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Alternative use of chromosome fragmentation sites in the ciliated protozoan *Oxytricha nova*

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ABSTRACT

During its life cycle, the hypotrichous ciliated protozoan Oxytricha nova transforms a copy of its micronucleus, which contains chromosome-sized DNA, into a macronucleus containing linear, gene-sized DNA molecules. A region of the micronuclear genome has been defined that gives rise to two distinct macronuclear DNA molecules during development. Through analysis of recombinant macronuclear and micronuclear clones, the generation of the two macronuclear DNA molecules was shown to be the result of alternative use of chromosome fragmentation sites. In addition, evidence was obtained that adjacent micronuclear precursors of macronuclear DNA molecules can overlap by a few base pairs. The significance of these findings in relation to developmental chromosome fragmentation is discussed.

INTRODUCTION

The hypotrichous ciliated protozoan Oxytricha nova contains two organizationally and functionally distinct nuclei. The micronucleus contains a complex genome with DNA organized as chromosomes, but is transcriptionally inactive during the vegetative growth of the cell. In contrast, the macronucleus is responsible for vegetative nuclear transcription, but has a highly unusual genetic organization. The macronuclear genome contains approximately 20,000 different linear DNA molecules (referred to as "macronuclear genes") with an average size of 2.2 kbp (1,2). On average, each of these macronuclear gene-sized DNA molecules is present in 1,000 copies per macronucleus.

Following the sexual phase of the life cycle, the macronucleus is destroyed and a new one is generated from a copy of the micronucleus through an extensive and complex series of DNA rearrangement events (reviewed in 2,3). In brief, the micronuclear chromosomes are first replicated to form polytene chromosomes. The polytene chromosomes are then fragmented and greater than 95% of the sequences originally present in the micronucleus are eliminated. Finally, the remaining linear gene-sized DNA molecules undergo

a number of rounds of DNA replication giving rise to the mature macronucleus. Two additional types of events are known to occur during macronuclear development. First, simple repeat sequences (C_4A_4 repeats) are added to the ends of macronuclear DNA molecules and serve as telomeres during vegetative growth (4,5). Second, blocks of DNA sequences (internal eliminated sequences; IESs) are removed from internal regions of the micronuclear chromosomal precursors of macronuclear DNA molecules by a nucleic acid breakage and joining, or splicing process (4,6).

We have been examining the micronuclear organization of particular macronuclear DNA molecules to learn more about the types of rearrangement events that occur during macronuclear development. In a previous study (7) we had identified a region of a cloned segment of micronuclear DNA (clone LMIC2-2) that, in hybridization analyses, shared homology with both 5.0 kbp and 2.6 kbp macronuclear DNA molecules (referred to as the C4 and C5 "genes", respectively; Fig. 1a). The macronuclear C4 and C5 genes appeared to share a common region of 2.6 kbp, with the C4 gene having a unique left end of 2.4 kbp. This observation suggested that one region of the micronuclear genome can give rise to two distinct macronuclear DNA molecules by some type of alternative processing. Based on what is known about the types of rearrangement events that occur during development, two types of alternative processing events can be envisioned: 1) alternative fragmentation of the micronuclear chromosome, and 2) differential removal of an IES. Since both of these rearrangement events are thought to occur after the polytene chromosome stage of development, a single cell could receive more than one type of macronuclear DNA molecule from a single region of the micronuclear genome.

In the case of C4 and C5, however, the situation is complicated. In addition to LMIC2-2, a second related cloned segment of micronuclear DNA (clone LMIC2-5; Fig. 1a) was also identified that shared homology with the two macronuclear DNA molecules (7). Thus, it was formally possible that distinct regions of the micronuclear genome separately give rise to the C4 and C5 macronuclear DNA molecules during development, and alternative processing is not operative.

