

FIG. 6. Structure of micronuclear rDNA (top) and macronuclear rDNA (bottom) in *Tetrahymena* species. The rDNA gene (stippled bar) in the micronucleus is flanked by sequences that are eliminated when the gene is excised (open bars). The expanded regions of the micronuclear rDNA show the positions of three 15-bp Cbs copies in the 5′ eliminated segment, and one 15-bp Cbs copy in the 3′ eliminated segment (Cbs copies are denoted by solid triangles). A pair of 42-bp inverted repeats separated by 28 bp in the 5′ end of the micronuclear gene becomes the center of the head-to-head palindromic dimer of macronuclear rDNA. Telomeric sequences are added to the ends of the macronuclear rDNA dimer and to the two excision/elimination points in the flanking DNA (cross-hatched). Redrawn from Yao et al. (362).

head copies of the rRNA gene (168) (Fig. 6). The 21-kbp palindrome is amplified in the anlage to 9,000 molecular copies, or 18,000 gene copies, which is ~300 times more than the average copy number for the other subchromosomal fragments in the anlage. Telomeric sequences consisting of 20 to 70 tandem copies of 5′-C_4A_2-3′ are added to each end of the palindrome by telomerase (176, 370). Each gene encodes 25S and 17S rRNA as a single transcript (91, 359); the two transcripts are made in opposite directions from start sites near the center of the palindromic molecule. The two arms of the palindrome are connected head-to-head at the center of the molecule by 24 to 28 bp depending on the *Tetrahymena* species (88, 163, 178).

Formation of the palindrome from the monomeric rDNA molecule depends on a pair of 42-bp inverted repeats at the 5′ end of the rDNA (Fig. 6) and a free end that is 5′ to the inverted repeats in the monomeric, micronuclear rDNA gene (364). The two repeats in a pair are separated by the 28-bp sequence that later becomes the spacer between the two arms of the rDNA palindromic dimer. Yasuda and Yao (364) present some models that explain the roles of the 42-bp inverted repeats and free 5′ end in the formation of a palindrome.

Fragmentation also occurs in a close relative of *Tetrahymena* species, *Glaucoma chattoni* (171). The macronuclear DNA ranges in size from greater than 100 down to 2.1 kbp, with many 5′-dC_4dA_4-3′ repeats at the molecular ends. rRNA is encoded by a 9.3-kbp molecule. Fragmentation also occurs in the *Paramecium* anlage, although less is known about it than in *Tetrahymena* species. The haploid micronuclear genome of *Paramecium* species contains 0.30 pg or 2.9×10^5 kbp and is made up of 40 to 45 chromosomes (79), which means an average DNA size of 700 kbp per chromosome. Macronuclear DNA molecules have an average molecular size of 300 kbp (70, 267), suggesting that only a few fragmentation events occur per chromosome, many fewer than in *Tetrahymena* species, but the end result is subchromosomal fragments of about the same size as in *Tetrahymena* species. Telomeric sequences composed of 5′-dC_4dA_2-3′ or 5′-dC_3dA_3-3′ tandem repeats are added to the new ends created by fragmentation in *Paramecium* species (35). Molecules encoding rRNA in the *Paramecium* macronucleus are not palindromic as in *Tetrahymena* species (98). They are of various lengths and contain correspondingly one to several copies of the rRNA transcription unit arranged head-to-tail in tandem and separated by spacers (99).

The many careful studies of *Tetrahymena* species have identified three global changes in DNA when a micronucleus is converted into a macronucleus: (i) repetitious sequences and some unique sequences dispersed throughout the genome are deleted and destroyed; (ii) the original five pairs of chromosomal DNA molecules are fragmented in a specific pattern into roughly 200 molecules; and (iii) each of the ~200 different subchromosomal molecules is amplified to an average of ~57 copies in the mature macronucleus. Both of the haploid genomes in the original diploid micronucleus are represented initially among the 57 copies of each subchromosomal fragment in a new diploid macronucleus, but cells eventually become homozygous because of random distribution of alleles by amitotic division (51), a process called phenotypic assortment.

What do these three global DNA changes in the germ line genome accomplish functionally? The single most profound functional change in the anlage is conversion from the complete or nearly complete transcriptional silence of the micronucleus to the highly active transcriptional state of the mature macronucleus. It is not apparent how fragmentation, deletion, or amplification of the genome might be related to the transcriptional activation in the anlage. Although the amplification of the subchromosomal DNA molecules makes possible a very high rate of RNA synthesis in the macronucleus, it is probably not involved in the activation of transcription. The high rate of RNA synthesis engendered by amplification is presumably necessary to support large cells, such as ciliates. In general, the bigger the ciliate, the greater is the amplification in its macronucleus(ei). Transcriptional activation might be brought about by changes in proteins associated with the DNA, perhaps including histones, rather than any change in the DNA molecules themselves. Histone changes that occur in the *Tetrahymena* anlage have been reviewed by Gorovsky (115) and Nomoto et al. (249).

It is possible that repetitious sequences throughout the micronuclear chromosomes provide for a global organization of chromatin that maintains genetic repression or that the deleted sequences have some other role in silencing the genome (353), but there is no evidence to support either idea. On the contrary, at least one micronucleus-specific repetitious sequence designated M has no general repressor action (or deleterious effect) when it is experimentally introduced into a macronucleus and specifically does not repress rDNA to which it has been experimentally spliced (114). It is also unclear what role amplification, deletion, or fragmentation might have in the switch from mitotic division in the micronucleus to amitotic division in the macronucleus. Perhaps among the deleted sequences are some essential to mitosis, e.g., centromeres (centromeric DNA has not yet been identified in *Tetrahymena* micronuclear chromosomes). In any case, fragmentation into subchromosomes would presumably create many acentric fragments and thereby make successful mitosis impossible even if the original five centromeres were still present. Fragments could undergo mitotic assortment if the five original chromosomes were holocentric, but observation of meiotic chromosomes shows no evidence of holocentricity.

In hypotrichs (discussed below), 95% or more of the micronuclear sequence complexity (and total DNA) is deleted. The elimination of so much sequence complexity, which is obviously superfluous to vegetative growth, may represent a significant reduction in the burden of maintaining and replicating unneeded DNA in the genome in the macronucleus. However this idea seems less compelling in *Tetrahymena* species, in which only 10 to 20% of the sequence complexity is eliminated. Perhaps solutions to these problems will begin to emerge when the eliminated repetitious and unique sequences of *Tetrahymena* species have been more thoroughly characterized.

MICRONUCLEAR AND MACRONUCLEAR DNAs OF HYPOTRICHS

Hypotrichs undergo the same three global genomic changes of elimination (deletion), fragmentation, and amplification of DNA sequences as do *Tetrahymena* cells during the conversion of a micronucleus to a macronucleus, but the phenomena are more extreme and differ in significant respects. Table 1 includes the macronuclear and micronuclear DNA contents and sequence complexities of the major species of hypotrichs studied so far. The total content of macronuclear DNA per cell exceeds the content of a single micronucleus in the different species by ratios that range from 27:1 for *Euplotes crassus* to 200:1 for *E. eurystomus* (19). These ratios, which reflect amplification of sequences in the macronucleus, become more meaningful when the total sequence complexities of macronuclear and micronuclear DNAs are compared. DNA sequence complexity is available for both the micronucleus and

macronucleus in two organisms, *Oxytricha* species and *Stylonychia lemnae*. The micronuclear-to-macronuclear sequence complexity ratio is an average of 24 for *Oxytricha* species and 47 for *S. lemnae*. These numbers mean that the *Oxytricha* macronucleus has ~4% of the DNA sequences present in the micronucleus whereas the *S. lemnae* macronucleus has ~2% of the sequences that are present in the micronucleus. The numbers can be restated as elimination of 96% of the micronuclear genomic DNA complexity in *Oxytricha* species and 98% in *S. lemnae* during macronuclear development.

As determined by reassociation kinetics, about 30% of the micronuclear DNA of *Oxytricha* species is composed of repetitious sequences and 70% consists of unique sequences (199). Micronuclear DNA in *S. lemnae* contains multiple repetitious components (21, 189, 311). Macronuclear DNA in both species consists of a single kinetic component derived from a subset of the unique sequences in the micronucleus. Thus, in the anlage all of the repetitious sequences and most of the unique sequences are eliminated. The sequence complexity in the macronucleus is ~14 times the complexity of *Escherichia coli* DNA for *Oxytricha* species and ~12 times for *S. lemnae*. The small subset of micronuclear unique sequences that become the macronuclear DNA encode all of the genes necessary for cell maintenance and proliferation. If the micronucleus does contribute genetic function to vegetative growth, such contribution must be minute at best, representing no more than a few of the thousands of genes in the micronucleus. In amicronucleate *Stylonychia* and *Oxytricha* strains, the macronuclear DNA unquestionably contains all the genetic information for vegetative growth. This is consistent with the idea that normally the vast majority of sequences in the micronucleus make no contribution to the vegetative life of the organism. Although a few micronucleus-specific sequences may contain genes that function in meiosis and mitosis, most micronuclear DNA in hypotrichs is apparently nongenetic.

The two macronuclei together in *Oxytricha* species contain 1.05×10^{11} bp, with a complexity of 5.5×10^{7} bp. Therefore, there is an average of ~1,900 copies of each sequence per cell or ~950 copies per macronucleus before DNA replication in the cell cycle. Replication produces an average of ~3,800 copies per cell of each sequence, which are distributed imprecisely between the two daughter cells at division. In *S. lemnae* each sequence is present in ~7,500 copies per macronucleus or ~15,000 per cell before DNA replication (312, 349). These very high copy numbers of genes provide for the high rates of transcription that are presumably required to drive the metabolism of very large cells. In their largest dimension *Oxytricha* species are 95 μm and *S. lemnae* is 140 μm.

Micronuclear DNA in hypotrichs has a very high molecular weight. As a rough estimate, the DNA molecule in each of the approximately 120 chromosomes contains on average $\sim 10 \times 10^{6}$ bp in *Oxytricha* species and $\sim 18 \times 10^{6}$ bp in *S. lemnae*. These are crude estimates, but there is no doubt that the DNA molecules are very large. Carefully prepared micronuclear DNA observed in the electron microscope exists as extremely long molecules (274).

The size of the DNA molecules in the macronucleus contrasts sharply with the size of micronuclear DNA (Fig. 7). The low molecular weight of macronuclear DNA was discovered first in *Oxytricha* species (initially misidentified as *S. mytilus*) in sucrose gradients and by electron microscopy (274). Molecules observed by electron microscopy are linear, and most of the molecules are in the 400- to 3,700-bp range, with a number-average size of 2,200 bp for the *Oxytricha* species and *O. nova*, 1,836 bp for *E. aediculatus*, and 2,514 for *S. pustulata* (273, 319). In large-scale cultures, macronuclei are rather easily and efficiently separated from micronuclei and purified by velocity sedimentation and/or isopycnic centrifugation for such organisms as *E. eurystomus*, *E. aediculatus*, *S. lemnae*, *S. pustulata*, and various *Oxytricha* species. Purified hypotrich macronuclear DNA separated by size by agarose gel electrophoresis shows molecules ranging in size from ~400 to ~15,000 bp, with the bulk of the DNA in the 1- to 8-kbp range (Fig. 8). There are rather few molecules with sizes between 8 and 15 kbp and a few molecules ranging in size from 400 to 1,000 bp. The smaller ones are not usually visible in a gel by ethidium bromide staining but can be detected by Southern blot hybridization.

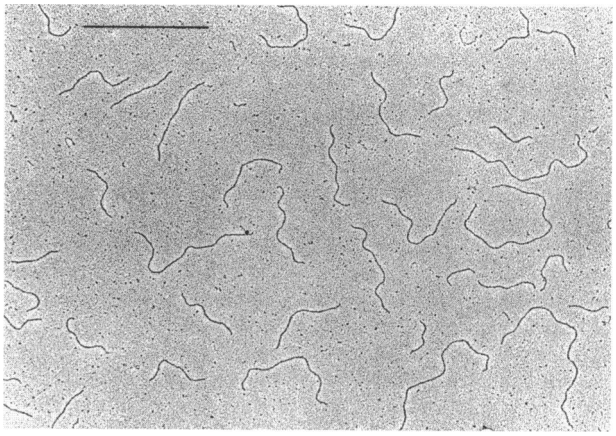

FIG. 7. Electron micrograph of macronuclear DNA isolated from an *Oxytricha* species. The average molecule is 0.8 μm long. Bar, 1 μm. Electron micrograph courtesy of K. G. Murti.

A band is often visible at ~7,400 bp; these molecules are present in a differentially higher concentration and encode 25S, 19S, and 5.8S rRNAs as a single transcript (213, 307, 318). In *O. nova* the rDNA is present in ~100,000 copies per macronucleus, compared with the average of ~950 for other molecules. This differential amplification of rDNA occurs in the anlage of hypotrichs as it does in *Tetrahymena* species (reviewed in reference 353).

A few bands appear at other molecular weight positions in the gel. These may represent other genes that are differentially amplified. These bands and the overall size distribution create patterns that are reproducible from one DNA preparation to

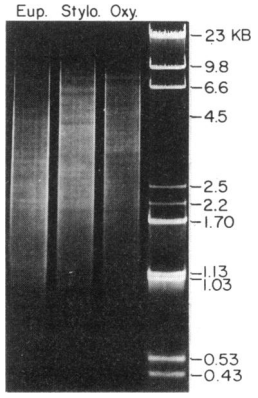

FIG. 8. Size distribution of macronuclear DNA molecules from *E. aediculatus* (Eup.), *S. pustulata* (Stylo.), and *O. nova* (Oxy.) shown by gel electrophoresis. The right lane contains size markers in kilobase pairs. The rDNA is visible as a band at ~7.4 kbp. From reference 318.

FIG. 9. Diagram of the main features of a hypotrich macronuclear DNA molecule. The single coding region usually occupies most of the molecule, and the leader and trailer are short in most cases. Telomere sequences, 5′-$(dG_4T_4)_n$-3′, are present on both ends.

another and differentiate one species from another (313, 319). For example, the size distribution of DNA is perceptibly smaller for *E. aediculatus* than for *Oxytricha* or *Stylonychia* species; the electron microscopy measurements confirm this. The sequence complexity of macronuclear DNA (5.5×10^7 bp in *Oxytricha* species) is carried by molecules that average 2,200 bp in length. This indicates that there are ~24,000 different molecules (each with an average copy number of 950). Thus, after DNA replication in the cell cycle, each of the two macronuclei in *O. nova* contains $\sim 46 \times 10^6$ DNA molecules. The number is even higher in *S. mytilus* and in *E. aediculatus*.

In general, changes of germ line DNA in the anlagen of hypotrichs resemble changes in *Tetrahymena* species: sequences are eliminated, subchromosomal fragments are created, and the retained sequences are highly amplified, but in hypotrichs the alterations are more extreme (discussed below). In addition, in both types of organisms repetitious sequences in the germ line are eliminated in the anlage and tandem telomeric repeats (3′-dG_4T_2-5′ for *Tetrahymena* species and 3′-dG_4T_4-5′ for hypotrichs) are added to the ends of the fragments. All these DNA changes are accompanied by a switch from mitosis to amitosis, which is characteristic of the macronucleus in all ciliate species, and by activation of transcription.

Very few ciliates in other major groups have been studied by molecular biological techniques. The macronuclear DNA of *Stentor coeruleus* (order Heterotrichida and separated from *Tetrahymena* species and hypotrichs by very large genetic distances) appears to occur in subchromosomal fragments. Most DNA ranges from 7.5 to 60 kbp as determined by sucrose gradients and 45 to 75 kbp by electron microscopy (261). Macronuclear DNA in two cyrtophorid ciliates, *Trithigmostoma* and *Chilodonella* species, which are very distant relatives of hypotrichs, ranges in size from 0.35 to ~70 kbp with an average size of 4 kbp and with numerous bands in electrophoretic gels, presumably representing particular, differentially amplified molecules (195, 229). rRNA in the two species is encoded by DNA molecules of 14.5 kbp in *Trithigmostoma* species and 15.5 kbp in *Chilodonella* species, and these appear to be dimers as in *Tetrahymena* species. Both organisms, like hypotrichs, have polytene chromosomes in their macronuclear anlagen (283–286), and those in *Trithigmostoma* species have been observed to break down, as in hypotrichs.

It is a reasonable conclusion that the fundamental strategies of sequence elimination, fragmentation, and amplification were established hundreds of millions of years ago, before the evolutionary split between the order Hymenostomatida (the genera *Tetrahymena*, *Paramecium*, etc.), hypotrichs, and other ciliate groups and may have facilitated the rich evolution among the ciliates.

STRUCTURE OF MACRONUCLEAR DNA MOLECULES OF HYPOTRICHS

Sequence elimination and fragmentation in hypotrichs produce small DNA molecules that can encode only one or a few genes. All of the ~75 macronuclear DNA molecules fully or partially characterized so far in various hypotrich species contain only a single transcription unit or gene. However, the tendency has been to study shorter molecules because such a study is easier; possibly some of the longer molecules (e.g., over 8 kbp) possess more than a single coding function.

