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Gene-Sized Macronuclear DNA Molecules Are Clustered in Micronuclear Chromosomes of the Ciliate *Oxytricha nova*

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Following the sexual phase of its life cycle, the hypotrichous ciliate *Oxytricha nova* transforms a copy of its chromosomal micronucleus into a macronucleus containing short, linear DNA molecules with an average size of 2.2 kilobase pairs. In addition, more than 90% of the DNA sequences in the micronuclear genome are eliminated during this process. We have examined the organization of macronuclear DNA molecules in the micronuclear chromosomes. Macronuclear DNA molecules were found to be clustered and separated by less than 550 base pairs in two cloned segments of micronuclear DNA. Recombinant clones of two macronuclear DNA molecules that are adjacent in the micronucleus were also isolated and examined by DNA sequencing. The two macronuclear DNA molecules were found to be separated by only 90 base pairs in the micronuclear genome.

Hypotrichous ciliated protozoa, such as *Oxytricha nova*, undergo a drastic genome reorganization process as part of their normal life cycle (25, 26). The ability of these unicellular organisms to alter their DNA stems from the fact that each cell contains two types of nuclei. The micronucleus contains an unrearranged genome composed of chromosome-sized DNA molecules, but is transcriptionally inactive. The second type of nucleus in the cell, the macronucleus, is responsible for nuclear transcription during vegetative growth of the organism, despite having an unusual genetic constitution. The macronuclear genome consists entirely of multiple copies of short, linear, gene-sized DNA molecules with an average size of 2.2 kilobase pairs (kbp) (33). Since most macronuclear DNA molecules are transcribed (23; J. Heumann, Ph.D. thesis, University of Colorado, Boulder, 1977), and current evidence is consistent with each molecule's specifying a single product (11), they are often referred to as macronuclear genes.

Following each sexual phase of the life cycle, the macronucleus is destroyed and a new one develops from a mitotic copy of the micronucleus. This process of macronuclear development involves a complex series of events that dramatically alter the micronuclear genome (1, 25). At the cytological level, the micronuclear chromosomes are first replicated a number of times to form polytene chromosomes. Vesicles then form within the developing macronucleus in association with the fragmentation of the polytene chromosomes. Most of the DNA within each vesicle is subsequently destroyed. Finally, the vesicle disappear and the remaining low-molecular-weight DNA molecules undergo multiple rounds of replication to form the mature macronucleus. Comparative studies on the macronuclear and micronuclear genomes indicate that macronuclear development does not simply involve fragmenting the chromosomes to generate the gene-sized DNA molecules, but also entails the elimination of more than 90% of the sequence complexity of the micronuclear genome (17).

In addition, studies on the chromosomal organization of particular macronuclear genes indicate that two additional types of rearrangement events occur during development. First, repeats of the octanucleotide 5′-CCCCAAAA-3′ (C_4A_4 repeats) are added to the ends of macronuclear genes following their excision from the chromosome (5, 7, 15). These terminal sequences appear to function as telomeres for the linear macronuclear DNA molecules (24). Second, short blocks of DNA, referred to as internal eliminated sequences (IESs), are removed from the internal regions of the chromosomal copies of genes by a nucleic acid breakage and joining, or splicing, process (15).

Understanding the molecular nature of this DNA rearrangement process requires knowledge of the organization of the micronuclear chromosomes, particularly the organization of macronuclear genes in the chromosomes. An early model of micronuclear chromosome organization suggested that individual macronuclear genes were situated at intervals along the chromosome and separated by large "spacer" DNA segments that are eliminated during macronuclear development (26). This model was based on the observation that large amounts of chromosomal DNA are eliminated during development and places the eliminated DNA between genes. This type of chromosome organization was also suggested by the polytene chromosome that are observed during development. The polytene chromosomes are similar to those observed in *Drosophila melanogaster*, in which individual band units have been suggested to represent individual genes (14).

Boswell et al. (4) have provided some evidence that this model may not be valid. Recombinant clones containing large segments of *O. nova* micronuclear DNA (>10 kbp) were isolated from a genomic library by their ability to hybridize with radiolabeled macronuclear DNA. When these micronuclear clones were hybridized to Southern blots of total macronuclear DNA, all but one showed homology to multiple macronuclear DNA molecules. These results suggested that macronuclear genes were clustered in the micronuclear genome and separated by short spacer segments. However, because of the screening method used, it is possible that the clones chosen in this analysis were not representative of the general organization of macronuclear genes in the chromosome. Because clones were selected by

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their ability to hybridize with total macronuclear DNA, regions of the genome containing macronuclear gene clusters may have been preferentially chosen.

In this report, we have examined the arrangement of macronuclear genes within the micronuclear chromosome in greater detail, using a strategy that avoids this bias. Our results indicate that macronuclear genes are clustered in the chromosome and separated by very small amounts of spacer DNA. The implications of our results for chromosome organization and the genome reorganization process of macronuclear development are discussed.

MATERIALS AND METHODS

Cell culture and isolation of DNA. *O. nova* strain H10 was grown under nonsterile culture conditions with *algae* as a food source as previously described (32). Macronuclei and micronuclei were isolated from starved cells, and DNAs were purified as described previously (15, 17).

Bacterial plasmid DNA was prepared by the sodium dodecyl sulfate (SDS) lysis procedure of Godson and Vepnek (10). Recombinant bacteriophage were purified on glycerol step gradients, and DNA was prepared as described previously (19).