In the present study we have examined the macronuclear and micronuclear organization of the C4 and C5 genes in detail. In addition to the regions of the micronuclear genome represented in clones LMIC2-2 and LMIC2-5, a third micronuclear region sharing homology with C4 and C5 has been defined. We demonstrate that all three of these micronuclear copies give rise to both

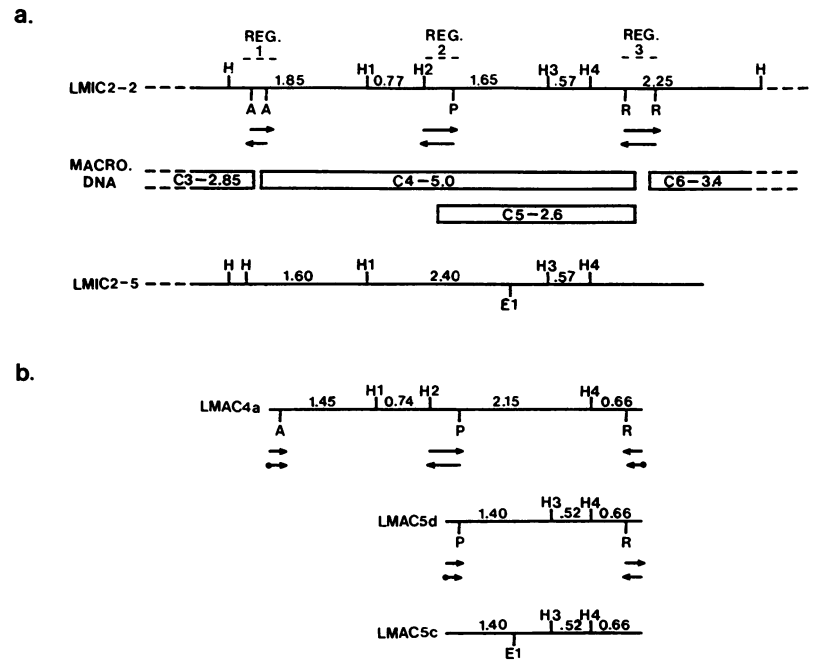

Figure 1. A.) Restriction maps of micronuclear clones LMIC2-2 and LMIC2-5. Also shown are macronuclear DNA molecules (boxes) which share homology with the micronuclear clones. The macronuclear DNA molecules are aligned to the regions of the clones with which they share homology. Regions of clone LMIC2-2 that were sequenced are indicated (regions 1, 2, and 3), and correspond to those shown in Figure 4. b.) Restriction maps of the macronuclear C4 clone LMAC4a and macronuclear C5 clones LMAC5d and LMAC5c. H = HindIII, E = EcoRI, A = HaeIII, P = PvuII, and R = RsaI. All HaeIII, PvuII, and RsaI sites are not shown. Regions of macronuclear and micronuclear clones sequenced by either the dideoxy method (⟶) or the Maxam and Gilbert chemical method (•⟶) using 3' end-labeled DNA are indicated.

a C4 and C5 gene in the macronucleus via alternative processing. Sequence analyses of micronuclear and macronuclear recombinant clones indicate that variable use of chromosome fragmentation sites is responsible for generating the C4 and C5 macronuclear DNA molecules. Finally, during this work, evidence was obtained indicating that the micronuclear precursor of a 2.85 kbp macronuclear DNA molecule (C3) overlaps the left end of the C4 gene.

MATERIALS AND METHODS

Cell Culture and DNA Isolation

O. nova, strain H10, was cultured using the algae Chlorogonium as a

food source (8). Macronuclei and micronuclei were isolated, and DNAs prepared, as previously described (4,9).

Recombinant plasmid and bacteriophage lambda DNAs were prepared using established procedures (10).

Gel Electrophoresis and Hybridization

Restriction endonucleases and DNA modifying enzymes were purchased from either Bethesda Research Laboratories or New England Bio-Labs, Inc., and were used according to the directions of the manufacturer.

DNA fragments were separated and sized on 0.75-2.00% agarose or low melting point agarose gels prepared and run in 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0. DNA was transferred to nitrocellulose filters using the method of Southern (11). Filter were hybridized under conditions previously described (6,12), using DNA probes labeled with ^{32}P by the method of Feinberg and Vogelstein (13).

DNA Sequencing

DNA fragments were cloned into bacteriophage M13 mp8, mp9, mp10, or mp11 and sequenced by the dideoxy chain-termination method (14). Alternatively, DNA fragments were 3' end-labeled, and sequenced by the Maxam and Gilbert chemical method (15).