The macronuclear DNA molecules in all the hypotrich species studied so far conform to one structural pattern (Fig. 9). Every molecule consists of a 5′ leader and a 3′ trailer that flank the transcription unit. Each end of a molecule has a telomeric sequence made of tandem repeats of 3′-dG_4T_4-5′ in *Oxytricha* (182, 262), *Stylonychia* (209, 252), and *Paraurostyla* (273) species, *Onychodromus quadricornutus* (273), and several related genera (Fig. 9). Unlike *Tetrahymena* telomeres, the length of telomeres in hypotrichs is rather constant, but a small percentage of molecules have one to several extra repeats of 3′-dG_4T_4-5′. For at least some of these molecules the added length is in the double-stranded region, as evidenced by sequencing of molecules cloned after removal of the single-stranded tails (132, 276). Telomere formation and function are discussed below.

Because hypotrich macronuclear DNA molecules are so small and contain single transcription units, they are referred to as gene-sized molecules or minichromosomes. The traditional definition of a chromosome is as a mitotic, meiotic, or polytene entity or as a genetic linkage group; hence the term "minichromosome" is less appropriate than the descriptive term "gene-sized molecule."

The 5′ leaders of hypotrich genes vary from 33 bp (phospholipase C-encoding molecule in *E. crassus*) (184) to ~1,500 bp (rDNA molecule in *Oxytricha* species) (318). Leaders for most of the gene-sized molecules in Table 2 are less than 200 bp. The 5′ leaders presumably contain sequences needed for transcriptional regulation, for specification of transcription start sites, and for origins of replication (replication of the gene-sized molecules in hypotrich macronuclei is discussed below).

TELOMERES

The presence of inverted terminal repeat sequences (telomere sequences) at the ends of macronuclear DNA in hypotrichs was first suggested by the electron-microscopic observation that the denatured gene-sized molecules form single-stranded circles when quickly renatured (337). The inverted repeats, shown by reassociation studies to be the same for all molecules (145), consist of the sequence of repeats of 5′-dCdCdCdCdAdAdAdA-3′ (182, 252) arranged to produce the terminal structure in *Stylonychia* and *Oxytricha* species

$$\begin{array}{r} 5'\text{-}dC_4dA_4dC_4dA_4dC_4\_\_\_\_\_ \\ 3'\text{-}dG_4\ T_4dG_4\ T_4dG_4\ T_4dG_4\ T_4dG_4\_\_\_\_\_ \end{array}$$

and in *E. aediculatus*

$$\begin{array}{r} 5'\text{-}dC_4dA_4dC_4dA_4dC_4dA_4dC_4\_\_\_\_\_ \\ 3'\text{-}dG_2\ T_4dG_4\ T_4dG_4\ T_4dG_4\ T_4dG_4\ T_4dG_4\_\_\_\_\_ \end{array}$$

The motif of 16 bases of single-stranded 3′-dG_4T_4-5′ followed by 20 bp of double-stranded 3′-dG_4T_4-5′ is characteristic of *S. pustulata*, *S. lemnae*, and *S. mytilus*; *O. nova*, *O. fallax* (73), and *O. trifallax*, *Onychodromus quadricornitus*, *Paraurostyla weissei*, and *Urostyla* species (273). The presence of a 14-base single-stranded tail, 3′-$dG_2T_4dG_4T_4$-5′, followed by 28 bp of 3′-

TABLE 2. Characteristics of 47 macronuclear genes in hypotrichs[a]

Gene	Organism	5′ LDR (bp)[b]	TATAA box[c]	CCAAT box[c]	ORF (bp)[d]	3′ TLR (bp)[b]	Poly(A) site[e]	Reference
Actin I	*E. crassus*	54	−27	Absent	1,140	53	+45	131
Actin I	*O. fallax*	183	−47, −30	Absent	1,074	186	+155	162
Actin I	*O. nova*	192	−31	Absent	1,128	212	+43	122
Actin I	*O. trifallax*	184	−47, −30	Absent	1,128	193	+31	84
Actin I	*Oxytricha* strain WR	184	−47, −30	Absent	1,128	190	+30	84
Actin II	*O. nova*	100	−44, −35, −30	Absent	1,128	124	+79	235
AS1	*O. nova*	87	Absent	Absent	129	209	+52	132
AS2	*E. crassus*	104	Absent	Absent	129	210	Absent	132
AS2	*O. nova*	104	Absent	Absent	129	210	Absent	132
Calmodulin	*S. lemnae*	130	−36	Absent	450	193	Absent	107
C2	*O. nova*	81	−45	Absent	249	416	+118	181
DNA polymerase α	*O. nova*	329	−280, −71	Absent	4,479	130	+109	218
EF-1α	*S. lemnae*	183	−44, −21	Absent	1,341	266	+59	38
81-MAC(IIIA)	*O. fallax*	88	−43	Absent	1,116	149	+11	342
HSP70	*O. nova*	394	−69, −45	Absent	1,818	382	Absent	23
Histone H1	*E. eurystomus*	636	−597, −553, −544, −54, −38	Absent	408	210	+17, +192	134
Histone H4	*O. nova*	1,153	−1094, −816, −749, −301, −280, −200, −27	−585, −370, −98	315	151	Absent	130
Histone H4G	*S. lemnae*	73	Absent	Absent	315	≥225	+41, +89	331
Histone H4K	*S. lemnae*	1,187	−998, −409, −19	−941, −870, −654, −572, −91	315	131	Absent	331
ORF1 and ORF2	*E. crassus*	348	Absent	Absent	333	80	Absent	191
ORF1	*S. lemnae*	498	−73, −67, −61	−374	582	96	+86	190
Pheromone 4	*E. octocarinatus*	134	−73, −49	−82	384	123	+14, +67	231
Phospholipase C	*E. crassus*	33	Absent	Absent	426	65	+61	184
POB4	*S. lemnae*	454	−101, −24, −18, −12	−332, −322	615	108	Absent	332
Polyubiquitin	*E. eurystomus*	140	−41, −17	Absent	690	68	+17, +31	135
Polyubiquitin	*O. trifallax*				690	273	+133	80
R1	*O. nova*	67	−24	Absent	885	37	Absent	292
RNA polymerase A2	*E. octocarinatus*	352	−204, −13	Absent	3,501	26	+16	172
RNA polymerase B2	*E. octocarinatus*	62	Absent	Absent	3,615	38	Absent	172
719	*S. lemnae*	65	−61	Absent	210	404	+26, +44, +382	107
TBP	*E. crassus*	40	Absent	Absent	1,341	64	Absent	330
TBP homolog	*E. crassus*	54	Absent	Absent	1,383	35	Absent	330
TBP(α)	*O. nova*	159	−47	−53, −18	1,488	447	+48, +118, +149	236
TBP(α)	*S. mytilus*	155	−111, −35	−41	1,482	401	+63, +154, +339	94
TBP(β)	*O. nova*	162	−117, −38	−111	1,158	285	+28, +169	146
TBP(β)	*S. mytilus*	177	−135	−129	1,179	277	Absent	94
α-Tubulin	*E. octocarinatus*	78	−42	Absent	1,353	100	Absent	204
α-Tubulin	*E. vannus*	86	−59	Absent	1,350	69	Absent	108
α-Tubulin	*O. granulifera*	170	−39	Absent	1,353	101	Absent	108
α_1-Tubulin	*S. lemnae*	192	−164, −135, −40	−76, −17	1,338	243	+87	137
α_2-Tubulin	*S. lemnae*	74	−68, −41	Absent	1,350	267	+68, +99	138
β-Tubulin	*E. crassus*	49	−23	Absent	1,341	81	Absent	131
β-Tubulin	*E. octocarinatus*	63	−29, −54	−5	1,335	70	Absent	204
β_1-Tubulin	*S. lemnae*	126	−56	−46	1,329	343	+107, +145, +175, +271, +282	67
β_2-Tubulin	*S. lemnae*	170	−67, −39	Absent	1,329	284	+71, +253	67
γ-Tubulin	*E. octocarinatus*	49	Absent	Absent	1,389	80	+8, +43	203
V2	*E. crassus*	395	−386	Absent	216	203	+56	27

[a] Macronuclear genes of the hypotrichs *E. crassus*, *E. eurystomus*, *E. octocarinatus*, *E. vannus*, *O. fallax*, *O. granulifera*, *O. nova*, *O. trifallax*, *Oxytricha* strain WR, *S. lemnae*, and *S. mytilus* that have been sequenced.

[b] LDR, leader, or 5′ noncoding region; TLR, trailer, or 3′ noncoding region.

[c] 5′ TATAA 3′ and 5′ CCAAT 3′ locations are with reference to the A of the ATG translational start codon.

[d] Open reading frame (ORF) lengths include the translational stop codon but exclude introns. AS1, AS2, C2, ORF1, ORF2, POB4, R1, 719, and V2 all have open reading frames of ≥129 bp encoding putative proteins of unknown function.

[e] 5′ AATAAA 3′; locations are with reference to the A of the TGA translational termination codon (or TAA in *Euplotes* species); some indicated poly(A) sites allow a one-base deviation (A to T or T to A) from the standard sequence. From reference 23.

dG_4T_4-5′ is present in *E. aediculatus*, *E. eurystomus*, *E. octocarinatus*, and *E. crassus* (28, 29, 182, 231, 295).

The similar sequence, $(3'\text{-}dG_4T_2\text{-}5')_{50-70}$, is present at the ends of the rDNA dimeric molecule (41) and the subchromosomal molecules (360, 367) in the *Tetrahymena* macronucleus. As in hypotrichs, the *Tetrahymena* telomere sequence ends in a single-stranded overhang of 16 to 20 bases of 3′-dG_4T_2-5′ (140). The telomere repeat sequences in the acellular slime mold, *Didymium* species, and in budding yeasts (335) end with single-stranded DNA (although the single-stranded tails may be present only at the end of S phase in yeasts), which suggests that short, G-rich, single-stranded tails may be a general

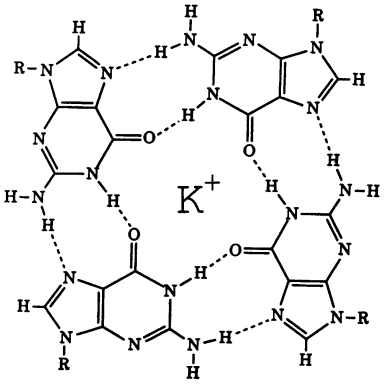

FIG. 10. Four guanine bases in a planar arrangement. Each guanine is a hydrogen bond acceptor and donor in Hoogsteen base pairing. K^+ is shown bound in the central cavity created by stacked layers of G quartets.

feature of telomeres. The *Paramecium* telomere consists of a random mixture of 3′-dG_4T_2-5′ and 3′-dG_3T_3-5′ (102), but whether a 3′ single-stranded tail is present is not known.

Native macronuclear DNA molecules from *S. mytilus* aggregate end-to-end when incubated with a high salt concentration, e.g., 150 mM NaCl (208, 253). At a low concentration of DNA in 150 mM salt, macronuclear molecules of *S. mytilus* (208) and *Oxytricha* species (240) form double-stranded circles. The telomere-telomere interaction responsible for the intermolecular and intramolecular end-to-end joining is mediated by the 3′ single-stranded tails. These form an unusual four-stranded structure consisting of four stacked quartets (for hypotrich telomeres) of guanine bases hydrogen bonded by Hoogsteen base pairing (Fig. 10) (31, 139, 299, 302, 317, 343). A single-stranded 3′ tail can form a hairpin, and two hairpins can interact to produce the G quartets (Fig. 11). The two macronuclear DNA molecular ends may be arranged side-by-side or may approach each other from opposite directions as in Fig. 11. The four-stranded structure forms rapidly in >100 mM Na^+ and is markedly stabilized by K^+ against melting by heat (1). A K^+ ion is believed to be chelated slightly below the center of a G-quartet plane. The hairpin G-quartet structure has been confirmed at 2.5-Å (0.25-nm) resolution with crystallized single-stranded telomeres (3′-$dG_4T_4dG_4$-5′) (165).

Pressing questions are whether the G quartet exists in vivo and, if so, what purpose it may serve in chromatin organization and function, e.g., transcription and replication. On lysis of purified *Oxytricha* macronuclei in 0.5 to 2 M NaCl, the DNA is released as high-molecular-weight aggregates containing about two molecules of a single major protein per DNA molecule (210). The integrity of the aggregates depends on both DNA-DNA interaction and DNA-protein interaction. Within the aggregates the ends of the DNA molecules are protected from exonuclease attack, suggesting that the aggregates are formed by end-to-end interaction of DNA molecules. Gentle, progressive dissociation of chromatin with proteinase indicates that the ends of DNA molecules are held together by interactions that also involve protein molecules to form rosettes of DNA molecules (243) discussed below. More direct evidence for the in vivo existence of the G-quartet structure has been the identification in yeasts of a nuclease activity that is specific for G-quartet four-stranded DNA (214). The activity is associated with partially purified telomere-binding protein (TBP). It binds to G-quartet DNA in vitro and cleaves the DNA in a neighboring single-stranded region. Another protein, called *QUAD*, isolated from liver chromatin, binds specifically to a four-stranded, G-rich oligomer but not to a single- or double-stranded oligomer (334).

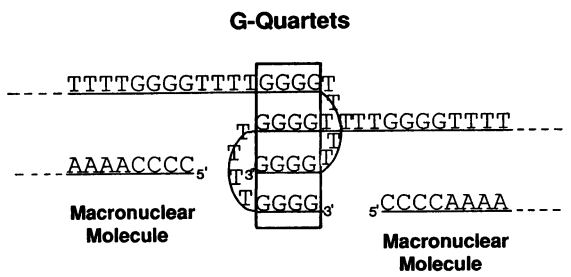

FIG. 11. Two macronuclear DNA ends bound by formation of four stacked G quartets (Fig. 10). The ends could belong to the same molecule, making a circle, or to different molecules, resulting in end-to-end aggregation. Redrawn from reference 96.

Proteins that remain bound specifically to *Oxytricha* telomeric sequence after extraction of chromatin with 2 M NaCl have been characterized (118, 119). The proteins, which protect telomeres from double-stranded exonuclease (*Bal* 31) digestion, can be dissociated with ionic detergent and are apparently bound to the DNA by hydrophobic interaction. The TBP in *Oxytricha* species consists of a heterodimer of two polypeptides of 56 and 41 kDa called α and β TBPs (280, 281, 288). Dimerization of the α and β polypeptides depends on binding of both subunits to single-stranded telomeric DNA (5′-$T_4dG_4T_4dG_4$-3′) (96, 97). The β subunit by itself greatly accelerates G-quartet formation in single-stranded telomeric sequence of both *Oxytricha* (5′-$T_4dG_4T_4dG_4$-3′) and *Tetrahymena* (5′-$T_2dG_4T_2dG_4$-3′) species, which strongly suggests that G quartets may exist in vivo (95, 96). This chaperone action of the β subunit, by promoting G-quartet formation, may be partially responsible for the observed inhibition of telomerase activity by TBP in vitro (373).

In *E. crassus* the TBP consists of a 51-kDa monomer that binds tightly in a very salt-stable complex (282). A homolog to the 51-kDa TBP with extensive sequence identity apparently also binds to telomeric 5′-T_4dG_4-3′ sequences (330).

TBPs in the macronucleus are most probably important in inhibiting recombination between telomeres and in protecting the telomeric ends of DNA molecules from nucleolytic attack. The issues to be resolved are the role of TBPs in the structural arrangement of telomeres, particularly the single-stranded, G-rich 3′ termini (G quartets), the role of the protein-telomere complex in establishing chromatin structure, and the disposition (does it change?) of the protein-telomere complex during replication of DNA ends, particularly telomerase-mediated extension of telomeres.

The telomeres of the micronuclear chromosomes of *O. fallax* are also composed of 3′-dG_4T_4-5′ repeats but in much longer blocks (74). Extensive digestion of micronuclear DNA with various restriction nucleases leaves blocks of 3 to 6 kbp of 3′-dG_4T_4-5′, suggesting that the telomeres may be heterogeneous in length. In undigested DNA, 90% of the 3′-dG_4T_4-5′ repeats are rapidly degraded by *Bal* 31 exonuclease, showing their terminal location in chromosomes. The remaining undigested 3′-G_4T_4-5′ repeats are interstitial within the micronuclear chromosomes, and most or all are associated with transposonlike telomere-binding elements (TBEs [discussed below]). In similar experiments with *O. nova*, only ~50% of 3′-dG_4T_4-5′ repeats are degraded by *Bal* 31, perhaps in part because the blocks of repeats in *O. nova* are much longer (5 to

20 kbp) (153). *O. nova*, like *O. fallax*, has interstitial 3′-dG_4T_4-5′ repeats, usually in blocks of five to eight, but it is not known whether these are associated with transposonlike elements. Whether the telomeres of micronuclear chromosomes have 3′ single-stranded tails and telomere-binding proteins is not known.