Recombinant libraries. The construction of the *O. nova* genomic macronuclear (LMAC) and micronuclear (LMIC) DNA libraries used in these studies has been described previously (15). The LMAC library was made by inserting blunt-ended macronuclear DNA molecules into the bacteriophage vector λgt10 (13) with synthetic *Eco*RI linkers. The LMIC library was constructed by inserting micronuclear DNA fragments generated by partial digestion with *Sau*3A into the *Bam*HI site of bacteriophage λ47.1 (18). Recombinant libraries were screened by the plaque hybridization method of Benton and Davis (2).

The recombinant clone pMACR1, containing a 1.1-kbp macronuclear DNA molecule, was chosen from a small macronuclear DNA library constructed in the plasmid pBR322 (15). It was radioactively labeled and used to screen the LMIC library, resulting in the isolation of micronuclear clone LMICR1-7.

To isolate a recombinant clone of the macronuclear *C3* gene, approximately 50,000 clones of the LMAC library were screened with a restriction fragment derived from the region of LMIC2-5 with homology to *C3*. One positive recombinant clone, LMAC3, was isolated. The macronuclear insert in LMAC3 was subcloned into the plasmid pBR325 (3) to generate the recombinant clone pMAC3. pMAC3 was then used to screen an additional 100,000 clones of the macronuclear library, resulting in the isolation of a second recombinant *C3* gene clone, LMAC3-G.

Gel electrophoresis and hybridization. DNA samples were size fractionated on 0.7 to 1.5% agarose or low-melting-point agarose gels (Bethesda Research Laboratories) with TBE (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA) as the gel and running buffer.

For hybrdization, the DNA was transferred to nitrocellulose filters by the method of Southern (30). Hybridizations were done at 65°C as previously described (5). Following hybridization, filters received two 30-min washes in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) plus 0.5% SDS, followed by two 30-min washes in 0.1× SSC plus 0.5% SDS, all at 65°C. In hybridizations using fragments of micronuclear clones to probe genomic macronuclear DNA, the final two washes were done in 1× SSC plus 0.5% SDS. The less stringent conditions were used to detect short regions of homology.

In cases involving hybridization of different probes to the same DNAs, nitrocellulose filters were reused in multiple hybridizations. For this purpose, blots were stripped of probe by two 15-min washes in 10 mM Tris (pH 8.0)–0.1 mM EDTA–0.5% SDS at 100°C Blots were reused up to five times with this procedure. This method also provided an internal control for the completeness of restriction endonuclease digestions of DNA. Experimental blots were probed with a DNA fragment whose hybridization pattern had been determined previously in order to rule out the possibility of partial restriction endonuclease digestions.

Probes were generally radioactively labeled with [α-^{32}P]dATP by nick translation (27). For this purpose, restriction fragments of micronuclear clones were purified from low-melting-point agarose gels (16). Alternatively, fragments were excised from gels and directly labeled by Klenow fragment synthesis primed with random synthetic hexanucleotides as described by Feinberg and Vogelstein (8, 9).

Restriction endonuclease mapping and DNA sequencing. Restriction enzymes were purchased from Bethesda Research Laboratories, Baltimore, Md. and used as specified by the manufacturer. Restriction maps were constructed by using single and double restriction endonuclease digestions, followed by sizing on agarose gels. In some cases, mapping of lambda recombinant clones was facilitated by subcloning *Eco*RI restriction fragments derived from the insert into the plasmid vector pBR325 (3). *Hin*dIII maps were constructed by generating a partial restriction digest series of lambda clone DNA (19). Southern blots of the partial digest series were then made and probed with terminal insert fragments to deduce the order of *Hin*dIII sites.

DNA sequencing was done by the Maxam and Gilbert chemical method (20) or by the dideoxy method (21, 29). The M13 bacteriophage strains M13mp8, M13mp9, M13mp10, and M13mp11 were used to subclone fragments for dideoxy sequencing.

RESULTS

Analysis of the *C2* gene cluster. To study the organization of macronuclear DNA molecules in the micronuclear chromosome, we wished to isolate cloned segments of micronuclear DNA without bias towards selection of clones with multiple macronuclear DNA molecules. Our approach was to use random individual cloned macronuclear DNA molecules to screen the *O. nova* LMIC micronuclear DNA library. Micronuclear clones were thus isolated that contained the micronuclear precursor of the gene plus flanking chromosomal DNA sequences. These micronuclear clones were then examined for the presence of percursors of additional macronuclear DNA molecules.

We first studied two cloned segments of the micronuclear genome that had been isolated in a previous study (15) by their homology to an 810-base-pair (bp) cloned macronuclear DNA molecule, referred to as the *C2* gene. These two clones, LMIC2-5 and LMIC2-2, contained micronuclear DNA inserts of 11.4 and 12.7 kbp, respectively. Both clones were restriction mapped, and the location of the *C2* gene in each was determined (15) (see Fig. 2A). The regions of the micronuclear genome contained in each clone were distinct, as indicated by their restriction maps, but both regions of the genome gave rise to 810-bp macronuclear DNA molecules that were 95% homologous at the DNA sequence level. We have suggested that these represent allelic forms of the same gene.