RESULTS

Restriction maps of the two micronuclear clones, LMIC2-2 and LMIC2-5, which share homology with the C4 and C5 macronuclear DNA molecules are shown in Figure 1a. The micronuclear DNA inserts in the two clones are very similar, but are distinguished by a number of variant restriction sites, including an EcoRI site and a HindIII site (E1 and H2; Fig. 1a). Restriction fragment probes from these two clones were used to screen a recombinant library of macronuclear DNA molecules (LMAC library; 4) to isolate clones of the C4 and C5 genes. Nine clones were isolated and their restriction maps determined. One of the nine clones, LMAC4a, contained a 5.0 kbp C4 gene (Fig. 1b). The remaining clones contained 2.6 kbp C5 genes, and could be classified into two types based on their restriction maps. The restriction maps of two representative C5 gene clones, LMAC5d and LMAC5c, are shown in Figure 1b. The restriction map of LMAC5d resembles that of LMIC2-2, in that it lacks an internal EcoRI site (Fig. 1). LMAC5c, on the other hand, resembles micronuclear clone LMIC2-5 in that it contains an EcoRI site. The C4 gene

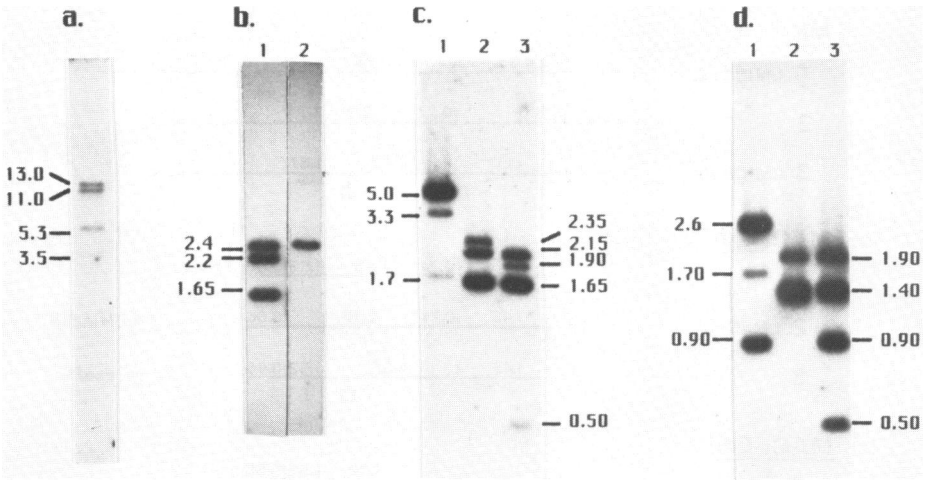

Figure 2. Southern hybridizations to genomic macronuclear and micronuclear DNAs. a.) Autoradiograph of hybridization of the 5.0 kbp insert of LMAC4a to a Southern blot containing EcoRI digested micronuclear DNA. b.) Hybridization of the 1.65 kbp HindIII fragment of LMIC2-2 to 1 µg HindIII digested micronuclear DNA (lane 1), and 5 x 10^{-3} µg of HindIII digested LMIC2-5 DNA (lane 2). The amount of LMIC2-5 DNA in lane 2 was calculated to be equivalent to a single copy sequence in the micronucleus, relative to the amount of DNA in lane 1. c.) Hybridization of the 1.65 kbp HindIII fragment of LMIC2-2 to macronuclear DNA (5.0 kbp size-class) digested with EcoRI (lane 1), HindIII (lane 2), and EcoRI + HindIII (lane 3). d) Hybridization of the 1.65 kbp HindIII fragment of LMIC2-2 to macronuclear DNA (2.6 kbp size-class) digested with EcoRI (lane 1), HindIII (lane 2), and EcoRI + HindIII (lane 3). In all cases, sizes of homologous DNA fragments are indicated in kilobase pairs.

clone, LMAC4a, lacks the H3 site, and thus differs from both micronuclear clones.*