REPLICATION OF MICRONUCLEAR AND MACRONUCLEAR GENOMES

The conversion of a micronucleus to a macronucleus not only brings about activation of transcription but also is accompanied by a major change in the regulation of DNA replication. In *Tetrahymena* and *Paramecium* species and hypotrichs, macronuclear DNA replication is initiated in early to middle interphase, depending on how rapidly the cells are growing, and ends just before amitotic division of the macronucleus (15, 160, 174, 175, 227, 291, 316). Micronuclear replication follows one of two patterns. In *Tetrahymena* species and *E. eurystomus*, micronuclear DNA synthesis lasts 10 to 15 min beginning immediately at the end of micronuclear telophase, even before cytokinesis is complete (175, 227, 316). DNA replication in the micronucleus and macronucleus is totally separate and nonoverlapping. The pattern is different in *O. nova*, *S. lemnae*, *S. pustulata*, *Urostyla weissei*, and *Onychodromus quadricornutus*. The multiple micronuclei replicate DNA in a short interval during mid to late macronuclear replication (15, 160, 206, 273). The multiple macronuclei replicate DNA with tight synchrony, but the micronuclei are somewhat asynchronous, e.g., in *Urostyla grandis*. In any case, in all these ciliates DNA replication is separately controlled in micronuclei and macronuclei since replication is initiated and terminated in the two kinds of nuclei at very different times.

Because micronuclear DNA molecules are very long and replicate so quickly, it is assumed that many origins of replication are present in every molecule, but little is known about replication origins in the micronucleus of any ciliate. Many clones of micronuclear DNA (fragments of 1.6 to 12 kbp) contain sequences that work as origins of replication in yeasts (autonomously replicating sequences) (65). Many or most of these origins are eliminated during macronuclear development. Macronuclear sequences that work as autonomously replicating sequences in yeasts are far less evident, occurring less often than once per cloned gene-sized molecule.

The replication of the gene-sized molecules in the macronuclei of hypotrichs is organized in an unusual chromatin structure called the replication band (Fig. 12). A replication band forms in a small area on the inner surface of the nuclear envelope, expands across the macronucleus, and then migrates through it, replicating the millions of short DNA molecules in its path. In *Euplotes* species replication bands ordinarily form perfectly synchronously at the ends of the very elongated, C-shaped macronucleus and move centrally, meeting in the middle of the macronucleus after some hours (104, 277). When the bands meet, they fuse and disappear, which signals the completion of DNA replication, and the macronucleus becomes nearly spherical as it begins the amitotic process. In *Aspidisca* species a single replication band originates at the middle of the very long macronucleus and develops as a crescent that expands across the macronucleus, and the middle of the crescent contacts the nuclear envelope on the opposite side. The crescent then splits into two bands that travel away from one another (the opposite direction to that in *Euplotes* species) to the two ends of the macronucleus, where they disappear. A *Euplotes* macronucleus occasionally shows the *Aspidisca* pattern of a centrally originating band or may have

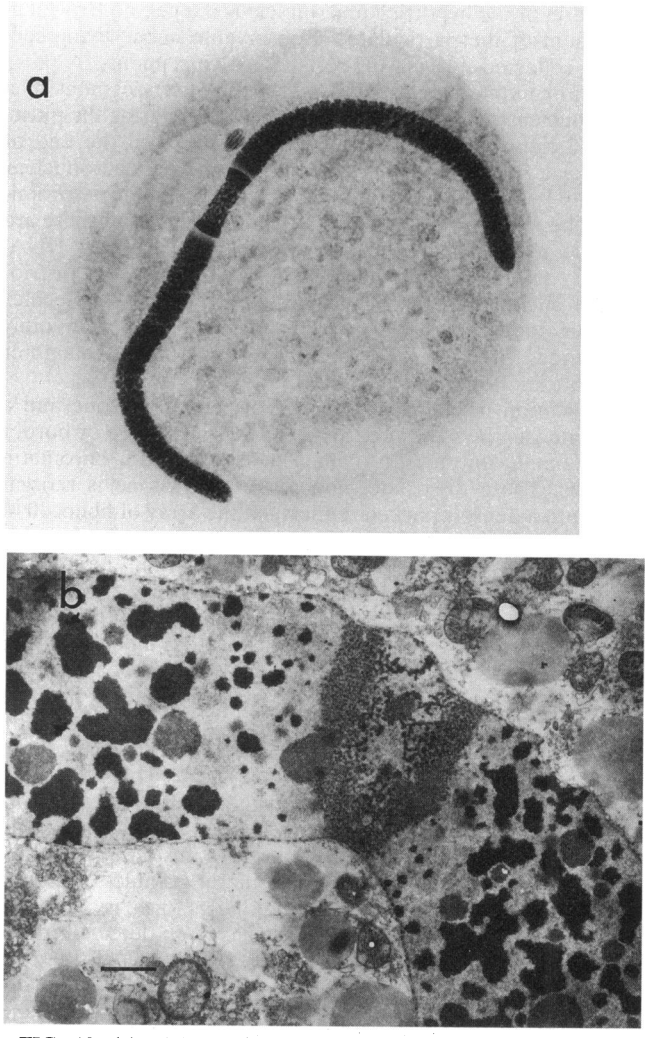

FIG. 12. (a) Light micrograph of *E. aediculatus* stained by the Feulgen procedure to show DNA. Two replication bands have nearly reached each other near the center of the long, rod-shaped macronucleus. Each band consists of a DNA-rich forward zone (dark staining) and a DNA-poor rear zone (very light staining). The micronucleus is arrested in late prophase or metaphase. The macronucleus is ~120 μm long. (b) Electron micrograph of *E. crassus*. Two replication bands (RB) have just reached each other in the center of the macronucleus. A nucleolus is passing through a replication band without alteration of its structure. Bar, 1 μm. From reference 206.

only a single band that originates at one tip and travels the full length of the macronucleus. In hypotrichs with two ovoid macronuclei (*Stylonychia*, *Oxytricha*, *Paraurostyla*, and *Holosticha* species), a single replication band forms at the outer end of each macronucleus and travels toward the inner end near the center of the cell. In *Urostyla grandis*, bands form synchronously in the hundreds of small macronuclei.

A heat shock blocks the migration of a replication band in *E. eurystomus* and stops DNA replication (93, 255). Bands resume replication when cells are allowed to recover, and additional bands often form, so that a single macronucleus may contain four or more bands. The consequence of the extra DNA replication is not known.

The millions of gene-sized DNA molecules are randomly distributed throughout a macronucleus. During interphase the

chromatin of the hypotrich macronucleus is a gel; no Brownian movement of the particulates is observable in the living cell. This is reflected in DNA pulse-labeling experiments. A pulse of [^{3}H]thymidine labels the DNA in the short segment of a macronucleus through which the band travels during the pulse, and the radioactive DNA remains in place until the end of interphase. At the beginning of amitosis the macronuclear contents become solated and Brownian movement is prominent. The macronucleus becomes nearly spherical (if there are multiple macronuclei, they fuse), and pulse-labeled DNA, formerly restricted to a short segment, becomes fully distributed throughout the macronucleus. Thus, the DNA molecules from any localized part of a macronucleus become randomly distributed throughout the entire volume of the daughter macronuclei at amitosis (16, 60, 175).

Replication bands are composed of a forward zone and a rear zone. As the band progresses, the front surface or border of the band continuously comes in contact with chromatin granules. These dissociate, and the DNA-histone is reorganized into a densely packed semicrystalline array of fibers 40 to 50 nm in diameter (254) (Fig. 12). At the point of this conversion, RNA synthesis stops and the DNA in the forward zone is transcriptionally inert (277). The forward zone, which in three dimensions has the form of a disc across the macronucleus, is up to several micrometers thick. At the rear border of the forward zone, the 40- to 50-nm fibers disappear into the rear zone, where a loose mesh of 10-nm diameter fibers is present (254). The concentration of both DNA and protein in the rear zone is extremely low, certainly more than an order of magnitude lower than in the forward zone. The gene-sized molecules are replicated in the highly hydrated rear zone (277). The rear border of the rear zone is not distinct; small chromatin granules appear and enlarge by fusion or accretion to the size of typical interphase chromatin granules. The rear zone, including the region in which granules form, is a disc less than 1 μm thick. No RNA synthesis has been detected in the region of replication; in the transition to granules in the rear zone, RNA synthesis resumes.

As a replication band progresses, it encounters nucleoli but does not visibly affect their structure (Fig. 12). [^{3}H]thymidine labeling of nucleoli does not occur in a band (or elsewhere in the macronucleus), which suggests that nucleoli do not contain rDNA, as in other eukaryotes. [^{3}H]rRNA used in situ hybridization labeled macronuclei of *O. fallax* uniformly throughout but did not appear to label nucleoli (263). Nucleoli in the hypotrich macronucleus are probably sites of assembly of ribosomal subunits, but the rRNA transcripts may be generated from rDNA genes outside the nucleoli.

The molecular interpretation of the chromatin changes in the replication band is not clear. The conversion of transcriptionally active chromatin to the transcriptionally inert semicrystalline fibers of the forward zone is presumably a required reorganization for DNA replication. Intriguing questions are how this preparatory transition and the transition to the replicative state in the rear zone are brought about. Clearly, all the enzymes and factors for DNA replication are present in the rear zone; do they migrate with the band as it progresses, or is replicative machinery present throughout the macronucleus and simply mobilized within the band as it advances? It is probable that they migrate with the band. Three proteins identified immunologically are highly localized in the replication band (2). Also, a protein known as proliferating cell nuclear antigen, believed to be an auxiliary protein of DNA polymerase δ, is heavily localized in the rear zone of the replication band in *E. eurystomus*, but there is little or none in the rest of the macronucleus (256). An antibody to a filament-forming protein in *Tetrahymena* species, possibly related to an intermediate filament protein, binds specifically to a 49-kDa protein localized in the replication band of *Euplotes* species (250). These observations suggest that at least some proteins in the replication band, particularly proteins involved in DNA replication, migrate with the band (256).

Macronuclear DNA molecules in hypotrichs initiate replication at or near one or both ends (3, 242). Replicating molecules are either Y forms or double Y forms with the replication fork(s) progressing inward from an end(s), and replication bubbles are not found. The model suggested by these observations is that replication is initiated at or near a molecular end as soon as it enters the rear zone from the forward zone. In molecules with double Ys, the arms of one Y are always longer than those of the second Y, presumably because one end of the molecule enters the rear zone before the other.

Replication bubbles observed by electron microscopy in the 21-kbp rDNA of the *Tetrahymena* macronucleus show that replication initiates not at ends as in hypotrichs but a few hundred base pairs to one side of the center of the molecule (58, 326). It is puzzling that only one origin to one side of the center is activated and that the corresponding origin symmetrically located on the other side of the center is not activated in the palindromic rDNA molecule. Sequences cloned from a region within 1.9 kbp of the rDNA center function as origins (autonomously replicating sequences) in yeast cells (11, 12, 177).

The macronucleus of *E. eurystomus* contains at least 10^8 DNA molecules. At the highest reproductive rate (generation time, 8 h; band transit time, 6 h), ~2,500 molecules enter the rear zone of each band and are fully replicated per second. It is probable that the replication band evolved as a mechanism to cope with tens of millions of individual small molecules, ensuring that every molecule is replicated. A replication band, at least in the form present in hypotrichs, probably could not cope with long subchromosomal DNA molecules such as those present in *Tetrahymena* and *Paramecium* macronuclei.

Replication of the ends of DNA molecules requires synthesis of new telomere sequences by telomerase (39, 40, 371, 372), a reverse transcriptase that carries its own RNA template that prescribes 5′-dG_4T_4-3′ repeats in the new telomere. One model, diagrammed in Fig. 13, proposes that to initiate replication, the 16-base single-stranded 3′ tail serves as a template for synthesis by DNA primase of an RNA primer consisting of $(5'\text{-}rC_4rA_4\text{-}3')_n$ (372). The requisite DNA primase is readily demonstrable in a preparation of isolated macronuclei. With the RNA primer in place, DNA replication could proceed by the conventional leading-strand–lagging-strand mechanism. Completion of replication consists of the addition of 16 bases of 5′-$T_4dG_4T_4dG_4$-3′ by telomerase (372). In this model the telomeres are origins of replication, which would agree with the electron-microscopic observation that replication begins near or at molecular ends.

In another version of this model, the existing 3′ single-stranded tail is first elongated by telomerase and then used as a template to synthesize an RNA primer (39, 40). This has the effect of elongating the double-stranded portion of the telomere, which would account for the lengthening of the telomeric sequence that can occur during *Tetrahymena* and yeast cell proliferation (3 to 10 bp per generation and up to 1,000 bp in *Tetrahymena* cells) (39, 40, 197, 374) but not during hypotrich cell proliferation.

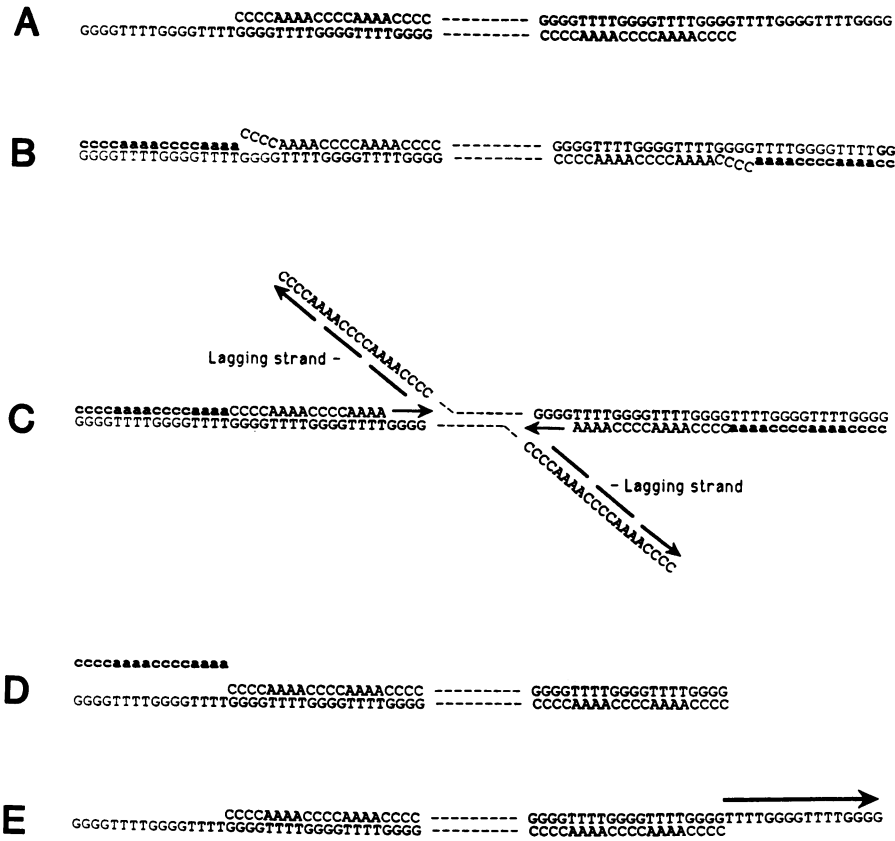

FIG. 13. Model for replication of gene-sized molecules in hypotrichs. (A) Macronuclear molecule before initiation of replication. (B) Synthesis of RNA primers by DNA primase with the 3′ single-stranded telomere repeats as templates. The RNA primers are in lowercase letters. (C) Extension of RNA primers by DNA polymerase in the leading strand, plus lagging-strand synthesis. (D) Removal of the RNA primer, releasing the 3′ single-stranded telomere (left end), and completion of replication to produce a blunt end at the right terminus. (E) Synthesis of a new 3′ single-stranded telomere at the right terminus by telomerase. Redrawn from reference 371.

REGULATION OF MACRONUCLEAR DNA CONTENT

There are two problems with the amitotic distribution of macronuclear DNA. The first is the unequal distribution of DNA to the two daughter macronuclei, and the second (discussed in the next section) is the nonmitotic, random distribution of particular DNA molecules to the daughter macronuclei. The maintenance of an average total DNA content is probably achieved differently in different ciliates. In *Tetrahymena* species a straightforward doubling of DNA between divisions seems to be the rule (22), although an extra round of DNA synthesis can occur in the daughter macronucleus that receives an amount of DNA below some limit (63, 81). Also contributing to small macronuclear size is elimination of chromatin from *Tetrahymena* macronuclei at division at a rate of ~4% per fission (64). The small size of macronuclei may be corrected by two full rounds of replication (64, 81). The amount of chromatin eliminated at mitosis is probably too small to make the full downward adjustment in the total DNA content in an oversized macronucleus (81). Instead, large macronuclei may skip an S period. In *Paramecium* species the unequal amitotic distribution of DNA is probably corrected by a variable amount of DNA synthesis in the next S period, with less than a doubling for large macronuclei and more than a doubling for smaller ones (37). *Paramecium* species do not extrude chromatin at division.

It is assumed that the traversal of replication bands in hypotrichs accomplishes an exact doubling of DNA, but this has not been directly measured. *Euplotes* macronuclei are occasionally observed with two sets of replication bands; in such a case the DNA content is presumably quadrupled. Extra replication bands have been observed in *Stylonychia*, *Oxytricha*, and *Paraurostyla* species and other hypotrichs. Chromatin extrusion during amitosis is not known to occur in hypotrichs. Hypotrichs apparently adjust macronuclear DNA content with an extra complete round of replication or skipping of a total round.

In summary, ciliates maintain a particular macronuclear DNA content (which may vary with culture conditions or clonal cell age), probably by a mechanism that depends on cell size. Adjustments may occur by chromatin extrusion, extra full or partial rounds of DNA replication, and/or skipped rounds of replication. The molecular basis for the regulation of total DNA content is totally unknown.