To determine whether these large cloned segments of micronuclear DNA also gave rise to other macronuclear

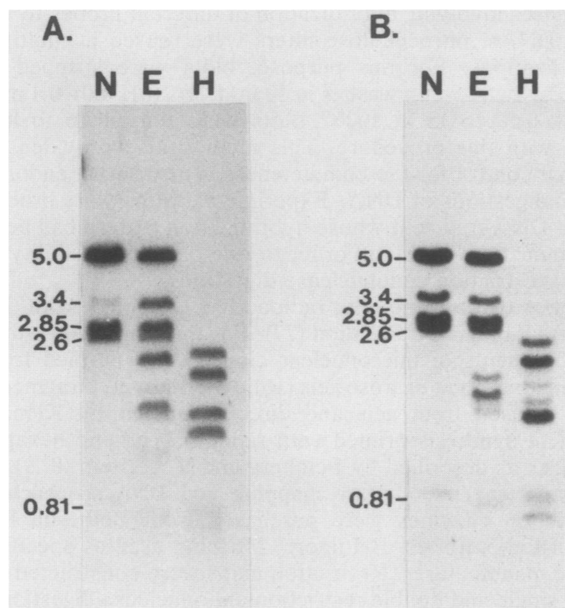

FIG. 1. Hybridization of micronuclear clones LMIC2-5 (A) and LMIC2-2 (B) to native macronuclear DNA (lanes N) and macronuclear DNA digested with restriction endonuclease *Eco*RI (lanes E) or *Hin*dIII (lanes H). Sizes of homologous DNA molecules in native macronuclear DNA are indicated (in kilobase pairs).

genes during development, each was radioactively labeled and hybridized to a Southern blot of native macronuclear DNA (Fig. 1). In addition to hybridizing to the 0.81-kbp *C2* gene, LMIC2-5 and LMIC2-2 shared homology with 5.0-, 3.4-, 2.85-, and 2.6-kbp macronuclear DNA molecules. One simple interpretation of this result is that each of these macronuclear genes is derived wholly or in part from the segments of micronuclear DNA contained in clones LMIC2-5 and LMIC2-2.

To determine the organization of these additional homologous macronuclear genes within the micronuclear chromosome, a series of restriction fragments derived from the micronuclear clone LMIC2-5 were individually hybridized to Southern blots containing native macronuclear DNA, as well as DNA digested with *Eco*RI and *Hin*dIII. The restriction fragments used in these hybridization experiments are shown in Fig. 2, along with the results of the hybridization analyses. The data obtained from the hybridizations to native macronuclear DNA will be considered first, as they allowed us to order the macronuclear genes along the cloned segment of micronuclear DNA. Each restriction fragment probe hybridized to more than one size class of macronuclear DNA. For fragments A, B, and C, a straightforward interpretation of the data was possible. Each of these restriction fragments hybridized to two size classes of native macronuclear DNA molecules, suggesting that these regions of the micronuclear clone contain the terminal regions of two adjacent macronuclear genes plus any spacer DNA that exists between them. For example, fragment A showed homology to the 5.0-kbp and 0.81-kbp macronuclear DNA molecules, indicating that this micronuclear restriction fragment spanned the ends of the chromosomal copies of these two macronuclear DNA molecules. The order of these two genes could then be deduced by determining which of the two hybridized to the adjacent restriction fragment probe B. As probe B hybridized to the 0.81-kbp macronuclear gene but not to the 5.0-kbp gene, the gene order must be 5.0 kbp-0.81 kbp from left to right. Overall, the hybridization results for fragments A, B, and C indicated a gene order of 5.0, 0.81, 2.85, and 5.0 kbp from left to right at the left end of LMIC2-5.

This would require that two distinct macronuclear DNA molecules 5.0 kbp long be derived from LMIC2-5. This appeared to be the case, as none of the probes derived from LMIC2-5 cross-hybridized (data not shown), including probes A and C, which detected the two 5.0-kbp macronuclear DNA molecules. (This interpretation is also supported by the hybridizations to macronuclear DNA digested with *Hin*dIII or *Eco*RI. Probes A and C detected different fragments that must be derived from distinct 5.0-kbp macronuclear DNA molecules.)

The hybridization results for the rightward fragments D, E, and F were more complex. All three fragments hybridized to one of the 5.0-kbp genes and a 2.6-kbp macronuclear DNA molecule, with the rightmost fragment F hybridizing to the 3.4-kbp macronuclear DNA molecule as well (Fig. 2B). The region of the clone represented by these probes was not large enough for all three macronuclear genes to be derived from distinct regions of the genome. One interpretation of these results is that two different macronuclear DNA molecules (the 5.0- and 2.6-kbp molecules) are derived from the same region of the chromosome during development by fragmentation of the chromosome at different sites. Such alternative processing has been observed in the related hypotrichous ciliate *O. fallax*, in which it appears to be common (6). More detailed studies of this region of LMIC2-5, which will be published elsewhere, indicate that alternative processing is responsible for generating the 5.0- and 2.6-kbp macronuclear DNA molecules.

Thus, the overall order of macronuclear genes along the micronuclear insert of LMIC2-5 was 5.0, 0.81, 2.85, 5.0 + 2.6, and 3.4 kbp. To faciliate the discussion of further analyses, we will refer to these macronuclear DNA molecules as the *C1*, *C2*, *C3*, *C4*, *C5*, and *C6* genes, respectively.

A similar series of hybridization analyses was performed with the micronuclear clone LMIC2-2 and the probes indicated in Fig. 2A (data not shown). The results indicated the same order of macronuclear gene precursors along the length of the cloned segment of micronuclear DNA. The single difference was that LMIC2-2 did not show homology to the 5.0-kbp *C1* gene, which was expected because this clone did not contain DNA to the left of the *C2* gene. These results reinforce our suggestion that LMIC2-5 and LMIC2-2 represent allelic forms of the same region of the micronuclear genome.