Additional screenings of the macronuclear library failed to yield C4 or C5 gene clones which differed from the three described above. Therefore, genomic Southern hybridizations were performed to determine the copy number of C4 and C5 gene sequences in the micronucleus and the macronucleus. Evidence for three regions of the micronuclear genome sharing homology with C4 and C5 was obtained by hybridization of the macronuclear insert of clone

*(In Figure 1, and elsewhere, the sizes of some micronuclear restriction fragments are indicated as being slightly larger than the corresponding macronuclear fragments. These differences have been confirmed by side-by-side electrophoresis of the relevant fragments and are real. They are due to the presence of IESs, which occur frequently in the micronuclear copies of macronuclear DNA molecules. See 4,6).

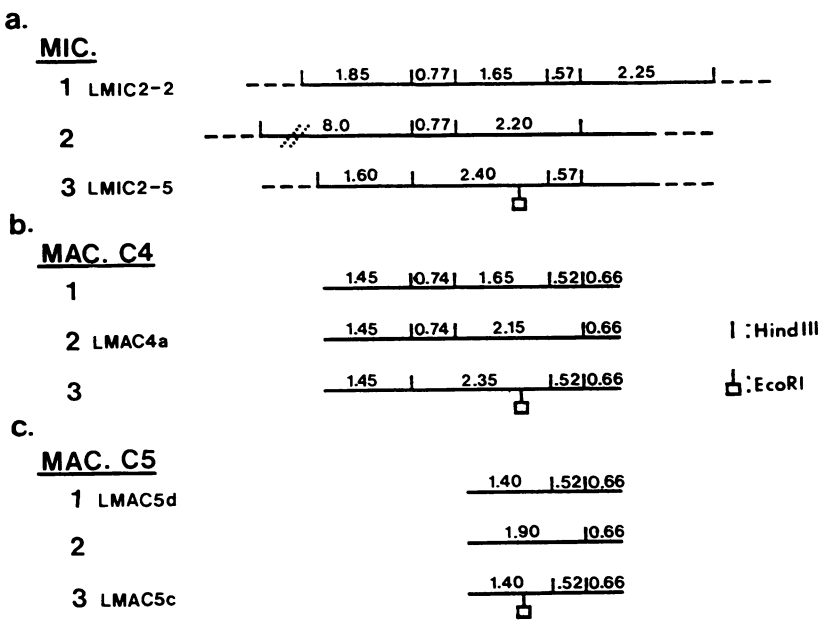

Figure 3. Genomic restriction maps determined through Southern hybridization analyses. a.) Restriction maps of the three micronuclear regions sharing homology with C4 and C5. b.) Restriction maps of the three macronuclear versions of the C4 gene. c.) Restriction maps of the three macronuclear versions of the C5 gene. Numbers (1, 2, & 3) indicate corresponding macronuclear and micronuclear versions. In cases where cloned examples of sequences have been isolated, the clone designation is also given.

LMAC4a to an EcoRI digest of micronuclear DNA. Four homologous fragments were detected in this hybridization (Fig. 2a). Based on the complete restriction maps of the micronuclear clones (see ref. 7), the 11.0 kbp fragment is derived from the region of the micronuclear genome represented by clone LMIC2-2, while the 5.3 and 3.5 kbp fragments are derived from the region represented by LMIC2-5. The remaining 13.0 kbp homologous fragment thus indicates that a third region of the micronuclear genome shares homology with C4 and C5.

Evidence for a third micronuclear copy was also obtained in a hybridization of the 1.65 kbp HindIII fragment of LMIC2-2 to HindIII digested micronuclear DNA (Fig. 2b). Homologous fragments of 2.4 and 1.65 kbp were detected, which are expected based on the maps of LMIC2-5 and LMIC2-2, as well as a 2.2 kbp fragment representing the third micronuclear region. This hybridization experiment also included a known amount of