COPY NUMBER PUZZLE

The multiple copies of each different subchromosomal (*Tetrahymena* and *Paramecium* species and others) or gene-sized (hypotrichs) molecule are assumed to be distributed randomly by amitosis to the two daughter macronuclei, because there is no known feasible mechanism by which the many copies can be assorted equivalently. For *Oxytricha* species, any hypothesized mechanism would be required to distribute equally the ~4,000

copies (the two macronuclei each contain an average of ~2,000 copies of each gene-sized molecule at the end of replication; they fuse just before amitosis to make a composite macronucleus) of ~24,000 different genes, i.e., to cope with assorting ~10^8 DNA molecules. Therefore, without macronuclear renewal through autogamy or conjugation, genetic imbalances would be expected to develop; these would result in a high rate of cell mortality and, eventually, in total extinction of the culture (48, 265, 267). However, this does not occur. The problem cannot be ameliorated by compensatory events that maintain a constant average macronuclear DNA content. *Tetrahymena* cells unable to conjugate or undergo autogamy and therefore incapable of macronuclear renewal, as well as amicronucleate strains, have been maintained in laboratory culture for decades with little mortality. Similarly, an amicronucleated clone of *Oxytricha* species has been maintained in laboratory culture for over 3 years with an average of 1.5 divisions per day, and an amicronucleated clone of *Paraurostyla weissei* has been maintained for over 1 year with an average division rate of 1.1 per day, both with almost no mortality (273). Therefore, the conclusion appears inescapable that ciliates have the ability to sense the copy numbers of their genes and carry out selective replication of particular DNA molecules to correct copy shortfall produced at amitosis (46, 267).

The hypothesis of selective replication to correct copy numbers is strongly supported by the maintenance of constancy in copy number for at least some genes over a long period of cell proliferation. In *E. crassus* the copy numbers measured for three gene-sized molecules (of unknown coding function) were ~900, ~1,400, and ~6,500 in cells less than 15 generations after cell mating and remained about the same in cell clones for more than 300 generations after mating (30). Similarly, the three genes belonging to the 81 macronuclear family in *O. fallax* are maintained in a constant ratio of 1:1:10 (143).

The copy numbers in young cells are set during or very soon after macronuclear development. Amplification of all DNA molecules occurs during macronuclear development, and differential amplification may set different copy numbers at this time. It is reasonable to expect that copy number control could not distinguish between alleles of a particular gene, and this is so (257). This has been shown specifically for a restriction fragment variant in *E. crassus* (28). In *Tetrahymena* species all loci eventually become homozygous during vegetative growth through phenotypic assortment (51, 228, 245).

The copy number of rDNA is regulated in *Tetrahymena* species separately from the rest of macronuclear DNA (see reference 198 for a review). The copy number of macronuclear rDNA is ~9,000, or 200-fold above the average copy number for other DNA molecules. After a shift from nutritionally poor to rich conditions, rDNA replicates preferentially, so that logarithmically growing cells have 30 to 40% more rDNA than do starved or stationary-phase cells (89, 90). Underreplication of rDNA apparently occurs when cells shift from nutritionally rich to poor conditions.

There are some clues in the rDNA molecule itself about regulation of its copy number. The control of rDNA copy number in *Tetrahymena* cells involves a *cis*-acting repeated sequence in the promoter region (196). Also, increased replicative ability of a particular rDNA allele, at the expense of a second allele, is conferred by artificially increasing the number of origins in the molecule, although the overall number of rDNA molecules remains constant (369). A mutation in the 3′ Cbs 15-mer that prescribes excision of rDNA results in a failure to amplify rDNA without much affect on excision, suggesting that the 15-mer is important in regulating rDNA copy number (165a). Copy numbers for non-rDNA, subchromosomal molecules in the *Tetrahymena* macronucleus can be different by as much as 10-fold (48). This suggests that more of some molecules are needed than others and is consistent with the hypothesis of copy number regulation.

The copy number control for several DNA molecules in *O. nova* has been observed to change spontaneously. rDNA molecules (7,400 bp), which are ordinarily amplified ~100 times more than other gene-sized molecules, increased abruptly in a clonal culture from ~10^5 copies per macronucleus to ~10^6 copies (83). Molecules of 490 and 520 bp, which are normally not detectable in an ethidium bromide-stained gel, also became amplified simultaneously and stood out as bands in a gel. The amplified copy numbers of all three molecules remained stable for many months and then returned to the usual, lower degree of amplification. The return may have been due to successful mating within the clone (selfing), with subsequent growth dominance of the recently mated cell. *O. nova* frequently undergoes selfing; most of the exmated cells do not complete macronuclear development and die, but an occasional (<1%) cell matures and proliferates. During 1 year of clonal growth of an *O. nova* culture, rDNA molecules and two other molecules of unknown coding function (450 to 500 bp) underwent differential amplifications, detectable in a stained gel, of 11-, 24-, and 107-fold, respectively (132). Other molecules, encoding two kinds of actin, histone H4, β-tubulin, and one molecule (C2) of unknown coding function, did not fluctuate significantly in the same clone over the same period.

In *S. lemnae*, a differential increase in the copy number of different molecules (of unknown coding function) occurs during aging of different clones (312). This is a strain-specific characteristic that is inherited through the micronucleus in Mendelian fashion. It shows that determination of the copy number of a particular kind of gene-sized molecule is somehow genetically controlled and that the "set point" for the copy number can be changed. An A+T-rich sequence in the end region of a 1,218-bp molecule that overamplifies during clonal growth has been identified as a potential *cis*-acting element involved in overamplification (332).

Many observations on *Tetrahymena* species and hypotrichs point to a mechanism for monitoring and correcting copy numbers of DNA molecules in the macronucleus. Given the staggering number of individual molecules whose copy numbers must be individually regulated, it seems plausible that the copy number of a gene is sensed in some manner through the function of the product of the gene. How sensing, whether operating through a gene product or through the DNA molecule itself, could create a signal that brings about corrective replication remains obscure.

5′ LEADERS (PROMOTERS) AND 3′ TRAILERS IN CILIATE GENES

Regulation of gene expression in *Tetrahymena* species is primarily transcriptional, as in other eukaryotes, although posttranscriptional regulation has been documented for a few genes. The transcription activity of 13 genes changes significantly, sometimes strikingly, in relation to different physiological states of the cell, i.e., growth, starvation, and conjugation (309). Expression of a 14th gene, encoding the serotype H3 surface antigen (Ser H3 gene) in *Tetrahymena* species, increases dramatically in response to increase in culture temperature (215) or in growing cells compared with starved cells (309) through stabilization of Ser H3 mRNA without a measurable change in the transcription initiation rate. Concentra-

tions of mRNAs for α- and β-tubulins increase in response to deciliation/reciliation of *Tetrahymena* cells (304). An identical 14-bp sequence occurs upstream of the initiation site of the α-tubulin gene (−30 bp) and a β-tubulin gene (−34 bp) that is a candidate for the coordinated control. (There are two β-tubulin genes in *T. pyriformis* [33]). Other conserved sequences of 8 and 12 bp that may be involved in transcriptional control occur much farther upstream of the two genes.

Response to heat shock in *Tetrahymena* cells (100, 126) is mainly transcriptional, although increased production of small heat shock proteins (25 to 29 kDa) may be posttranscriptional (103), as with the Ser H3 gene above. The 5′ regions of at least some heat shock genes in *Tetrahymena* cells may be expected to have heat shock elements (HSE) as in other eukaryotes. Copies of the highly conserved HSE, GC--GAA--TTC--GC (260), occur in the upstream region of the *Tetrahymena* ubiquitin genes (246), and a transcription factor called heat shock factor (HSF) (322) that binds specifically to the HSE has been partially purified from *Tetrahymena* species (26). Two HSEs occur in the 5′ region (at −121 and −140 relative to the transcription start site) of an RNA polymerase III-transcribed gene that encodes a small, cytoplasmic RNA molecule (G8) (127). It appears that the mechanism for transcriptional regulation of *Tetrahymena* heat shock genes is conventional, but TATAA, CCAAT, and GC boxes, which are frequent features of eukaryotic promoters, are not found in the promoter regions of the ubiquitin or G8 genes. An analysis of the 5′ upstream regions of histone H3 and H4 genes in 22 *Tetrahymena* species revealed a single, 13-bp consensus sequence, TATCCAAT TCARA (49), at −151 bp (H3) and −141 bp (H4) that could serve to bind a transcription factor. This consensus sequence has been found in the 5′ regions of six other histone genes (32, 248, 348), a ribosomal gene (71), and an α-tubulin gene (34). This 13-mer contains a CCAAT box, but TATAA and GC boxes are generally absent. A gene encoding an apparent TATA-binding protein has been cloned and sequenced from *Tetrahymena thermophila* (308b). Antibodies to the protein stain macronuclei but not micronuclei in vegetative cells, which is consistent with the different transcriptional activities of the two kinds of nuclei. The TATA-binding protein is transiently present in the micronucleus at conjugation, a time when this nucleus shows transcriptional activity. Whether the protein recognizes a TATA sequence or some variation of it remains to be determined.

In hypotrichs, because most leaders are less than 200 bp, the gene-sized molecules in macronuclei offer an unusual opportunity to study promoter functions. Thus, the excision of genes from micronuclear chromosomes by the organism places an upper limit on the length of the 5′ upstream *cis*-acting sequences that may function in transcription control. Many of the molecules contain putative TATAA boxes in about the expected position upstream of the ATG translation start codon. For the actin I gene in *O. nova*, transcription starts at 31 or 34 bp upstream of the ATG codon (122), but transcription start sites are not known for most of the genes. CCAAT boxes are present in only 11 of 47 genes in Table 2. Some of these CCAAT boxes that occur in genes with long leaders may be spurious because they occur too far upstream of the probable transcription start sites. Others are too close to the start site to fit the usual convention for CCAAT location (usually 80 to 160 bp 5′ to the transcription start site).

The 5′ leaders and 3′ trailers are A+T rich, usually exceeding 70% A+T. Through most of their length, they appear to change rapidly in evolution. The 1,125-nucleotide coding region of the actin I gene of *O. nova* has high homology with an identically sized coding segment of the actin I gene in the closely related species *O. fallax* (85% nucleotide identity and 91.5% amino acid identity), but the 5′ leaders in the two species (192 and 183 bp) and 3′ trailers (212 and 186 bp) are almost totally different from each other. A nearly identical 18-mer occurs just upstream of the ATG codon in the actin I gene in *O. nova* and *O. fallax* (122), as well as in *O. trifallax* and *Oxytricha* sp. strain WR (84) and may be involved in transcriptional events. A 6-mer is present in all four species just upstream of the 18-mer, but the rest of the 183- to 191-bp leaders in the four species show no sequence similarities. The trailers of many genes do not contain the ubiquitous polyadenylation signal, AATAAA, of higher eukaryotes (Table 2). It may be that a majority of the sequences in leaders and trailers have no sequence-specific function, although length itself may be important since it seems to be maintained for particular genes, at least among closely related species.

O. nova has at least four macronuclear genes encoding histone H4 (130). Three of these have short 5′ leaders; the fourth has a 5′ leader of 1,153 bp. Similarly, *S. lemnae* has two macronuclear H4 genes, one with a 216-bp leader and the other with a 1,207-bp leader (331). The coding regions of the H4 genes in the two species are 92% identical in nucleotide sequence. The comparison of the 1,153- and 1,207-bp leaders reveals a distal region of 243 or 244 bp, respectively, that is 70% identical in the two species, a more proximal region of 167 bp with 70% identity, and a region between the two of much lower identity (141). The remaining ~550 bp of the A+T-rich (65%) leaders show no significant similarity in sequence, but it may be important that they are similar in total length. Transcription of histone genes is coordinated with DNA replication in eukaryotes in general. In *E. eurystomus*, synthesis of histones is coordinated with DNA replication (270); presumably this also represents transcriptional regulation. The long 5′ leaders for the H4 histone genes in *O. nova* and *S. lemnae*, and particularly the conserved regions, are perhaps important for the cell cycle regulation of histone gene transcription.

Regulation of transcription by leader sequences has been shown for the gene encoding the heat shock protein HSP70 in *O. nova* (23). This gene is transcribed at a very low rate in nonshocked cells, but heat shock rapidly induces massive transcription. The macronuclear gene encoding HSP70 is contained in a 2,650-bp molecule with a 394-bp leader with putative TATAA boxes 45 and 69 bp upstream of the ATG codon. At 134 bp upstream of the ATG codon are two copies of the eukaryotic, palindromic consensus element (HSE), 5′-C--GAA--TTC-G-3′, separated by 19 bp (the copies differ in the first nucleotide). The presence of the HSEs implies the presence of the transacting transcription factor, HSF, that regulates transcription of the gene encoding HSP70 in other eukaryotes. No function has been identified for the remaining 215 bp of leader sequence upstream of the HSEs.

It is clear from the data so far that hypotrich and *Tetrahymena* genes are different in their promoters. TATAA boxes are present in most hypotrich genes and generally absent in *Tetrahymena* genes. CCAAT boxes are absent in most hypotrich genes but may be present more frequently in *Tetrahymena* genes, although the *Tetrahymena* sample is still small (13 different genes). Both lack GC boxes, and both have HSEs in heat shock response genes. The TATAA box is a binding site for transcription factor TFIID, but some nonciliate genes lack TATAA boxes. At least some of these contain a non-TATAA sequence 15 to 35 bp upstream of the major transcription sites to which TFIID binds with a much lower affinity than to a TATAA sequence (341). Thus, the transcription initiation rate may be lower for genes that are TATAA-less.

INTRONS IN CILIATE GENES

Most hypotrich genes characterized so far lack introns. Of the 47 hypotrich genes listed in Table 2, only 11 have introns. In general, ciliate macronuclear introns are rather short. The macronuclear gene encoding the 41-kDa β polypeptide of the TBP (the βTBP gene) in *O. nova* contains a 110-bp intron (146), and the gene encoding αTBP has an intron of 52 bp (120). The same two genes in *S. mytilus* have identically placed introns, but they are shorter, 48 and 46 bp (94). *E. crassus* has two macronuclear genes encoding two 51-kDa telomere binding proteins that are 35% identical in amino acid sequence (330). One gene contains a 101-bp intron, and the other has two introns of 49 and 24 bp. If the two TBP genes are truly homologs (derived from the same ancestral gene), at the very least one intron has been gained or lost in evolution and the other has changed in size and position as the genes diverged. Two mating-hormone (pheromones) genes in *E. octocarinatus* contain two introns each (45, 231). The gene encoding γ-tubulin in *E. octocarinatus* has two introns of 36 and 26 bp (202) that occupy the same position as two of the three introns in the γ-tubulin gene of *Aspergillus nidulans* (251), which suggests that the two introns existed before fungi and ciliates diverged in evolution $\sim 10^9$ years ago. Many other hypotrich genes, such as those encoding histones, α- and β-tubulins, actins, and polyubiquitin, have no introns.

The three β-tubulin genes in *P. tetraurelia* all contain two introns of 27 bp each (85), and the hemoglobin gene in *P. caudatum* contains an intron of only 23 bp (350), which is the shortest intron reported to date for any eukaryote. Introns may be more common in *Tetrahymena* species than in hypotrichs. In some *Tetrahymena* species, an intron of ~400 bp interrupts the coding sequence for 26S rRNA (340), but in other species the rRNA gene is uninterrupted (59, 77, 78). An isoleucyl-tRNA synthetase gene in *T. thermophila* contains eight introns (222), and a gene (*cnjB*) of unknown function transcribed during conjugation in *T. thermophila* is interrupted by perhaps as many as 30 introns (69, 224). Genes encoding ribosomal proteins (reviewed in references 69 and 247), histone H1 (348), a calcium-binding protein (321), and phosphoglycerate kinase (328) all have one or more introns. The actin gene in *Tetrahymena* species, like that in hypotrichs, lacks introns (71, 147).

A+T AND G+C CONTENT OF CILIATE DNA

Micronuclear and macronuclear DNAs in *Tetrahymena* cells are unusually A+T rich. The A+T content calculated from buoyant density (357) and from melting temperature (4) is 76% for both nuclei. Coding regions of genes have, on average, much less A+T, ~54% (4, 32, 71, 149). Most of the DNA in the *Tetrahymena* (and probably *Paramecium*) micronuclear and macronuclear genomes is noncoding, existing as "spacer" DNA between genes and also making up transposonlike elements. These sequences are very A+T rich (>76%). Introns in *Tetrahymena* genes are even more A+T rich (>85%) (69). Hypotrich macronuclear DNA is not as A+T rich. *O. fallax* macronuclear DNA is ~68% A+T determined by direct chromatographic analysis (287). It is possible to calculate separately the A+T and G+C content of leaders, trailers, and coding regions in the gene-sized molecules in hypotrichs. DNA flanking micronuclear genes is 70% A+T. The average A+T content in macronuclear gene-sized molecules in several hypotrich species is 75% for leaders, 69% for trailers, and 52% for coding regions. Thus, hypotrich DNA resembles *Tetrahymena* DNA with a high A+T content for noncoding sequences and a 50 to 60% A+T content for coding sequences. The significance of the differences in A+T content in leaders and trailers in hypotrich genes is unexplained. The evolutionary drifting of noncoding DNA toward A+T richness is a widespread phenomenon among eukaryotes.