Spacing of genes in the *C2* cluster. The hybridizations of probes derived from LMIC2-5 and LMIC2-2 to *Hin*dIII- and *Eco*RI-digested macronuclear DNA allowed us to more precisely localize the precursors of macronuclear DNA molecules along the lengths of the cloned DNA segments. The approach relied on equating restriction fragments detected in macronuclear DNA with the restriction maps of the micronuclear clones.

In many cases the hybridization patterns were complex (Fig. 2A). The sums of the lengths of the macronuclear fragments hybridizing often exceeded the lengths of the undigested macronuclear genes homologous to the same restriction fragment probe. This type of result suggests that there are multiple, or polymorphic, forms of some of the macronuclear genes. The results can be explained, however, if LMIC2-5 and LMIC2-2 both give rise to macronuclear genes during development. For example, two forms of the

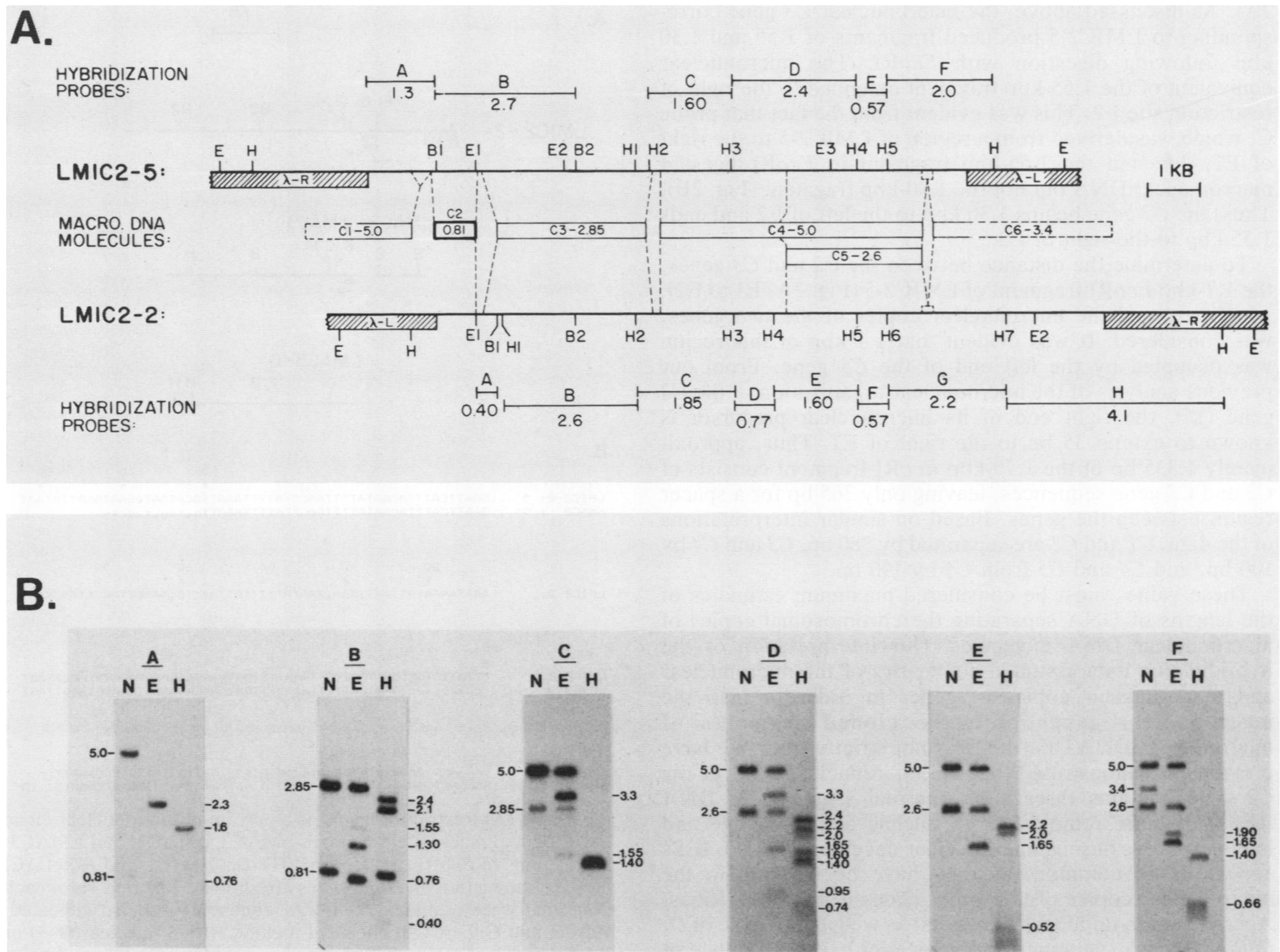

FIG. 2. (A) Restriction maps of micronuclear clones LMIC2-5 and LMIC2-2, indicating positions of *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B) sites. Restriction sites in the micronuclear DNA insert are numbered consecutively from left to right for reference in the text. The restriction fragments (A through F) used to probe macronuclear DNA are shown along with their sizes (in kilobase pairs). Also shown are the regions of the cloned micronuclear DNA segments that give rise to the six macronuclear DNA molecules (*C1* through *C6*) during development. KB, kilobase. (B) Autoradiograms of hybridizations of subfragments of LMIC2-5 to native macronuclear DNA (lanes N) and macronuclear DNA digested with *Eco*RI (lanes E) or *Hin*dIII (lanes H). The fragments used in each hybridization are shown above each set of lanes (see panel A). Sizes of homologous native macronuclear DNA molecules are indicated to the left of each autoradiogram, and the sizes of additional DNA fragments appearing in restriction digests of macronuclear DNA are indicated on the right (in kilobase pairs).