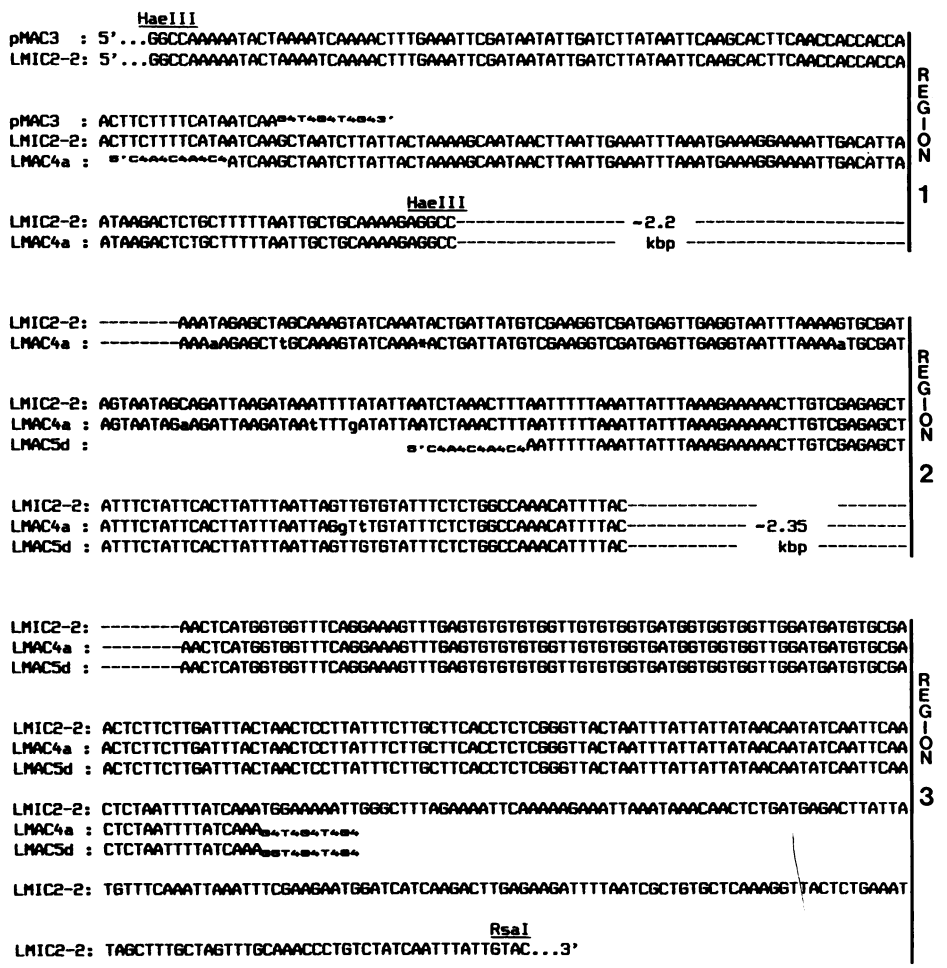

Figure 4. DNA sequences of selected regions of recombinant clones LMIC2-2, LMAC4a, and LMAC5d. Also shown is the right terminal sequence of pMAC3, a recombinant clone of a 2.85 kbp macronuclear DNA molecule that is adjacent to C4 in the micronucleus. Regions 1, 2, and 3, correspond to those indicated in Figure 1. Dashes (-) denote regions not sequenced, along with their approximate sizes. Base mismatches in the LMAC4a sequence are shown in lower case and a single nucleotide deletion is indicated by an asterisk.

LMIC2-5 DNA equivalent to a single-copy sequence in the micronucleus. Comparison of the intensities of the hybridization signals in genomic DNA with the copy number standard was consistent with each of the three micronuclear sequences being present in a single copy in the micronucleus. Through additional genomic hybridization experiments using various

restriction fragments derived from the clones as probes (data not shown), it was possible to construct restriction maps of the three micronuclear regions bearing homology to C4 and C5 (Fig. 3a). These three micronuclear DNA segments, which vary in their restriction maps, will be referred to as versions 1, 2, and 3.