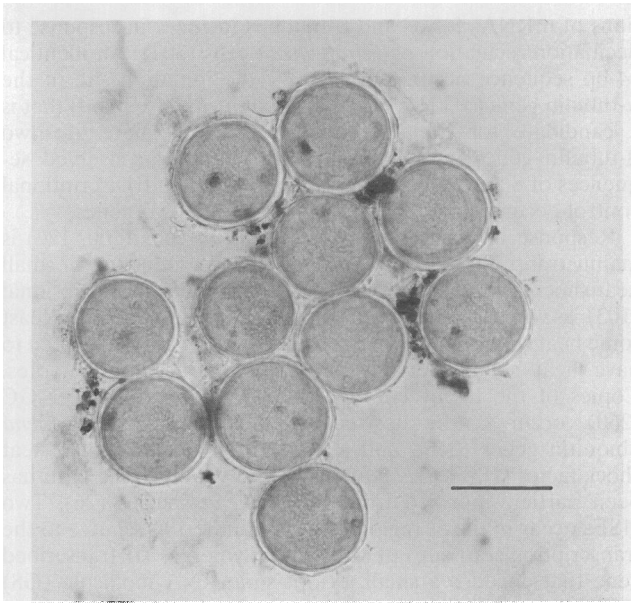

FIG. 14. Cysts of *Oxytricha* strain WR. Bar, 30 μm.

ENCYSTMENT AND EXCYSTMENT OF HYPOTRICHS—A PROBLEM IN GENE EXPRESSION

In addition to transcriptional regulation during the cell cycle, in response to stress (heat and heavy metal ions), and during meiosis of the micronucleus (in *Tetrahymena* species) (220, 221, 223), major regulation probably occurs during encystment. Encystment is a potentially important opportunity to study transcriptional regulation of gene-sized molecules in hypotrichs. In many hypotrichs, including *Oxytricha* and *Stylonychia* but not *Euplotes* species, starvation induces cyst formation (Fig. 14) (68, 327). This is accomplished by rapid synthesis of a thick cell wall composed of glycoproteins, carbohydrates, and other substances (see reference 125 for review). No trace of such a wall is present in proliferating cells; its presence, even if slight, would almost certainly interfere with ciliary action. Production of the cell wall must require synthesis of specialized components, which, in turn, probably means that transcription of many genes is induced, as in bacteria. The synthesis of the cell wall is accompanied eventually by total shutdown of transcription and translation, and cysts may remain inert for many months. Cysts of *Stylonychia* species must be kept wet, but cysts of *Oxytricha* species can be stored dry. Cysts (wet or dry) can be kept frozen at −70°C for at least several years with little loss of viability. As an organism encysts, it reabsorbs all of its cilia, loses all its cortical structures, and becomes smaller. Excystment can be induced by adding food organisms such as the unicellular alga *Chlorogonium elongatum* and occurs within 15 min in some *Oxytricha* species. In response to food organisms, the encysted ciliate forms new cilia, becomes motile within the cyst, digests an opening in the cyst wall, and emerges. Nothing is known about the molecular biology of the signaling mechanism for encystment or of excystment. Encystment and excystment, which probably involve tens of macro-

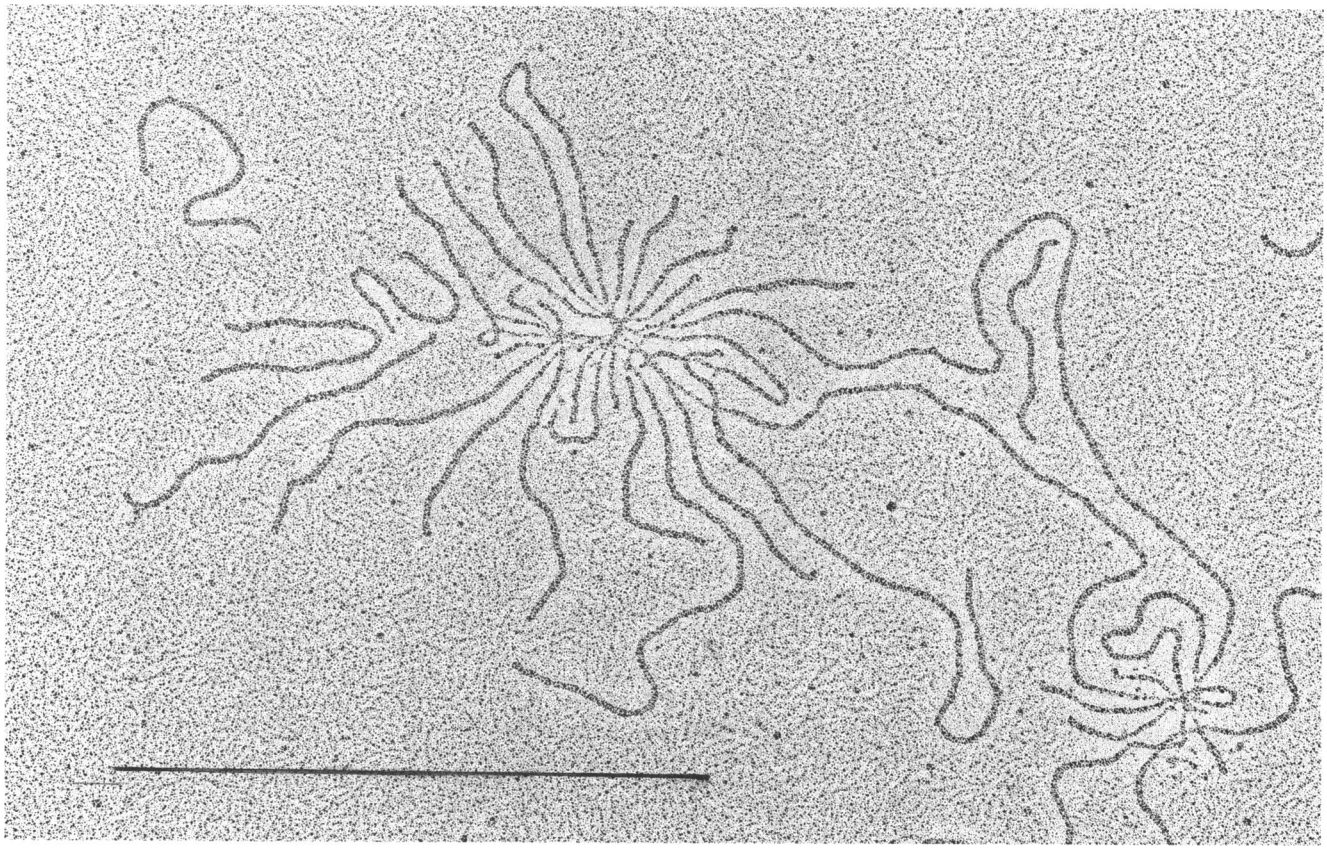

FIG. 15. Rosettes of macronuclear DNA obtained by a 10-s treatment of chromatin with protease K. The two rosettes are connected by two molecules, which suggests that two rosettes may have arisen by splitting of a single rosette. Bar, 1 μm. Micrograph courtesy of K. G. Murti.

nuclear genes, thus provide a special opportunity to study transcriptional regulation.

ORGANIZATION OF HYPOTRICH GENE-SIZED MOLECULES IN CHROMATIN

The macronuclei of hypotrichs contain many millions of gene-sized molecules that are aggregated in groups to produce the chromatin granules seen by electron microscopy (Fig. 12). Some insight about how the gene-sized molecules are organized in chromatin granules has been gained by electron-microscopic observations of the progressive dissociation of isolated granules incubated with protease (243). Initially the chromatin appears as a mass of very large interconnected rosettes in which DNA molecules extend as loops from compact centers (Fig. 15). As protein digestion proceeds, smaller individual rosettes become visible (Fig. 15). A rosette consists of a dense center to which some DNA molecules are anchored by both ends, forming a loop, and to which other DNA molecules are attached by only one end. Some molecules extend from the center of one rosette to the center of another, suggesting that these smaller rosettes represent breakup products of larger rosettes. DNA molecules in a single rosette are of many different sizes, which implies that many different kinds of genes are present in a single aggregate. As protein digestion proceeds, more and more DNA molecules are freed from rosettes at both ends. These observations are interpreted to mean that the telomeres of gene-sized molecules are anchored together by protein into a compact central knot. TBP may participate in the aggregation of ends. Immobilization of the ends of gene-sized molecules may be important for inducing negative supercoils that facilitate transcription, although supercoiling in macronuclear DNA has not been reported.

ARRANGEMENT OF GENES IN HYPOTRICH MICRONUCLEAR CHROMOSOMES

The existence of gene-sized DNA molecules in the macronucleus provides a unique opportunity to determine how precursor genes are arranged in micronuclear chromosomes. In such a study it is assumed (i) that each different-sized macronuclear DNA molecule represents a different gene or version of a gene (for example, the two genes encoding αTBP and βTBP occur in two versions each that differ by a single amino acid in their coding function) and (ii) that only one gene is present per macronuclear DNA molecule. (The word "gene" is used here to describe a gene-sized molecule in the macronucleus, which consists of a 5′ leader, a coding region, a 3′ trailer, and telomeres.) Both assumptions have proven correct for the ~55 macronuclear molecules encoding known genes (those in Table 2 plus rRNA genes) that have been characterized so far. Most of these molecules are less than 3 kbp long. The largest macronuclear DNA molecule sequenced to date in *O. nova* is 4,938 bp long, with a single open reading frame of 4,476 bp encoding a large subunit of α DNA polymerase (218). On the basis of these observations, it is reasonable to use macronuclear DNA molecules to map the positions of genes in micronuclear DNA. In addition to coding regions, macronuclear DNA molecules contain 5′ leaders (mostly <200 bp) and 3′ trailers (mostly <300 bp), and these are included in the

mapping strategy. Large, cloned fragments of *O. nova* micronuclear DNA (>15 kbp) containing unique sequence typically hybridize with roughly equal efficiency to three to six different sized macronuclear molecules in DNA prepared by the Southern technique (42, 157, 185). The macronuclear sequences account for most of the unique sequence within the micronuclear clones. Thus, macronuclear genes occur in clusters of at least three to six within micronuclear DNA, with rather short spacers composed of unique sequence between genes in a cluster.

Spacers between six genes in one cluster and five genes in another in *O. nova* range from 90 bp to, at most, a few hundred base pairs (185). The actin I gene in *O. nova* is separated from an adjacent macronuclear precursor by 15 bp (124). In *E. crassus*, genes D7 and D8 are separated by a spacer of 36 bp and genes V2 and V3 are 400 bp apart (27). Genes V1 and V2 overlap by 6 bp. These two genes may become physically separated from one another during macronuclear development by a staggered cut in the DNA duplex followed by fill-in of 5′ overhanging tails, although there are other possibilities (29). Staggered cuts are made in other DNA-processing steps in the anlage (discussed below), which makes this explanation of gene disjoining attractive. These studies provide minimum estimates for the numbers of genes in clusters; it is likely that some clusters contain more than five or six genes. Micronuclear clones containing many kilobase pairs of unique sequence or containing an extremely large repeated sequence (25 kbp) appear to dominate in an *O. nova* micronuclear DNA library (157). The micronuclear genome contains 2,000 to 3,000 copies of the 25-kbp repeat element (157). These long, nongene stretches appear to be dispersed throughout the genome and presumably occur as spacers between clusters of genes; genes have been found associated with the repeat element 40 to 50% of the time. Only one case of a long, nongenic unique sequence adjacent to a gene has been found so far: the gene encoding the actin II protein is interrupted near its 5′ end in micronuclear DNA by at least 4.5 kbp of unique, micronucleus-specific sequence (235).

The global sequence pattern is similar in cloned, micronuclear DNA of *E. crassus* (156). One-quarter of randomly chosen clones contained only unique sequence devoid of genes. One-half contained a large repetitive element, and about one-third contained both the repetitive element and a gene cluster. The juxtaposition of a copy of the large repetitive element with gene clusters may be a significant feature of the micronuclear genome in *E. crassus* (27, 156) and *O. nova* (157). What function is provided by stretches of nongenetic unique sequence and large repetitive elements, other than forming spacers between gene clusters, remains obscure.

DIFFERENCES IN THE GENETIC CODE OF CILIATES

Sequencing of genes in *Paramecium* and *Tetrahymena* species and hypotrichs has revealed that the three codons, TAA, TAG, and TGA, which are universal stop codons for nuclear genes in other eukaryotes, have evolved different codon meanings in ciliates (Table 3). In the two α-tubulin genes and one of the two β-tubulin genes of *S. lemnae* (67, 137) and in the actin gene (71, 147) and two histone H3 genes (149) in *Tetrahymena* species, TAA is used as a glutamine codon. Consistent with this, in *O. nova* TAG and TAA are not used as stop codons. TAG encodes an amino acid in *Oxytricha* species, probably glutamine (120, 122), but it is infrequently used. TAA and TAG are infrequent amino acid codons in an *O. fallax* gene (144). In genes encoding the giant surface antigen proteins of *Paramecium* species (56, 264, 268), TAA and TAG are likewise used as glutamine codons. Thus the only codon used for stop in *Stylonychia*, *Oxytricha*, *Tetrahymena*, and *Paramecium* species is TGA. The conversion of TAA and TAG to amino acid codons is reflected in the tRNA complement (192). *Tetrahymena* species contain three tRNAs for glutamine (129). Two are unusual because their anticodons recognize the universal stop codons TAA and TAG. The third glutamine tRNA is conventional and presumably reads the normal glutamine codons CAA and CAG. Sequence homologies among the tRNAs suggest that genes encoding the two unusual tRNAs for glutamine evolved from the gene that encodes the normal glutamine tRNA.

TABLE 3. Use in ciliates of the three codons that specify stop in most other organisms[a]

Genus	Use of:		
	TAA	TAG	TGA
Tetrahymena	Gln	Gln	Stop
Paramecium	Gln	Gln	Stop
Paraurostyla	Gln	Gln	Stop
Oxytricha	Gln	Gln	Stop
Stylonychia	Gln	Gln	Stop
Euplotes	Stop	Stop	Cys

[a] See text for references.

The genetic code has taken another twist in *Euplotes* species. TAA is used as a stop codon instead of as a glutamine codon as in other hypotrichs and *Paramecium* and *Tetrahymena* species (131). In *Euplotes* species, TGA, the only stop codon in other ciliates, encodes cysteine in the pheromone 3 gene (232) and in RNA polymerase genes (173) instead of stop. TAG has been encountered as a stop codon in 2 (92a, 259) of 21 *Euplotes* genes sequenced so far (see, e.g., references 131, 135, and 136). *Blepharisma* species, like *Euplotes* species, use TAA as a stop codon instead of using it as a glutamine codon (201). Whether TGA is a cysteine codon in *Blepharisma* species is not known. The genus *Blepharisma* is thought to be the earliest ciliate to have diverged in the phylum Ciliophora after the ciliates diverged from the main line of eukaryotes (121).

The differences between *Euplotes* (and *Blepharisma*) species and other ciliates raise questions about evolutionary relationships among the organisms. One model that is consistent with evolutionary relationships defined by sequences of small-subunit rRNA (Fig. 1) is that TAA and TAG dropped out as stop codons in early ciliates before *Tetrahymena* and *Paramecium* species and hypotrichs diverged. Subsequently, TAA and TAG reemerged as glutamine codons. Early after their evolutionary appearance, the genus *Euplotes* (and *Blepharisma*), which is distantly related to the genera *Stylonychia* and *Oxytricha*, reestablished TAA as a stop codon and converted TGA to cysteine. The switching of TAA and TAG to glutamine may have originated with mutation of a glutamine tRNA gene to a weak suppressor tRNA (131). This could have been followed by loss of TAA and TAG as stop codons and their appearance as glutamine codons in coding regions of genes. TAA and TAG have been adopted unevenly as glutamine codons in *Tetrahymena* species. In 11 *Tetrahymena* genes, TAA and TAG combined appear on average once per gene. However, in a partially sequenced tRNA synthetase gene (*crjB*), eight adoptions of TAA plus TAG are present, and in the partial sequence of a conjugation-specific gene (*cupC*), there

are 14 TAA and 19 TAG codons (reviewed in reference 219, where the original references to the earlier gene-sequencing papers can be found). *Paramecium* species use TAA for glutamine frequently and TAG more rarely, and *Stylonychia* and *Oxytricha* species use both codons very sparingly (120, 122, 146; reviewed in reference 219). What might have driven the switch in assignment of the stop codons TAG and TAA to glutamine in *Oxytricha* and *Stylonychia* species and the stop codon TGA to cysteine in *Euplotes* species is not known.

The unusual genetic code may be the reason that no virus has ever been found in ciliates, although bacterial symbionts are common (reviewed in reference 269). The changes in the ciliate code could conceivably be a barrier to adaptation of nonciliate viruses to ciliates.

CODON USAGE IN CILIATES

Codon usage in ciliates is strongly biased (219). *Tetrahymena* species and hypotrichs resemble budding yeasts, using the codon AGA for arginine but rarely using the arginine codons CGT, CGC, CGA, CGG, and AGG. Codon usage in *Paramecium* species, based on three cell surface antigen genes (219), is different from that in other ciliates and in yeasts. *Paramecium* species use the threonine codons ACT and ACA preferentially, whereas *Tetrahymena* species, hypotrichs, and yeasts use ACT and ACC about equally for threonine. GCG (alanine), GGG (glycine), CCG (proline), TCG (serine), and ACG (threonine) are very infrequently used in the hypotrich genes sequenced so far. CCG and ACG are not used at all in 12 *Stylonychia* genes to encode 161 prolines (CCG) and 234 threonines (ACG). There is no indication yet that codon usage in ciliates is different in genes expressed at high rates from that in genes expressed at low rates, as is the case in other eukaryotes.