C3 gene are expected from the restriction maps of the two micronuclear clones. LMIC2-5 contained an *Eco*RI site (E2) in the region with homology to the *C3* gene, while LMIC2-2 lacked this site (Fig. 2A). When probe B of LMIC2-5, which spanned the *Eco*RI site, was hybridized to *Eco*RI-digested macronuclear DNA, hybridization to a 2.85-kbp macronuclear DNA molecule was seen as well as to fragments 1.55 and 1.30 kbp in length (Fig. 2B; the 0.76-kbp fragment that was also detected is known to be derived from the *C2* gene). The hybridization to the 2.85-kbp DNA molecules represented a macronuclear version of the *C3* gene which lacked an *Eco*RI site (*Eco*RI$^-$ version) and was presumably derived from the region of the micronuclear genome represented by LMIC2-2 during development. The 1.55- and 1.30-kbp fragments were derived from a second macronuclear version of the *C3* gene which contained a single *Eco*RI site (EcoRI$^+$ version), as expected from the restriction map of LMIC2-5. Controls for partial restriction endonuclease digestion were performed (see Materials and Methods), eliminating this as an explanation for these results.

Similarly, the hybridization analyses indicated that both LMIC2-5 and LMIC2-2 gave rise to polymorphic forms of the *C4* and *C5* genes in the macronucleus, although additional *Hin*dIII fragments were present, which suggests that a third copy or version of these genes exists in both genomes. In the case of the *C1* and *C6* genes, no polymorphic *Hin*dIII or *Eco*RI sites were detected, and it was thus not possible to determine whether two regions of the micronuclear genome were active in generating these macronuclear DNA molecules.

Taking into account the existence of polymorphic forms of some of the genes in the *C2* cluster, it was then possible to localize the genes and estimate the amount of DNA separating macronuclear genes in the micronuclear chromosome. For example, the locations of the ends of the *C3* gene in LMIC2-5 were determined relative to the *Eco*RI site E2 (Fig.

2A). As discussed above, the macronuclear *C3* gene corresponding to LMIC2-5 produced fragments of 1.55 and 1.30 kbp following digestion with *Eco*RI. The micronuclear equivalent of the 1.55-kbp fragment extended to the right of restriction site E2. This was evident from the fact that probe C, which was derived from a region of LMIC2-5 to the right of E2, detected the 1.55-kbp fragment in *Eco*RI-digested macronuclear DNA but not the 1.30-kbp fragment (Fig. 2B). Thus, the *C3* gene begins 1.30 kbp to the left of E2 and ends 1.55 kbp to the right of E2.

To determine the distance between the *C2* and *C3* genes, the 1.7-kbp *Eco*RI fragment of LMIC2-5 (Fig. 2A, E1 to E2), which spanned the micronuclear copies of the two genes, was considered. It was evident that 1.3 kbp of this region was occupied by the left end of the *C3* gene. From our previous analysis of the micronuclear organization of the *C2* gene (15), the right end of its micronuclear precursor is known to extend 35 bp to the right of E1. Thus, approximately 1,335 bp of the 1.70-kbp *Eco*RI fragment consists of *C2* and *C3* gene sequences, leaving only 365 bp for a spacer region between the genes. Based on similar interpretations of the data, *C1* and *C2* are separated by 380 bp, *C3* and *C4* by 100 bp, and *C4* and *C5* from *C6* by 190 bp.

These values must be considered maximum estimates of the lengths of DNA separating the chromosomal copies of macronuclear DNA molecules. Our interpretation of the hybridization data assumed colinearity of the micronuclear and macronuclear copies of genes in order to map the macronuclear genes onto the cloned segments of micronuclear DNA, but this was not strictly true. We have previously demonstrated that the micronuclear copy of the *C2* gene contains three short internal segments of DNA (IESs) that are removed by a nucleic acid breakage and joining process during macronuclear development (15). IESs appear to be common, as they have been found in the micronuclear copies of two other genes in *O. nova* (Ribas-Aparicio et al., unpublished results) as well as in a gene of *O. fallax* (Herrick, personal communication). The existence of IESs in other genes within the *C2* gene cluster would have the effect of exaggerating intergenic distances.

Therefore, for one pair of adjacent genes, the precise size of the intergenic spacer was determined by DNA sequence analysis. To determine the distance between genes in the chromosome, recombinant clones of two macronuclear DNA molecules that are adjacent in the micronucleus were analyzed by DNA sequencing of their terminal regions. By then sequencing the corresponding regions of the micronuclear clone, it was possible to precisely determine intergenic distances. The macronuclear DNA molecules *C2* and *C3* were chosen for this analysis.

The isolation and DNA sequence analysis of the macronuclear *C2* gene clone used in these studies, pMAC2 (Fig. 3A), has been reported previously (15). Two macronuclear *C3* gene clones, pMAC3 and LMAC3-G, were isolated (see Materials and Methods) and their restriction maps were determined (Fig. 3A). Comparison of the restriction maps indicated that pMAC3 was very similar to a region of the micronuclear clone LMIC2-2 and was probably derived from the corresponding region of the micronuclear genome during development. The restriction map of LMAC3-G, on the other hand, more closely resembled that of LMIC2-5 and represented its macronuclear version of the *C3* gene.