Genomic hybridization experiments were also performed to determine the number of different types of C4 and C5 genes in the macronucleus. In this instance, it was not possible to simply perform hybridizations to total macronuclear DNA, as the C4 and C5 genes often yielded identical restriction fragments confusing the analysis. Therefore, we first subjected total macronuclear DNA to agarose gel electrophoresis and isolated DNA in the 5.0 kbp size range, as well as DNA in the 2.6 kbp size range. Each of these DNA samples were then separately digested and used to construct Southern blots to map the C4 and C5 genes. The existence of three macronuclear forms of the C4 gene was clearly demonstrated in a hybridization of the 1.65 kbp HindIII fragment of LMIC2-2 to HindIII digested macronuclear DNA of the 5.0 kbp size class (Fig. 2c; lane 2). Fragments of 2.35, 2.15, and 1.65 kbp were detected in this hybridization. The 2.35 kbp genomic HindIII fragment contains an EcoRI site, while the other two fragments do not (Fig. 2c; lane 3). In the case of the 2.6 kbp macronuclear DNA, two HindIII fragments of 1.4 and 1.9 kbp were detected using this same probe (Fig. 2d; lane 2), indicating that at least two forms of C5 exist. Evidence for a third form of C5 was obtained in the EcoRI + HindIII double digest of the 2.6 kbp macronuclear DNA (Fig. 2d; lane 3). In comparison to the HindIII digest, two new fragments of 0.9 and 0.5 kbp were seen, indicating that some of the 1.4 kbp Hind III fragments contain a single EcoRI site, thus defining a third form of the macronuclear C5 gene.

Similar hybridizations were performed using a series of probes spanning the C4 and C5 genes (data not shown). This allowed us to construct genomic restriction maps of the three forms of both the C4 and C5 genes (Fig. 3b & c). As is the case with the three micronuclear regions, the three macronuclear versions of the C4 and C5 genes are defined by the presence or absence of two HindIII restriction sites as well as an EcoRI site (Fig. 3). Furthermore, the restriction maps of the three macronuclear versions of both the C4 and C5 macronuclear DNA molecules match the three versions of the homologous micronuclear regions. It is thus evident that each of the three micronuclear versions gives rise to both a macronuclear C4 and C5 gene through alternative chromosome processing.

Alternative Fragmentation Generates C4 and C5

Either alternative fragmentation or alternative splicing could conceivably be used to derive two different-sized macronuclear DNA molecules from a single region of the micronuclear genome. To differentiate between these alternatives, DNA sequence analyses of relevant regions of the micronuclear and macronuclear clones were performed. The version 1 micronuclear clone LMIC2-2 and the corresponding version 1 macronuclear C5 clone LMAC5d were analyzed. Because macronuclear and micronuclear clones of the same version of C4 were not available, LMAC4a was also included in this analysis. The terminal regions of the cloned C4 and C5 macronuclear DNA molecules were sequenced, as well as the corresponding regions of the micronuclear clone assuming colinearity of the macronuclear and micronuclear clones (Fig. 1a & 1b).

The right ends of the C4 and C5 genes were found to be identical in sequence, and perfectly matched the corresponding region of the micronuclear clone (Fig. 4; region 3). In contrast, the left ends of C4 and C5 were completely different in their sequences. The left end of the C4 gene is, however, identical in sequence to a region of micronuclear clone LMIC2-2 that is essentially colinear with its restriction map (Fig. 1a & 4; region 1). The left end of the C5 gene is generated from a region approximately 2.4 kbp downstream (region 2; Figs. 1a & 4) of the fragmentation site that gives rise to the C4 gene left end. Thus, the results indicate that a common chromosome fragmentation site generates the right ends of the C4 and C5 genes, while variable use of two other fragmentation sites is responsible for generating their distinct left ends.

It has been suggested that splicing and fragmentation events during macronuclear development may be related processes (16). That is, incomplete splicing may result in chromosome fragmentation. In the current example, an IES could exist in micronuclear DNA at the site corresponding to the left end of the C5 gene. Correct removal of this IES would result in the macronuclear C4 gene, while abortive splicing would generate the C5 gene left end. To test this hypothesis, the sequence of the internal region of LMAC4a corresponding to the C5 left end fragmentation site was also determined. Comparison of the sequence of this region of LMAC4a with the corresponding region of LMIC2-2 indicates that no splicing events occur in this area (Fig. 4; region 2). It is thus unlikely that abortive splicing is involved in this alternative processing event. A number of base mismatches are apparent, however, providing further evidence that the LMAC4a clone is a

different version of the C4 gene.