DNA PROCESSING: CONVERSION OF MICRONUCLEAR DNA INTO MACRONUCLEAR DNA

Ciliates of opposite mating types can be induced to mate by starvation. In mating, two cells make a transient cytoplasmic connection, and the micronucleus(ei) undergoes meiosis. The two cells exchange haploid micronuclei through the cytoplasmic connection, and the migratory micronucleus fuses with a nonmigratory micronucleus to generate a new diploid micronucleus in each cell. The two cells separate, and all micronuclei, except the new diploid micronucleus, and the old macronucleus(ei) degenerate over an interval of many hours in the newly separated cells. Destruction of the old macronucleus is a regulated event in which the chromatin becomes pycnotic and ceases transcription and the DNA is digested into oligonucleosome-sized fragments typical of programmed death or apoptosis (72). The destruction of micronuclei presumably is a similarly programmed event.

The new diploid micronucleus divides mitotically without cell division, and one of the daughters develops into the new macronucleus(ei) over many hours or several days depending on the species.

PROCESSING IN *TETRAHYMENA* AND *PARAMECIUM* SPECIES

The first indication that a micronucleus has committed itself to development into a macronucleus is the start of DNA amplification. Sequence deletion then occurs very early in the DNA amplification period for no more than a few hours, when the amount of DNA increases from $4X$ to $8X$ (where X represents the amount of DNA present in a haploid genome), although the replication process is not directly linked to deletion (6, 25, 47).

There are ~6,000 sites in *Tetrahymena* micronuclear DNA at which sequences are deleted and the adjacent sequences are spliced (355). The deletion and splicing occur in most cases with high precision, generating the same rearranged DNA molecules in each anlage (150, 355). At least one exception occurs in *Tetrahymena* species; deletion events in a single anlage can produce two different products differing in size by 300 bp (25). The deleted sequences do not persist for some time in the anlage after their excision from the chromosomes (25); they become undetectable by in situ hybridization by the time the old macronucleus has totally disappeared and before the first postmating nuclear division of the new macronucleus (365).

The ~6,000 deleted sequences are spliced out with efficiency and precision. This is directed in part by direct-repeat sequences of 8 bp or less at the ends of the deleted segments (114). In addition to the direct repeat, in the case of a deleted sequence designated M, an essential element for correct cutting-splicing is a 5′-dA_5dG_5-3′ tract 44 to 51 bp away from the cut-splice site (113). At one end, the M segment can be spliced out at two different sites 0.3 kbp apart, and both sites possess copies of a short repeat and a 5′-dA_5dG_5-3′ tract. Splicing releases either a 0.9- or a 0.6-kbp fragment. A second deleted segment designated R (1.1 kbp) has 6-bp direct repeats at each end but lacks the 5′-dA_5dG_5-3′ tract in both flanking regions. The cutting-splicing of perhaps different families of deleted sequences must be achieved with different recognition mechanisms, involving perhaps both *cis* sequences and *trans*-acting factors.

The breakage mechanism that produces subchromosomal fragments in *Tetrahymena* species differs from the cutting-splicing mechanisms (361). A 15-bp sequence designated as the chromosome breakage sequence, or Cbs, identified as the signal sequence for excision of rDNA, is necessary and sufficient to specify a breakage site. The Cbs 15-mer occurs in the flanking DNA less than 20 bp from the cutting site and is eliminated by the fragmentation event (354, 363). Fragmentation creates subchromosomal molecules, including the rDNA, to which telomeres are added (370). The synthesis of telomere sequence onto broken ends appears to be closely coupled to fragmentation, since fragments lacking telomeres are not detectable (361).

Some deletion events in the *Tetrahymena* anlage represent the excision of transposonlike elements (the Tel-1 family) (61). These elements are somewhat variable in length but fall into a major size class of 9.7 kbp and a secondary class of 13.2 kbp. They possess blocks of 5′-dC_4dA_4-3′ at their extremities, and these blocks account for most and perhaps all of the interstitial 5′-dC_4dA_4-3′ sequences in *Tetrahymena* micronuclear DNA. The 5′-dC_4dA_4-3′ blocks are eliminated and do not contribute to formation of telomeres on the ends of the subchromosomal molecules retained in the macronucleus. Interstitial 5′-dC_4dA_4-3′ blocks are not present in macronuclear subchromosomal molecules (50).

The deletion program in the *Tetrahymena* anlage is sometimes variable. Some sequences persist as macronuclear DNA in some *Tetrahymena* cell clones but are eliminated from the anlage in other clones (338). Splicing out of this DNA from chromosomes in cell clones that lose it occurs at variable positions, resulting in polymorphisms in a subchromosomal DNA molecule that ultimately disappears by phenotypic assortment during vegetative growth.

The subchromosomal molecules with their new telomeres

created in the early *Tetrahymena* anlage replicate repeatedly until the average copy number of ~57 is reached. Some subchromosomal molecules amplify more than others (48), the extreme case being the rDNA molecule, which replicates to 9,000 copies instead of 57. At the end of the DNA replication phase, macronuclear development is complete, and cells resume vegetative proliferation.

Two fragmentation events in *Paramecium* cells have been shown to be under genetic control, perhaps mediated by a *trans*-acting factor. In the presence of a mutation known as d48, the micronuclear gene encoding a functional copy of the A antigen, which normally resides near a subchromosomal end in the wild-type macronucleus, fails to fragment properly, and part or all of the A antigen gene is deleted during macronuclear development (92, 102, 187). Other fragmentation events are normal in the presence of the d48 mutation. The participation of a *trans*-acting factor (encoded by the gene in which the d48 mutation has occurred?) is indicated by the restoration of normal processing (retention) of the A antigen gene in the anlage by injection of macronucleoplasm from a wild-type cell or injection of cytoplasm from a wild-type cell undergoing macronuclear development (133, 188). It appears that the old macronucleus produces a gene product that is required for correct processing of the A antigen gene in the anlage. The A antigen gene product itself is not the required factor, but it may be a product encoded by a gene closely associated with the A antigen gene (368), perhaps downstream of the A antigen gene (296). Similarly, the correct processing of a second gene, the B gene, requires the presence of a functional copy of the gene in the old macronucleus (298). This startling behavior indicates that processing of each gene in *Paramecium* species is gene specific, with each gene in the old macronucleus acting in some essential way for processing of the same gene during formation of a new macronucleus. Experiments with the G surface antigen gene provide another striking example of influence of the old macronucleus on processing of a specific gene in the anlage of *P. primaurelia* (230). Injection of a high copy number of the G gene into the macronucleus disrupts development of the anlage at a later time by modifying the site at which the telomere forms on the particular DNA subchromosomal fragment, resulting in almost complete elimination of the near terminal G gene in the new macronucleus. These findings with the A, B, and G genes provide an extraordinary insight into the regulation of DNA processing. Identification of the molecular nature of the signal from the old macronucleus to the anlage is enormously important but is probably also a very difficult problem and is not easily solved.

At least some fragmentation sites are normally variable. The telomere addition sites created when subchromosomal fragments form can vary by several hundred base pairs in *Paramecium* species (35, 55). The vegetative macronucleus of *Paramecium* species, and presumably the anlage, contains telomerase activity capable of synthesizing telomeric repeats on the ends of foreign, linear DNA that lacks telomeres (110). The telomerized foreign molecules are then maintained during subsequent proliferation. Molecules that fail to gain telomeres are degraded (44). A 15- to 30-fold-larger number of foreign molecules are retained and replicated if they possess telomeres before injection.

PROCESSING IN HYPOTRICHS

The study of macronuclear development in hypotrichs was initiated by Ammermann, who made thorough cytological and cytochemical observations in *E. aediculatus* and in *S. lemnae*

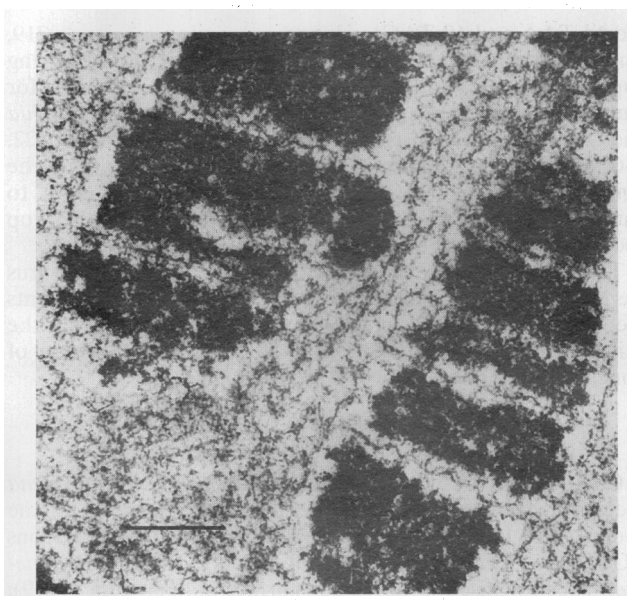

FIG. 16. Electron micrograph of short regions of polytene chromosomes in the anlage of *Oxytricha* species. The anlage is filled with proteinaceous material that surrounds the polytene chromosomes and forms fibrous septa through all interbands, transecting the DNA. Micrograph courtesy of K. G. Murti. Bar, 1 μm.

(originally misidentified as *S. mytilus*) (14). The micronuclear DNA content varies by ~100% from clone to clone in populations of *S. lemnae*, and this is reflected in the variation in the diploid chromosome number from 170 to 250. It appears that *S. lemnae* can carry variable numbers of extra chromosomes, which do not participate in anlage development but are eliminated without undergoing polytenization (17). In the micronucleus that is programmed to become a macronucleus, the 170 to 250 chromosomes (depending on the clone) become condensed and localized at the inner surface of the nuclear envelope (18, 21). Between 70 and 72 of these diploid number move inward into the anlage, where they despiralize and polytenize (13, 18). The condensed chromosomes at the anlage envelope, whose number varies from clone to clone, become pycnotic and are degraded during the following several hours. Such chromatin elimination from the very early anlage does not occur in *Oxytricha* species but may occur in *E. patella* (297).

A polytene chromosome stage is characteristic of the anlage in hypotrichs in general (9). The polytene chromosomes in *Stylonychia*, *Oxytricha*, *Holosticha*, and most *Euplotes* species contain bands with a variety of band morphologies and thicknesses. At about 50 h into anlage development, the chromosomes reach their maximum degree of polytenization: 64 copies of the DNA-protein fiber in *S. lemnae* (21) but less in *Oxytricha* and *Euplotes* species (probably 32 copies). During polytenization in the *Oxytricha* anlage, abundant proteinaceous material observed by electron microscopy accumulates on the inside of the nuclear envelope and subsequently disperses among the polytene chromosomes (Fig. 16) (238, 279). At the height of polyteny (40 to 50 h of development), the proteinaceous material forms septa (a fibrous mesh) through all the interbands of the polytene chromosomes (186, 239) (Fig. 16). The fibrous material then forms a capsule around each band in polytene chromosomes (278). The capsules, or vesicles, are physically separate since they freely disperse when the living anlage is broken open at this stage. Concomitant with vesicle formation, the molecular weight of the DNA falls markedly

(294), although not to the small size of the DNA in the mature macronucleus. Nothing is known about the protein that makes the vesicles. Conceivably it contains enzymes responsible for the transection of the DNA that runs through the interbands. This material is so abundant that it should be rather easy to isolate and characterize. It could hold an important clue about the mechanism of chromosome transection.

A curious example of the polytene chromosomes occurs in the marine organism *E. crassus*. These chromosomes lack bands and interbands; instead, they are uniform in structure throughout their lengths, as observed by light and electron microscopy, yet they are transected at the usual time (293). The strong impression that transection in the polytene chromosomes in other species is guided by the band-interband structure alone must be questioned. It may be that transection occurs at particular sequences and that these sequences are sequestered to interbands by whatever directs the band-interband organization of the polytene chromosomes. A comparative study of the chromosomes of *E. crassus* and other hypotrichs might answer some fundamental questions about the basis of band-interband organization and perhaps about chromosome transection.

Most of the DNA in each vesicle is now destroyed during the next several hours (278). This destruction reduces the total content of DNA in the anlage of *S. lemnae* by well over 90% (16). At the end of DNA destruction, the vesicles disappear and the remaining DNA mixes within the anlage. This begins the DNA-poor stage, which lasts ~40 h in *S. lemnae*, after which a series of replication bands rapidly traverse the anlage (290) until the average DNA molecule (gene) copy number for the particular species is reached and macronuclear development is complete.

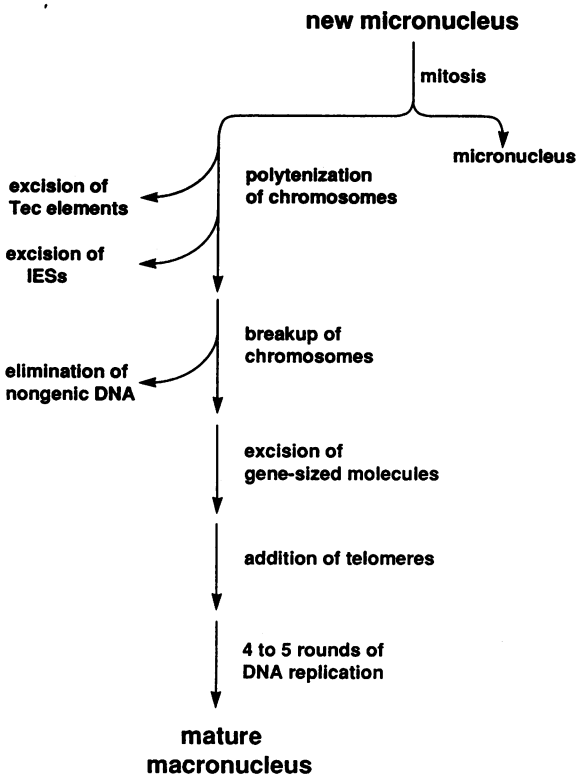

FIG. 17. The major events of macronuclear development in hypotrichs. The excision of Tec elements is well characterized in *E. crassus*. The timing of the excision of TBEs in *O. fallax* (not shown) is not known, but perhaps, like Tec elements, excision occurs during the polytene chromosome stage. The entire course of events occupies about 4 days. Redrawn from reference 271.

EXCISION OF TRANSPOSONLIKE ELEMENTS

Underlying the cytological and cytochemical events in the hypotrich anlage is a most unusual complex of DNA-processing events (Fig. 17). As in *Tetrahymena* species, the elimination of sequences in at least some hypotrichs begins in the early anlage; however, it lasts longer than in *Tetrahymena* species. In *E. crassus* the first event is the excision of families of transposonlike elements, called Tec*1* and Tec*2* (for transposonlike *E. crassus*), from the polytene chromosomes (27, 155, 156, 191). Tec*1* and Tec*2* elements are both ~5.3 kbp long and have inverted terminal repeat sequences of ~700 bp that are most similar between the two families in the most-terminal 150 bp, but their internal sequences do not readily cross-hybridize (191). About 30,000 copies of each Tec element are dispersed throughout the micronuclear genome of *E. crassus*, and some interrupt gene precursors in the micronucleus (27). There are no copies in the macronucleus. Open reading frames within the two Tec elements could possibly encode transposases that participate in Tec excision (154).

All copies of the Tec elements in both families are excised coordinately over 2 to 4 h (27, 155, 191, 325). After excision the Tec elements are destroyed. Within micronuclear DNA the two ends of Tec elements are immediately flanked by an excision target site of 5′-TdA-3′, and excision is precise. The sequence 5′-TTdGdAdA-3′, which occurs in the DNA flanking the Tec element 16 bp from the target site 5′-TdA-3′, is undoubtedly a crucial signal recognition site for factors involved in excision (158). This same 5-mer sequence may be involved in the eventual excision of gene-sized molecules from chromosomal DNA in *Euplotes* species at a later anlage stage (discussed below). The 5-mer is not involved in excision of gene-sized molecules in *Stylonychia* or *Oxytricha* species. During excision, one copy of the dinucleotide 5′-TdA-3′ remains at the splice site in the chromosome (325) and two copies appear in the circular, excised Tec element; the 5′-TdA-3′ dinucleotide is therefore duplicated during the circularization-excision process (158, 323). Ten additional nucleotides are present between the two copies of 5′-TdA-3′ in the excised circular Tec, which almost certainly means that excision occurs by staggered cuts in the DNA duplex at each end. Sequencing of excised Tec elements provides a consistent model in which one cut is made between the T and the A (but in the complementary chain) and another cut is made in the other DNA chain 10 bp into the flanking DNA (158) (Fig. 18). In the model the cut creates an 11-bp 5′ single-stranded tail at each end of the excised Tec element. The 11-bp tails from the two ends join to form a heteroduplex of 10 bp, leaving a 1-bp gap that is filled by DNA polymerase, and the heteroduplex is ligated by DNA ligase. The fill-in by DNA polymerase creates a duplication in the excised Tec element of the original 5′-TdA-3′ target site. In the excised Tec element the inverted repeats are arranged head-to-head and are separated by the two 5′-TdA-3′ target sites with 10 bp between them. In the model the excision site in the chromosome is then healed by filling in by DNA polymerase of the 11-bp 5′ single-stranded tails, followed by ligation. The fill-in process forms a single complete copy of the 5′-TdA-3′ known to be present at the excision site.