The macronuclear *C3* gene clone pMAC3 was chosen for sequence analysis, as it appeared to be derived from LMIC2-2, which also gave rise to the pMAC2 version of the *C2* gene.

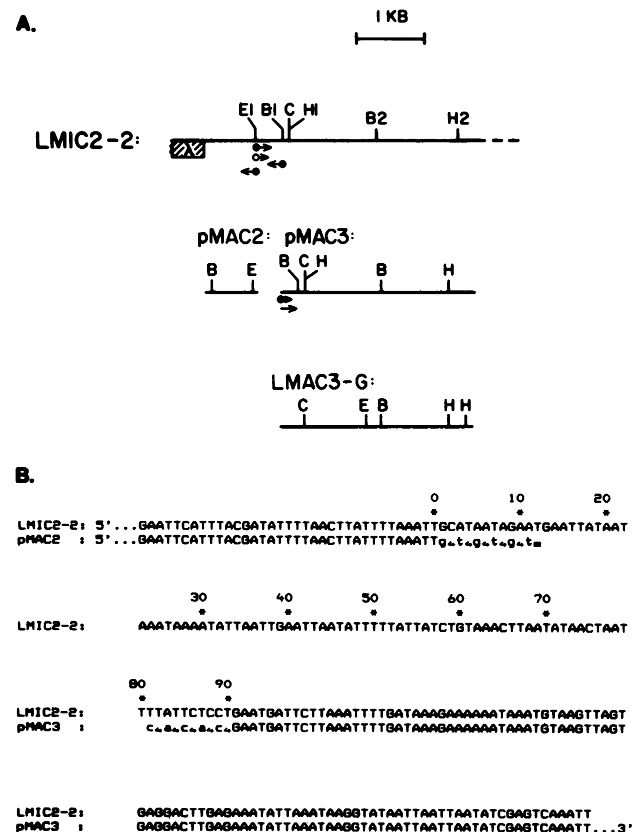

FIG. 3. (A) Restriction maps of a portion of micronuclear clone LMIC2-2 and macronuclear clones pMAC2, pMAC3, and LMAC3-G. Positions of *Eco*RI (E), *Hin*dIII (H), *Bam*HI (B), and *Hin*cII (C) sites are shown (not all *Hin*cII sites are shown). The regions of each clone that were examined by DNA sequencing are also indicated: Maxam and Gilbert (20) chemical method with 3'-labeled (●→) or 5'-labeled (○→) DNA, and dideoxy sequencing (21, 29) following subcloning into M13 bacteriophage vectors (→). The clone pMAC2 was completely sequenced in a previous study (15). KB, Kilobase. (B) DNA sequences of the adjacent termini of macronuclear clones pMAC2 and pMAC3, as well as the DNA sequence of the corresponding region of micronuclear clone LMIC2-2. The 90 bp of DNA sequence that separate the two macronuclear genes in the micronucleus are numbered. The terminal C_4A_4 repeats of the macronuclear clones are shown in lowercase letters. The difference in the lengths of the macronuclear terminal repeats is a function of the cloning method used in each case.

Approximately 200 bp of DNA sequence at the terminus of the pMAC3 insert adjacent to the *C2* gene in the micronucleus were determined by the strategy shown in Fig. 3A. In addition, the region of the micronuclear clone LMIC2-2 containing the *C2* and *C3* gene termini and intervening spacer sequences was also sequenced.

For both the *C2* and *C3* genes, the C_4A_4 repeats present at the ends of the macronuclear copies of these genes were found to be completely absent in the micronucleus (Fig. 3B). This confirms previous reports (5, 7, 15) that these terminal repeat sequences are added to macronuclear DNA molecules during the course of development. However, beginning at the first base following the C_4A_4 repeats, perfect matches of the macronuclear and micronuclear DNA sequences could be found (Fig. 3B). The results indicate that the *C2* and *C3* genes are separated by only 90 bp in the micronuclear chromosome. Southern hybridizations have demonstrated