The Micronuclear Copies of the C3 and C4 Genes Overlap

The precursor of a 2.65 kbp macronuclear DNA molecule (C3 gene) had been mapped to a region very near the left end of the C4 gene precursor in micronuclear clone LMIC2-2 (7; Fig. 1a). In addition, recombinant clones of the macronuclear C3 gene were isolated and their terminal sequences determined (6). The sequence of the right end of the C3 gene clone pMAC3 (which corresponds to the same version as micronuclear clone LMIC2-2) is compared to those of LMAC4a and LMIC2-2 in Figure 4a. Suprisingly, the sequences of the micronuclear C3 and C4 gene precursors were found to overlap by 5 bp.

DISCUSSION

Three regions of the micronuclear genome that share homology with the C4 and C5 macronuclear DNA molecules have been identified by genomic hybridization analyses. Each of these micronuclear regions has been demonstrated to give rise to both a C4 and C5 macronuclear gene through alternative processing.

The existence of three forms, or versions, of the C4/C5 region in the micronuclear genome is unexplained, especially considering that the strain of O. nova used in this study is clonal. However, this observation is not without precedent. Evidence has been obtained for three micronuclear copies of other macronuclear gene precursors which exist in the vicinity of the C4/C5 region (4,6), as well as for macronuclear gene precursors in O. fallax (17).

On the basis of DNA sequence analyses, the alternative processing involves variable use of chromosome fragmentation sites. Specifically, our data indicate that a single chromosome fragmentation site is used to generate the right ends of the C4 and C5 genes, while variable use of two chromosome fragmentation sites is responsible for generating the distinct left ends of these macronuclear DNA molecules. It should be noted that these conclusions are based on the analysis of a C4 gene clone (LMAC4a) that is of a different version than the micronuclear clone (LMIC2-2) and C5 gene clone (LMAC5d) analyzed. It is formally possible that the macronuclear C4 gene that actually corresponds to the region of the micronuclear genome represented by clone LMIC2-2 is derived in a different manner. There are, however, a number of reasons that indicate this is an unlikely possibility. First, in previous instances where multiple

versions have been encountered (4,17), they all show the same general sequence organization, differing only at a small number of nucleotide positions (<5%). Second, in the specific case of the macronuclear C4 genes, restriction fragments derived from their left termini share homology and show no major length differences, indicating that they are both derived and organized in a similar manner. Third, and most importantly, the sequence of the left end of LMAC4a is identical to that of the micronuclear clone studied, suggesting that its micronuclear precursor is processed in the same manner.

Alternative processing is possible during macronuclear development in the hypotrichs, as an early step of development involves the formation of polytene chromosomes. Thus, some DNA strands can be fragmented so as to yield C4 macronuclear DNA molecules, while others generate C5 macronuclear genes. Alternative processing has also been observed during macronuclear development in other ciliated protozoa. In Tetrahymena, alternative processing has been seen in regard to the removal of sequences from internal regions of macronuclear chromosomes (18,19). Alternative processing to generate distinct chromosomal ends, similar to what is described here, has recently been observed in Paramecium (20,21). This is the first defined example of alternative processing in O. nova, where this phenomena appears to be rare. Most cloned macronuclear DNA molecules share homology only with themselves in total macronuclear DNA in this organism (2). In contrast, the related hypotrich species Oxytricha fallax appears to use alternative processing much more frequently. Approximately one-third of its macronuclear DNA molecules share homology with other size-classes of macronuclear DNA molecules (22). One case of alternative processing has been analyzed in detail in O. fallax and appears to be based on a system similar to that described here (17,22,23). In this instance three related macronuclear DNA molecules (the 81-MAC family) of 1.6, 2.9, and 4.9 kbp are involved. The 1.6 kbp macronuclear DNA molecule constitutes a common sequence block present in all members of the family, while the 2.9 kbp and the 4.9 kbp macronuclear genes have 1.3 kbp and 3.6 kbp of additional DNA at their left and right ends, respectively. All three macronuclear DNA molecules are derived from the same region of the micronuclear genome through the use of alternative chromosome fragmentation sites.