The experimental observations make the model in Fig. 18 compelling. A critical element concerns the factors, presumably proteins, that recognize the signal sequences, make the

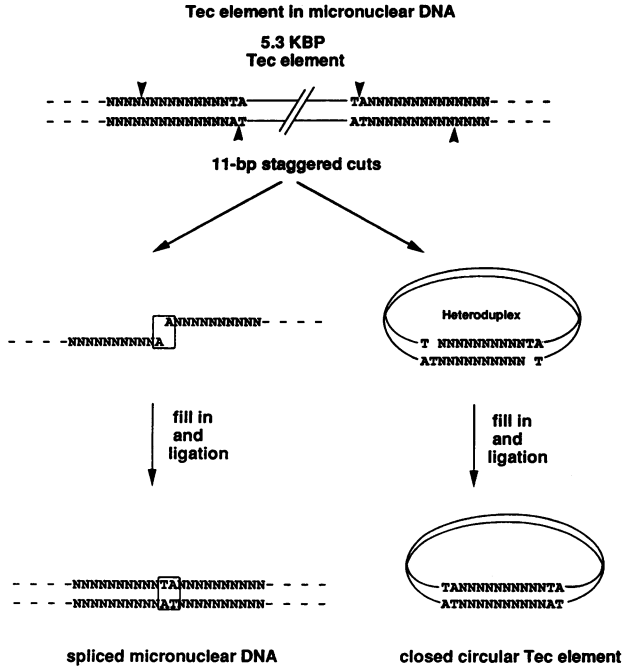

FIG. 18. Model of Tec excision. A TA dinucleotide is present immediately outside each end of the Tec element. N represents any nucleotide flanking the TA. Staggered cuts indicated by arrowheads excise the Tec element with an 11-base single-stranded 5′ extension at each end. The two 11-base single-stranded extensions are held in a heteroduplex, presumably by a protein(s). The nucleotide gaps at each end of the heteroduplex are filled in with an A and ligated, producing a covalently closed Tec circle with a heteroduplex junction. The junction contains two copies of the TA direct repeat and 10 bp derived from 10 bases in micronuclear DNA flanking each side of the Tec element. The two 5′ 10-base single-stranded copies remaining in micronuclear DNA after removal of the Tec element are held end-to-end, filled in by DNA polymerase, and blunt end ligated to close the excision site. The extra 10 bp (N) present in the closed-circular Tec element could be produced by variations in this model involving changes in the length of the 5′ extension in the staggered cut and the number of bases forming the heteroduplex. Redrawn from reference 158 with permission.

staggered cuts, and hold the ends of the excised Tec element together as a heteroduplex until ligation of the two chains has occurred. A similar model, involving staggered cuts and heteroduplex junctions, removes internally eliminated sequences (IESs) later in development of the anlage from chromosomal segments destined to become gene-sized molecules in *Euplotes* species (183) (discussed below).

Whether transposonlike elements are a general feature of ciliates or hypotrichs is not known. Transposonlike elements flanked by interstitial telomeric repeats occur in *Tetrahymena* species (61). A family of transposonlike elements called TBEs is present (probably dispersed) in the micronuclear genome of *O. fallax* and is totally removed from the anlage and destroyed (142). The 1,900 members of the family are not identical but share a considerable amount of conserved sequence. A TBE has a 77- or 78-bp inverted repeat at its termini, and the final 17 bp of the inverted repeat consists of the telomere sequence 5′-$dC_1dA_4dC_4dA_4dC_4$-3′. Immediately outside the inverted repeats is the direct repeat 5′-dAdAT-3′, which may serve as part of the target in the excision process. The TBEs are

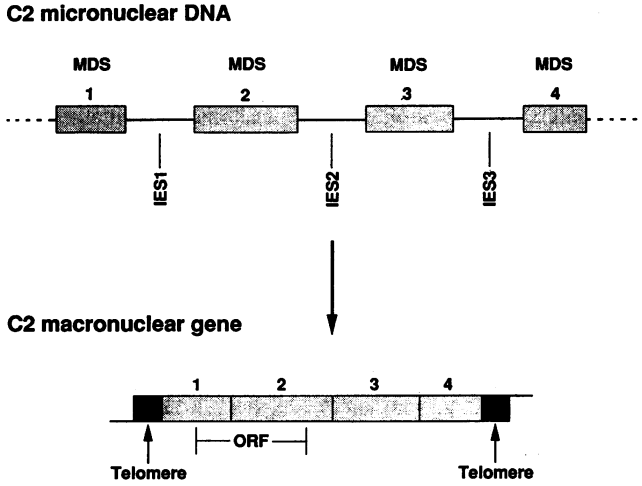

FIG. 19. Diagram of the C2 gene in the micronucleus and macronucleus of *O. nova*. The four MDSs are separated by three IESs in the micronuclear gene and are spliced to make the macronuclear gene-sized molecule. Telomeric sequences are added when the gene is excised from the chromosome. Redrawn from reference 181.

removed precisely from anlage DNA, but the timing of excision in development is unknown (151).

Interstitial telomeric repeats do not always necessarily represent transposons. Over 1,000 interstitial telomere repeats (18-mers of $dC_4dA_4dC_4dA_4dC_2$) occur in the polytene chromosomes of *S. lemnae* associated with two 2-kbp direct repeats that surround a 2.6-kbp segment (315). The central 2.6-kbp segment shows strong homology to a 2.6-kbp macronuclear gene. This repeated, telomere-containing element in polytene chromosomes does not resemble a transposon, and its significance is unknown. Interstitial 5′-dC_4dA_4-3′ blocks are present in micronuclear DNA of *O. nova*, but the few that have been cloned do not appear to represent ends of transposonlike elements (123). The DNA fragments in the macronucleus of *P. primaurelia* contain 25 to 60 blocks of interstitial telomeric repeats (170). About 30% of DNA molecules injected into the macronucleus integrate next to or into these telomeric repeats, demonstrating that the repeats are hot spots for recombination. An interstitial telomere repeat has been identified as a recombination (crossover) hot spot in a hamster chromosome (24).

EXCISION OF INTERNALLY ELIMINATED SEQUENCES

An obvious approach to the question of how gene-sized molecules are excised from micronuclear chromosomes is to clone the macronuclear and micronuclear versions of a gene and examine the junctions of the gene with its flanking DNA in the chromosome for sequences that might signal excision. The first analysis of cloned macronuclear and micronuclear versions of a gene-sized molecule was done with C2, a molecule with an open reading frame encoding a putative polypeptide of 82 amino acids. The micronuclear and macronuclear versions are identical, except that the micronuclear version contains three IESs, which are A+T-rich unique sequences of 49, 49, and 32 bp (181) (Fig. 19). One of these IESs interrupts the open reading frame, the second interrupts the 3′ end of the transcribed region outside the open reading frame, and the third interrupts the 3′ trailer downstream of the transcription unit. The four segments of the micronuclear precursor gene

separated by the three IESs are called macronuclear-destined sequences (MDSs). When the IESs are deleted from the gene, the MDSs are spliced to make the future macronuclear gene. The sequences flanking the micronuclear version of C2 contain no telomere sequences, which confirms the earlier finding that telomeric sequences are absent from micronuclear versions of genes and therefore do not signal excision (43). The absence of telomeric sequence at junctions is now a well-established fact for micronuclear genes in general (see, e.g., references 75, 124, 236, and 293). Potential target sequences for excision of gene-sized molecules are discussed below.

The original discovery of IESs in the C2 gene was followed by identification of IESs in every hypotrich micronuclear precursor of a macronuclear gene-sized molecule (with one possible exception [27]) that has been sufficiently characterized (~16 genes altogether in *Euplotes*, *Oxytricha*, and *Stylonychia* species). IESs are very A+T rich. The average A+T content calculated from 27 IESs in four micronuclear genes in *O. nova* is 82%. Extrapolation from three genes gives an estimate of 60,000 IESs in the micronuclear genome of *E. crassus* (292), and extrapolation from five genes in *O. nova* provides an estimate of 170,000 IESs in its micronuclear genome (272). It is likely that IESs appeared in evolution before the split between the genus *Euplotes* and the other hypotrichs, at least several hundred million years ago.

IESs were originally defined as unique A+T-rich sequences ranging in size from 31 to 374 bp (27, 183; also reviewed in reference 180). However, at least some transposonlike elements (TBEs) in *O. fallax*, which are several kilobase pairs long, interrupt regions of micronuclear DNA that become macronuclear gene-sized molecules and hence qualify as IESs (151). Similarly, some Tec elements (5.3 kbp long) in *E. crassus* appear to interrupt micronuclear versions of gene-sized molecules and technically are IESs (27, 156).

Because many IESs interrupt coding regions of genes, their removal is necessarily precise. Short direct repeats immediately flank the ends of every IES, whether the IES is a transposonlike element or a much shorter unique sequence. In *E. crassus* the direct repeats are 2 to 4 bp long and always include the dinucleotide 5′-TdA-3′. In *Oxytricha* and *Stylonychia* species the direct repeat may be 2 to 6 bp of any composition and the 5′-TdA-3′ dinucleotide is present no more often than expected randomly. In *E. crassus*, at least some larger IESs (a few hundred base pairs) are removed as circles (323), but it is theoretically impossible to remove IESs shorter than ~100 bp as conventional double-stranded circles because the DNA double helix cannot bend sufficiently. Unwinding of the double helix in a short IES or assisting circularization with proteins in some other way (single-strand nicking) would solve the problem.

IESs are excised similarly to Tec elements in *E. crassus*. The terminal direct repeat is duplicated so that the excised circular IES has two copies, and one copy is present at the splice site in the MDS DNA (323). Between the two direct 5′-TdA-3′ repeats in the circular IES is 10 bp derived by duplication from the MDSs flanking the original IES. This led to the hypothesis that the IES is removed by 10-bp staggered cuts in such a way that a duplication of the 5′-TdA-3′ repeat takes place with 10 bp between repeats in the excised IES (Fig. 20) (180, 323). The two single-stranded 5′ overhangs in the flanking MDS are filled in, and the two ends are ligated. The model has been strongly supported by the demonstration that the junction in the circular IES contains a heteroduplex of 6 bp (183). The 10-bp sequence between the direct repeats in the IES is completed by 2-bp fill-in reactions on each side of the central 6-bp heteroduplex, and then the chains are ligated.

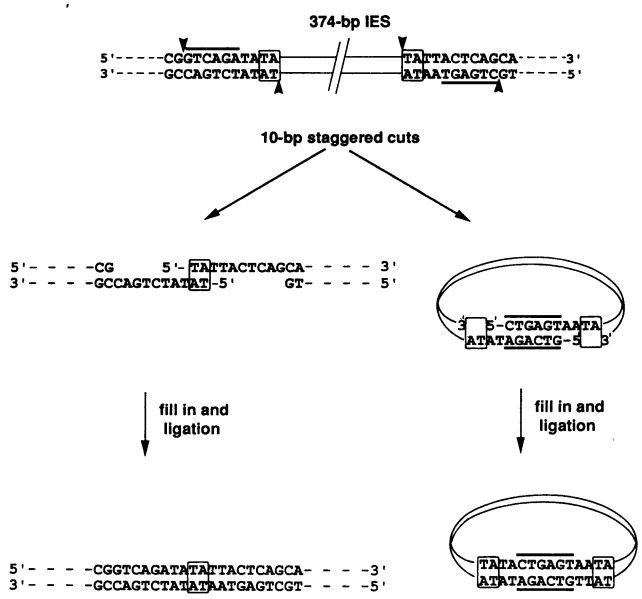

FIG. 20. Model of IES excision based on a 374-bp IES in the V1 gene of *E. crassus*. The positions of the 10-bp staggered cuts on each side of the IES are shown by arrowheads. The repeat dinucleotides at each end of the IES are boxed. The two 5′ single-stranded ends remaining after IES excision associate by the 5′-TdA-3′ dinucleotides (left side). The single-stranded gaps are filled and ligated; one copy of the TA repeat is then present in the junction. The 5′ single-stranded ends of the excised IES (right side) form a 6-bp heteroduplex (heavy bars over nucleotides). The two gaps are filled in and ligated, yielding a covalently closed circle with a 6-bp heteroduplex in the center of the junction. Redrawn from reference 323.

Although Tec elements and IESs appear to be removed by a similar mechanism in *E. crassus*, probably involving the same or nearly identical molecular machinery (enzymes and other proteins) (151, 155, 158, 183, 323), remarkably the two kinds of excision events occur at very different times in anlage development. Tec elements are removed very early in chromosome polytenization, but IESs are excised ~14 h later toward the end of the polytene stage (155, 191, 324) and just prior to fragmentation of the polytene chromosomes (324).

IESs may also be common in the *Paramecium* micronuclear genome, although very few genes have been analyzed for this phenomenon so far. The micronuclear gene encoding the A surface antigen protein contains nine IESs, and remarkably these are flanked by an AT direct repeat as in the distantly related *Euplotes* species (310).

CHROMOSOME FRAGMENTATION, EXCISION OF GENE-SIZED MOLECULES, AND TELOMERE ADDITION

Excision first of Tec elements and then of IESs in hypotrichs leaves the polytene chromosomes intact. Indeed, the DNA molecules in the polytene chromosomes continue to replicate after excision of TEC elements and possibly during and for a brief period after IES removal (324).

The transection of polytene chromosomes is accompanied by a sharp drop in molecular weight of the DNA (294), but it is uncertain what this represents in terms of gene excision. The initial excision step may release clusters of genes, a cluster corresponding to a band of a polytene chromosome (185). This idea is consistent with the proven clustering of genes in

micronuclear DNA but also with the estimate of 2,700 vesicles in the anlage in *O. nova*, in which one vesicle corresponds to one chromosome band (308). Thus the 24,000 genes occur in clusters that average 9 genes, i.e., 9 genes per polytene band.

Tec element and IES excisions account for a small part of the reduction in total sequence that occurs in the anlage. The main diminution is presumed to occur in the DNA destruction phase that begins with chromosome transection and vesicle formation. Thus, the fragments of DNA created by transection must contain not only clusters of genes but also very large amounts of nongenic unique and repetitious sequences that are destined for destruction. This is consistent with the detection of a DNA fragment in the vesicle stage of *E. crassus* that is more than twice as long as the gene-sized molecule to which it eventually gives rise (294). However, according to the model, much larger intermediates, containing several genes and much nongenic DNA, should be detectable.

The destruction of nongenic sequences accounts for the massive decrease in total DNA during the vesicle stage and the concomitant release of the gene-sized molecules, but the major question of how the organism discriminates between sequences to be destroyed and sequences to be preserved remains.

The excision of gene-sized molecules from micronuclear DNA appears to be imprecise in *Oxytricha* species, varying over a few base pairs in *O. nova* (29) and somewhat more in *O. fallax* (143). Unlike *Tetrahymena* species with their chromosome breakage sequence (Cbs) of 15 bp (361), no consensus sequence that might guide the excision process has been identified in *Oxytricha* species. In *E. crassus* a 14-bp consensus sequence occurs near the cutting site either 10 bp into the gene or 4 bp into the flanking DNA (29). This sequence perhaps guides the cutting of gene excision, which may account for the observation that, unlike in *Oxytricha* species, the excision of a particular gene always occurs at the same base pair. However, it is puzzling that the consensus sequence can direct cutting from either side of the cutting site.

Between 60 and 130 bp of leaders and trailers just inside the telomeres in 27 cloned macronuclear gene-sized molecules of *S. lemnae* commonly contain 6- to 9-bp inverted repeats; a 10-bp palindromic consensus sequence is present in leaders and trailers of all 27 molecules, usually at positions between 40 and 70 bp inward (217). The significance of these sequence regularities is unknown. They may have a function in excision of gene-sized molecules from chromosomes, as proposed by Maercker and Lipps (217), or they may serve in the initiation of replication.

The addition of telomere sequence to the ends of newly excised genes is presumably catalyzed in hypotrichs as it is in *Tetrahymena* species by telomerase, which is an abundant enzyme activity in hypotrichs (371). This addition must occur very rapidly, because ends without telomeres are not detected in anlagen around the vesicle stage (294). The initial telomeres in *E. crassus* are several times longer (371) and highly variable in length compared with the mature form (327a). These are pared to yield the standard length of 14-bp 3′ tails with a 28-bp double-stranded segment before anlage development ends. Telomere shortening occurs at a specific time in macronuclear development and is an active process that is not linked to DNA replication (327b). A model of telomere synthesis presented by Vermeesch and Price (327a) suggests that once the G-rich strand is synthesized by telomerase on a newly excised molecule, a single-stranded protein binds beginning at the 5′ end of the G-rich strand. This protects the single-stranded DNA and sets the G-rich strand length. RNA primase synthesizes a 14-ribonucleotide RNA primer on the protein-protected G-rich strand. The primer is extended with deoxynucleotides to create the 28-bp C-rich strand. Finally, the RNA primer is removed to yield the 14-nucleotide, G-rich 3′ single-stranded overhang.