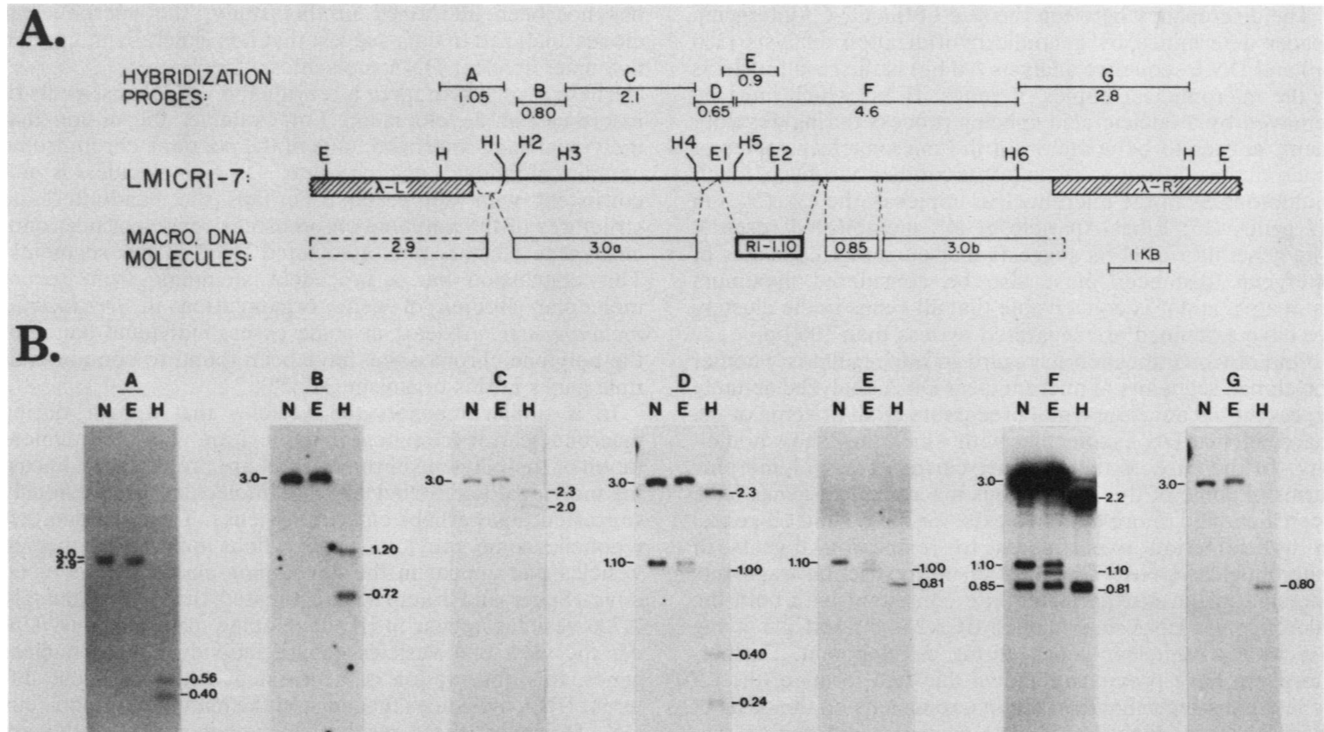

FIG. 4. (A) Restriction map of micronuclear clone LMICR1-7, showing positions of *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B) sites. Restriction fragments used to probe Southern blots of macronuclear DNA are indicated above the map. Also shown below the map are the macronuclear DNA molecules that are derived from the region of micronuclear DNA represented in LMICR1-7 during development. KB, Kilobase. (B) Hybridization of subfragments A through G of LMICR1-7 to native macronuclear DNA (lanes N) and macronuclear DNA digested with *Eco*RI (lanes E) or *Hin*dIII (lanes H). Sizes of homologous macronuclear DNA molcules are indicated on the left, and new DNA fragments appearing after digestion with a restriction endonuclease are indicated on the right (in kilobase pairs).

that the *C2-C3* junction region in the clone LMIC2-2 was a faithful representation of the micronuclear organization of this region (data not shown). Thus, the observed close spacing of the *C2* and *C3* genes was not an artifact of the cloning process.

Analysis of the *R1* gene cluster. A second cloned segment of micronuclear DNA was examined to determine whether the close spacing results obtained in analyzing the *C2* gene cluster represented the general organization of macronuclear genes in the micronuclear genome. The micronuclear clone LMICR1-7 was selected from the micronuclear library by its homology to a 1.1-kbp macronuclear DNA molecule contained in the clone pMACR1. A restriction map of the 9.6-kbp micronuclear DNA insert of LMICR1-7 is shown in Fig. 4A. When this clone was used as a hybridization probe against native macronuclear DNA, the 1.1-kbp *R1* gene was detected along with macronuclear DNA molecules 3.0 (doublet), 2.9, and 0.85 kbp long (data not shown).

The order and spacing of these five macronuclear DNA molecules was again deduced by individually hybridizing restriction fragments derived from LMICR1-7 to native macronuclear DNA and macronuclear DNA digested with *Eco*RI and *Hin*dIII (Fig. 4B). The results of this analysis indicate the following order of macronuclear DNA molecules in the micronucleus: 2.9, 3.0a, 1.1, 0.85, and 3.0b.

In determining the spacing of genes within this cluster, our analysis made use of information obtained from a related study in which the micronuclear and macronuclear copies of the 1.1-kbp *R1* gene were completely sequenced (Ribas-Aparicio, unpublished results). The left end of the *R1* gene begins 245 bp to the left of the fifth *Hin*dIII site (H5, Fig. 4A) of the micronuclear insert and ends approximately 1,400 bp to the right of this site. Taking this into account, the 2.9- and 3.0a-kbp macronuclear DNA molecules were separated by a maximum of 550 bp, and the intergenic distance between the 3.0a and *R1* gene was at most 400 bp. Our results did not allow us to separately define the intergenic distances between the *R1*, 0.85, and 3.0b macronuclear DNA molecules, because a restriction site has not been identified in the 0.85-kbp macronuclear DNA molecule. However, it could be deduced that there was a total of 150 bp in the two spacers between these three genes.

DISCUSSION

We have examined the organization of macronuclear DNA molecules in two regions of the micronuclear genome contained in recombinant clones. In each case the cloned segments of micronuclear DNA showed homology to multiple size classes of DNA molecules that were retained during the genome rearrangement process that occurs during macronuclear development. Hybridization experiments indicated that the macronuclear genes reside very near each other in the micronuclear chromosome. A total of eight adjacent pairs of macronuclear DNA molecules have been examined, and in no instance did the amount of DNA separating them exceed 550 bp. In one case, the precise distance between genes in the chromosome was determined by isolating macronuclear clones of genes adjacent in the chromosome and determining their terminal DNA sequences as well as the sequence of the corresponding region of the micronuclear clone. The two genes, *C2* and *C3*, were found to be separated by only 90 bp in the micronuclear genome.