The purpose of alternative processing is unclear. It could conceivably be used to generate distinct protein products, in a manner

analogous to alternative RNA processing (24) or immunoglobulin gene rearrangement (25). This would require that both the C4/C5 common region and the C4-specific region contain coding sequences. On the other hand, alternative processing could be used to place a single coding region under multiple forms of transcriptional control. This would require a coding region in the C4/C5 common region, and a unique set of transcriptional control sequences in the C4-specific region. Each of these models remains hypothetical, as we have been unable to detect transcripts homologous to either the C4/C5 common region or the C4-specific region in RNA isolated from vegetative cells (data not shown). These macronuclear DNA molecules could, however, be transcribed at a low level, only in response to particular environmental conditions, or only during particular periods of the organism's life cycle.

It is also conceivable that alternative processing serves no function. Chromosome fragmentation sites may be both appearing and disappearing in the genome on an evolutionary time scale. For instance, the sequences that specify fragmentation to generate the left end of the C5 gene may be in the process of degenerating, so that this site is only partially utilized, resulting in the alternative processing phenomenon. Perhaps the only constraint on variability in the locations of fragmentation sites is that they do not disrupt the coding and associated control regions of macronuclear DNA molecules.

Unexpectedly, our data also indicate that the micronuclear precursors of the C3 and C4 genes overlap by 5 bp. This conclusion is again based on the comparison of macronuclear and micronuclear clones which are of different versions, but, as we have discussed above, the macronuclear C4 gene analyzed appears to be very similar to the one that is actually derived from the region represented by micronuclear clone LMIC2-2. In addition, we have recently obtained evidence for a 6 bp overlap for the precursors of two macronuclear DNA molecules in the related hypotrich Euplotes crassus (Klobutcher and Fino, unpublished results). In this case, there is no evidence for multiple versions, and partial DNA sequence analysis indicates complete identity of all clones involved.

We had previously provided evidence that the precursors of macronuclear DNA molecules are clustered and separated by less than 500 bp on the basis of restriction mapping studies (7). In one case, DNA sequence analysis demonstrated that two adjacent macronuclear gene precursors were separated by only 90 bp of eliminated DNA. The overlap in

C3 and C4 indicates that the presence of an intergenic spacer segment is not required and raises the question of how these two molecules could be derived during development. One obvious model is that the fragmentation process involves a staggered double-stranded cut of the DNA, much like a restriction enzyme, so that both C3 and C4 initially receive the overlapping 4 bp in single-stranded form. The single-stranded tails could then be filled-in prior to, or in concert with, telomere formation. However, because of the existence of polytene chromosomes during development, other models are equally plausible. For instance, it is possible that fragmentation results in blunt-ended DNA molecules, but the process is not precise to the nucleotide so that cuts are made over a small region of the micronuclear chromosome. Under this scenario, C3 and C4 genes with slightly different ends would be generated from different polytene DNA strands, and the clones we have isolated would represent the products of different fragmentation events. In support of this model, variability in chromosome fragmentation sites has recently been found in O. fallax (23), as well as in Paramecium (20,21), and we have evidence that the termini of macronuclear DNA molecules can vary by 2-9 bp in O. nova (Klobutcher, unpublished results).

The observation of overlapping genes also has implications in regard to sequences which direct the chromosome fragmentation process. No conserved DNA sequences have yet been defined near chromosome fragmentation sites in the hypotrichs, but a 15 bp sequence has been associated with chromosome fragmentation sites in the ciliate Tetrahymena (26). The location of the 15 bp conserved sequence in relation to fragmentation sites has been determined in three cases, and it uniformly resides outside of macronuclear-retained sequences, that is, in DNA eliminated from the micronuclear genome. The observation that there is no intergenic spacer DNA between the C3 and C4 genes suggests that sequences specifying fragmentation in the hypotrichs need not reside in eliminated DNA and may, at least in some instances, be within the precursors of macronuclear DNA molecules.

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