ALTERNATIVE PROCESSING OF GENE-SIZED MOLECULES

At least some macronuclear gene-sized molecules form families in which the members of a family are derived from a single micronuclear precursor. One extensively analyzed family in *O. fallax*, known as 81-MAC, consists of three gene-sized molecules of 4.9, 2.9, and 1.6 kbp (57, 143, 144, 342). All three have a region of ~1.6 kbp in common. The 4.9-kbp member has an additional 3.3-kbp arm on one end of its 1.6-kbp segment, and the 2.9-kbp member has a different sequence arm of ~1.3 kbp at the opposite end of its 1.6-kbp commonality. There appear to be three versions of the micronuclear precursor of the 81-MAC family, which differ in a small percentage of nucleotide positions, and each generates its own set of three macronuclear DNA molecules for a total of nine. All three members of an 81-MAC set representing one micronuclear precursor version are produced in a single anlage by alternative processing among the multiple copies present in the polytene chromosome. A single transcript of 1.3 kb corresponding to most of the 1.6-kbp common region encodes a homolog of mitochondrial solute carriers. Three introns are present. No transcripts to either arms in the 4.9- and 2.9-kbp members of the family are detectable (143), and the function of these sequences is unknown.

In another clear case of alternative processing, two different-sized macronuclear DNA molecules (2.6 and 5.0 kbp) in *O. nova* are derived from the same micronuclear precursor (179). The two molecules are identical on one end, but one molecule is 2.4 kbp longer at the other end. As in the previous case, there are three versions of the micronuclear precursor, each giving rise to two genes by alternative processing. The significance of these cases of alternative processing is not understood.

SCRAMBLED GENES

The search for a consensus sequence that might direct gene excision and telomere addition led to the cloning and sequencing of additional matched pairs of micronuclear and macronuclear genes (the first was the C2 gene, which led to the discovery of IESs). Two actin genes, actin I and actin II, are present in *O. nova*. They each encode an actin of 375 amino acids but are only 63% identical in amino acid sequence (122, 237); hence they are very different genes. (Both are also very different from the conserved actin gene in yeasts through mammals.) Two versions of the actin I gene in micronuclear DNA have been cloned. They differ in ~3% of nucleotides but encode identical polypeptides (124). A macronuclear actin I gene that is a perfect base pair match to one of the two micronuclear versions, except for the presence of IESs, has been cloned. Some of its characteristics are given in Table 2.

The micronuclear actin I gene in *O. nova* is part of a gene cluster. At its 5′ end it is separated by 15 bp from an adjacent macronuclear precursor of 3.3 kbp of unknown function. More genes are present upstream and downstream separated by spacers, but these have not been mapped or defined. Both micronuclear versions of the actin I gene are in gene clusters of the same global structure, e.g., separated from the adjacent 3.3-kbp gene by a 15-bp spacer, and are therefore presumed to be alleles. The actin I gene consists of nine MDSs separated by eight IESs ranging in size from 20 to 122 bp (124, 275). The

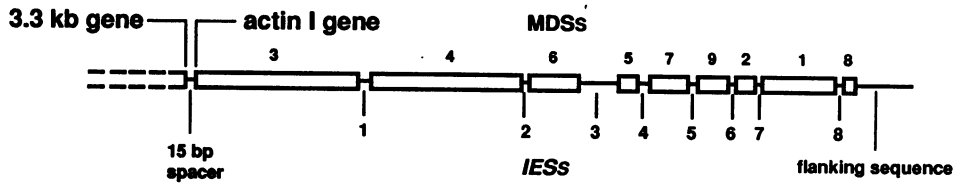

FIG. 21. The actin I gene in the micronucleus of *O. nova* occurs in a cluster of genes. A 3.3-kbp gene is present to the left, separated by 15 bp. One or more genes are present to the right, but these have not been defined. The micronuclear actin I gene consists of nine MDSs and eight IESs. The MDSs are numbered according to their order in the macronucleus. Only MDSs 3 and 4 in the micronucleus are in the orthodox order relative to one another. The other seven MDSs are scrambled.

nine MDSs are in the unorthodox or scrambled order 3-4-6-5-7-9-2-1-8, the proper order being defined by the 1 through 9 arrangement of the spliced MDSs in the functional macronuclear gene (Fig. 21). A search that included selection of 13 actin I gene clones from two micronuclear DNA libraries and PCR of native micronuclear DNA yielded only scrambled versions of the actin I sequence. Only the nonscrambled sequence is detectable in native macronuclear DNA or macronuclear DNA libraries.

It could be argued that the actin I gene has become scrambled since the last successful mating of *O. nova* by some random process of DNA breakage and rejoining, making the micronucleus nonfunctional. However, it is extremely improbable that random DNA breakage and splicing could have occurred in a 1,532-bp segment of DNA without introducing a single base pair change in the overall sequence. The micronuclear gene matches perfectly a cloned macronuclear gene, except that the micronuclear gene is scrambled and has IESs. Can an organism with scrambled micronuclear genes mate and produce a new macronucleus? No mating types of *O. nova* have been isolated, but selfing occurs from time to time in laboratory cultures. Usually all of the exmated cells that result from selfing die without resuming vegetative growth; thousands have been observed. An occasional exmated cell selected with a well-developed anlage survives if mating cells are kept at 12°C instead of the usual 23 to 24°C. In addition, PCR experiments with micronuclear DNA isolated from an exmated clone showed only the scrambled form of the actin I gene. Therefore, the scrambled gene must be processed into an unscrambled copy during macronuclear development. In addition, the actin I gene is similarly scrambled in *O. trifallax*, in which mating yields viable exmated cells.

Instructions for unscrambling the micronuclear actin I gene are apparently carried in the gene itself. At each MDS-IES junction is a sequence of 9 to 13 bp that is repeated at another MDS-IES junction somewhere else in the gene. Aligning the two members of a repeat pair occurs by folding the scrambled gene as shown in the model in Fig. 22. In the model, homologous recombination between the repeats would then join MDSs in the correct order and eliminate one copy of each repeat pair. All the pairs of repeats are different sequences. MDS2 has reversed polarity from the other eight MDSs and has inverted rather than direct repeat pairs between its MDS-IES junctions and two junctions elsewhere. The inverted repeats become direct repeats when that part of the molecule is folded into an inverted configuration; recombination would then join MDS2 to MDS1 and MDS3 in the orthodox orientation.

The gene encoding the αTBP is also scrambled in the micronucleus of *O. nova* (236), but, unlike in the actin I gene, the scrambling pattern is nonrandom. The 14 MDSs are arranged in the order 1-3-5-7-9-11-2-4-6-8-10-12-13-14. Again, pairs of repeats 6 to 19 bp long are present at MDS-IES junctions for scrambled MDSs. In this case they are all direct; folding the molecule to align pairs of repeats produces a concentric pattern (Fig. 23).

A third gene, encoding a large subunit of DNA polymerase α, contains 44 MDSs and is scrambled in a complex but nonrandom pattern including a large inversion (148), with pairs of repeats that could instruct unscrambling during macronuclear development.

The model of unscrambling by homologous recombination does not result in excision of a gene from its chromosomal location. When in development recombination might occur is not known, but gene excision presumably takes place after recombination, probably during the extensive breakdown of DNA in the vesicle stage of the anlage. Recombination as proposed would excise some of the eight IESs from the actin I gene as circles, but several others would simply be displaced to right and left flanking regions outside the gene (275). These IESs are presumably removed and destroyed later, perhaps at the time of gene excision. Nothing is known about the molecular machinery that accomplishes unscrambling.

So far six micronuclear genes (R1, C2, αTBP, βTBP, two alleles of actin I, and DNA polymerase α) have been sequenced in *Oxytricha* species and three (the two alleles of actin I, αTBP, and DNA polymerase α) have proven to be scrambled. These few data suggest that scrambled genes are a common phenomenon in the micronuclear DNA of *O. nova*, perhaps affecting half of all genes.

A few data are available for micronuclear genes in other species. According to a PCR experiment, the actin I gene is scrambled in *O. trifallax* with the same general pattern as in *O. nova* (124), but the αTBP gene is not scrambled in *O. trifallax* (84). It appears that the actin I gene became scrambled before the two species diverged in evolution but that the αTBP gene became scrambled in *O. nova* after the divergence. Therefore, scrambling may be an ongoing process in *Oxytricha* species. The gene encoding elongation factor EF-1α is not scrambled in the micronucleus of *S. lemnae* (38). Southern blot hybridization to macronuclear DNA with restriction fragments of two micronuclear DNA clones from *S. lemnae* suggests that segments of two macronuclear genes may be interspersed with one another in micronuclear DNA (314). Confirmation of such a remarkable kind of micronuclear gene scrambling will require a complete nucleotide sequence analysis.

The significance of gene scrambling in evolution is unknown, but the proposed folding pattern of DNA that facilitates unscrambling may be a clue to the scrambling event. Originally, genes such as the actin I and αTBP genes must have been orthodox. The folding pattern of the segment of DNA containing the gene in chromatin may have determined how it became reordered by recombination, i.e., the same folding pattern proposed for unscrambling. Given the folding pattern in chromatin, three events were necessary to produce scrambling: IESs had to be introduced; pairs of repeat sequences had to

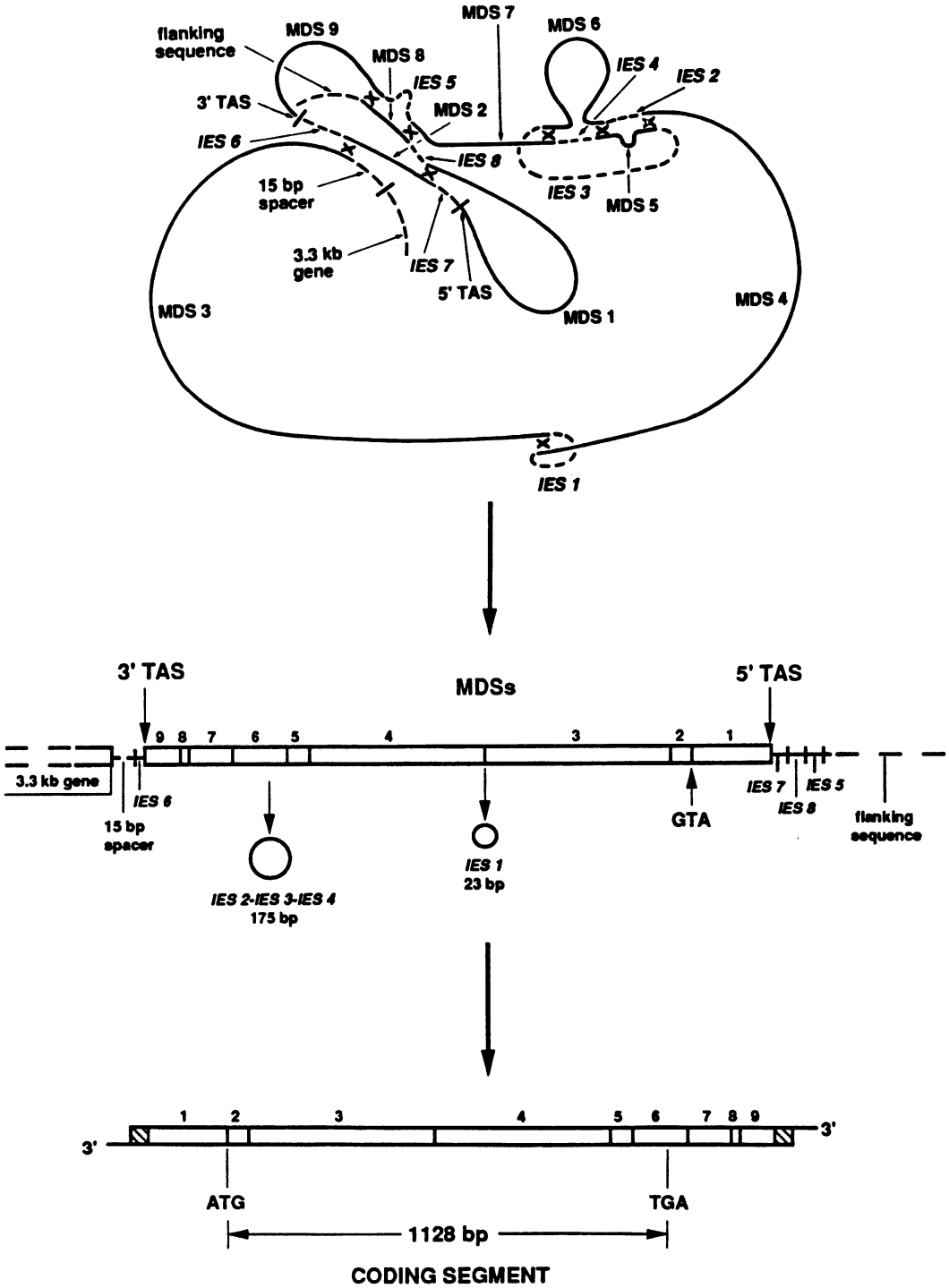

FIG. 22. In the recombination model for unscrambling of the actin I gene in *O. nova*, the micronuclear DNA is folded to align pairs of repeats at IES-MDS junctions (top). Recombination between the repeats in each pair eliminates some IESs, displaces others to flanking positions, accomplishes splicing of MDSs into the correct genetic order, and reverses the orientation of the gene (middle). Excision of the actin I gene and addition of telomeres produces the exact sequence determined for the version in the macronucleus (bottom). The gene has been inverted to conform to the convention of left-to-right transcription.

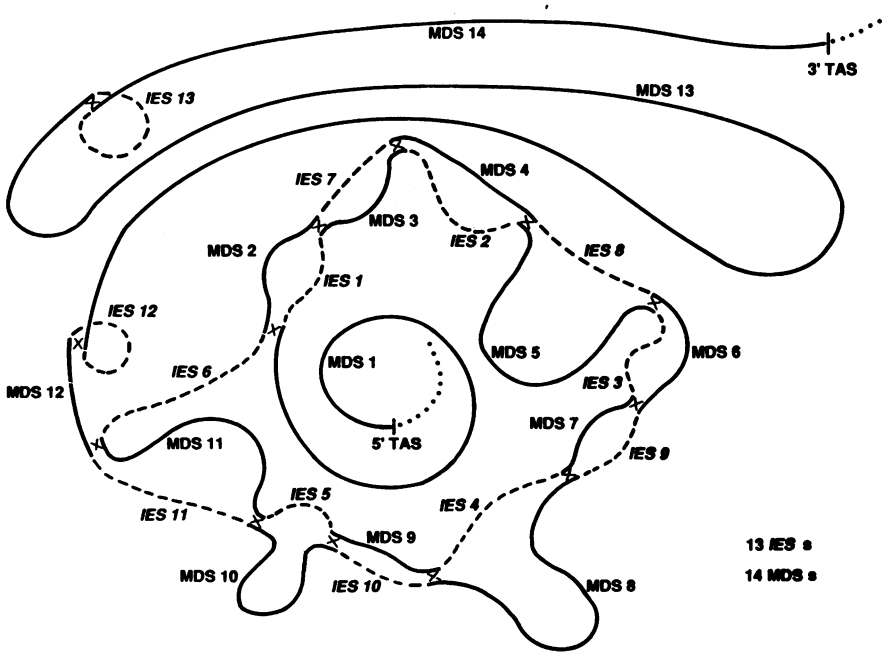

FIG. 23. Folded model of micronuclear DNA containing the gene encoding αTBP. The nonrandom scrambling pattern gives rise to concentric circles when pairs of repeats are aligned. Recombination between the pairs of repeats would remove IESs 1 to 11 as a single circle, remove IESs 12 and 13 as separate circles, and splice the 14 MDSs in the order present in the functional macronuclear gene. From reference 236.

form by duplication of terminal segments of MDSs (these terminal segments are often gene coding, and their existence must have preceded scrambling); and MDSs had to be reordered, presumably by recombination. How might the three events be related? Conceivably IESs were introduced into a gene before it became scrambled. However, pairs of repeats were probably created in the folded DNA molecule as a part of the MDS reordering event. There is no easily conceived model to explain how the three events might have been coordinated.

FINAL DNA AMPLIFICATION

The gene-sized molecules that are the final result of IES removal, unscrambling, chromosome breakup, gene excision, and telomere addition undergo several rounds of replication as the last phase of macronuclear development. This amplification occurs by means of replication bands, which are believed to replicate all DNA molecules once each time they pass through the maturing macronucleus and do not contribute to the differential copy numbers of genes observed in the vegetative macronucleus. Different copy numbers of genes may be established earlier in the anlage by differential replication. Such clearly seems to be the case for *Tetrahymena* rDNA (genes encoding rRNA). Whether this occurs for other genes, for example during chromosome polytenization in hypotrichs, is not known. Differential amplification of most genes may be achieved in the finished macronucleus by the mechanism that maintains gene balance.

Research on ciliates in general and on *Tetrahymena* species and hypotrichs in particular has uncovered an extraordinary array of DNA-processing events whose biological, evolutionary, or genetic significance ranges from poorly understood to totally mysterious. The study of the phenomena of transposon-like elements, IESs, differential amplification, gene scrambling and unscrambling, chromosome fragmentation, sequence elimination, gene excision, telomere synthesis, telomere function, and control of gene copy number will ultimately reveal new principles about molecular manipulations of DNA.

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