The discrepancy between the size of the *C2-C3* intergenic spacer determined by genomic hybridization analysis (360 bp) and DNA sequence analysis (90 bp) is the result of IESs in the micronuclear copies of genes. IESs, which must be removed by a nucleic acid splicing process during development, appear to be common in the micronuclear copies of macronuclear genes, and in other studies we have found multiple IESs in the micronuclear copies of the *C2*, *C3*, and *R1* genes (15; Ribas-Aparicio et al., unpublished results). The generality of IESs suggests that our other estimates of intergenic distances must also be considered maximum estimates, and it is conceivable that all genes in the clusters we have examined are separated by less than 100 bp.

One obvious question in regard to our results is whether the cloned segments of micronuclear DNA analyzed actually represent the developmental precursors of all or some of the macronuclear DNA molecules with which they show homology. In the case of the *C2* gene cluster, two polymorphic forms of some of the homologous macronuclear genes have been identified (more than two exist for the *C4* and *C5* genes) in hybridization experiments to restriction digests of macronuclear DNA. The sizes of the restriction fragments detected in these experiments are consistent with both the micronuclear DNA inserts of LMIC2-2 and LMIC2-5 giving rise to macronuclear genes during development. Furthermore, we have previously shown that two forms of the *C2* gene are distinguishable in the macronucleus on the basis of their DNA sequences (15). The sequence differences that define these two forms, or versions, are, however, precisely duplicated in LMIC2-2 and LMIC2-5, indicating that both are involved in generating macronuclear genes during development. Similarly, in the present study partial DNA sequence analysis indicated that the pMAC3 clone of the macronuclear *C3* gene was derived from LMIC2-2. Sequence studies on the second macronuclear *C3* gene clone, LMAC3-G, showed that it was a distinct second version of this gene (data not shown). The sequence of LMAC3-G was, however, an exact match of the corresponding region of LMIC2-5, again indicating that this second region of the micronuclear genome is involved in generating macronuclear genes. Our analysis of LMICR1-7 was less extensive, but was also consistent with this region of the micronuclear genome giving rise to macronuclear DNA molecules during development.

Our results thus indicate that sequences retained in the macronucleus exist very close to each other in the micronuclear genome. In combination with the results of Boswell et al. (4), close spacing of macronuclear genes appears to be the common motif in the micronuclear genome. In that study (4), cloned segments of micronuclear DNA were examined which showed homology to multiple size classes of macronuclear DNA, and only one micronuclear clone that shared homology with a single macronuclear gene was isolated. Although there was a potential bias in the method used to select micronuclear clones in that study (see Introduction), our own studies have avoided this bias by selecting micronuclear clones by their homology to single macronuclear genes. Thus, these combined results indicate that the original model of micronuclear chromosomal organization—macronuclear DNA molecules separated by large spacer or eliminated DNA sequences—is incorrect. Instead, our results suggest that macronuclear DNA molecules are clustered in the chromosome and furthermore that large regions of the genome are completely devoid of macronucleus-destined sequences. Although the maximum number of macronuclear genes within a cluster has not been addressed in this study, the micronuclear clones analyzed to data suggest that it is generally more than five macronuclear DNA molecules.

These results also force a reevaluation of other aspects of macronuclear development. For example, the notion that individual band-interband units of the polytene chromosome contain individual macronuclear DNA molecules is not consistent with our results. Perhaps the band-interband structures of the polytene chromosome represent macronuclear gene clusters plus associated large spacer segments. This conclusion has a precedent stemming from recent molecular studies of gene organization in *Drosophila melanogaster*. At least in some cases, individual bands of the polytene chromosome have been found to contain multiple genes in this organism (12, 22).

In a similar manner, the vesicles that appear during macronuclear development in association with the fragmentation of the polytene chromosomes are probably not encasing individual macronuclear DNA molecules, as previously suggested, but perhaps clusters of genes. This would in fact reconcile some previous observations on the number of vesicles that appear in the developing macronucleus of *O. nova*. Spear and Lauth (31) estimated that approximately 2,700 vesicles appear in the developing macronucleus. Under the view that vesicles encase individual macronuclear genes, this observation is inconsistent with the 20,000 different DNA molecules that exist in the mature macronucleus (25). However, these results can be reconciled if clusters containing about seven genes on average are partitioned into vesicles. If this is the case, the chromosome fragmentation that occurs during macronuclear development would appear to be a two-step process, involving first excision of the gene cluster and later of individual macronuclear genes. It is interesting that Roth and Prescott (28) have observed a putative DNA intermediate in the developing macronucleus of *Euplotes crassus* cells that is more than twice as large as the macronuclear gene to which it ultimately gives rise. Such an intermediate suggests that the initial fragmentation event occurs at some distance from the macronuclear gene and could encompass a gene cluster.

The close spacing of macronuclear genes also have some implications in regard to DNA sequences specifying the fragmentation sites. Sequence signals at the ends of macronuclear DNA molecules or in nearby flanking sequences have been presumed to specify where cuts are to be made. If such chromosome fragmentation signals reside in flanking micronuclear DNA, our results on the close spacing of genes limit their distance from the actual fragmentation site. For example, in the cases of the *C2* and *C3* gene, the putative sequence signal(s) must reside in the 90-bp intergenic spacer.

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