
Organization of gene and non-gene sequences in micronuclear DNA of *Oxytricha nova*

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ABSTRACT

In order to study the derivation of the macronuclear genome from the micronuclear genome in *Oxytricha nova* micronuclear DNA was partially digested with EcoRI, size fractionated, and then cloned in the lambda phage Charon 8. Clones were selected a) at random b) by hybridization with macronuclear DNA or c) by hybridization with clones of macronuclear DNA. One group of these clones contains only unique sequence DNA, and all of these had sequences that were homologous to macronuclear sequences. The number of macronuclear genes with sequences homologous to these micronuclear clones indicates that macronuclear sequences are clustered in the micronuclear genome. Many micronuclear clones contain repetitive DNA sequences and hybridize to numerous EcoRI fragments of total micronuclear DNA, yielding similar but non-identical patterns. Some micronuclear clones containing these repetitive sequences also contained unique sequence DNA that hybridized to a macronuclear sequence. These clones define a major interspersed repetitive sequence family in the micronuclear genome that is eliminated during formation of the macronuclear genome.

INTRODUCTION

Ciliated protozoans contain two kinds of nuclei, micronuclei and macronuclei. In the micronucleus the DNA has a high molecular weight and is organized into a diploid set of chromosomes that undergo mitosis at cell division and meiosis during cell mating. The micronucleus does not produce any RNA and is genetically inert, at least during vegetative cell proliferation. Thus, the micronucleus is strictly a germline nucleus, participating in genetic exchange between cells during conjugation. The macronucleus contains a large amount of DNA, all of which is in gene-sized molecules (1). A large number of these gene-sized molecules has been cloned in a plasmid. With these clones we have shown that a given coding function is always carried by the same sized DNA molecule in a given hypotrich species (reviewed in 2).

The gene-sized molecules are formed during macronuclear development after cell conjugation. During conjugation haploid micronuclei are exchange-

ed and new diploid micronuclei are formed. All unused haploid micronuclei and the macronuclei degenerate. The new micronucleus divides, and one of the daughters develops into a new macronucleus through a complex series of changes involving formation of polytene chromosomes (3), destruction of the chromosomes, and elimination of all repetitious sequences and ~95% of the unique sequences (4). In the process the gene-sized molecules are generated; these undergo 4 to 5 rounds of replication to produce the mature macronucleus with its multiple copies of each gene. At this point the cell resumes proliferation and may go through hundreds of cell generations before conjugating again. All transcription during vegetative existence takes place on the gene-sized molecules. Hypotrich strains lacking micronuclei derived in the laboratory or isolated from natural populations proliferate indefinitely (but cannot conjugate), proving that the micronucleus plays no essential role in transcription in vegetative cells.

As part of an effort to understand how genes are arranged in hypotrich chromosomes and to learn how gene-sized molecules are created during macronuclear development, we have prepared a partial library of cloned micronuclear DNA fragments in lambda bacteriophage and selected clones that contain macronuclear DNA sequences. We report here on the preparation, selection, and characterization of clones. We have mapped the location of particular macronuclear DNA sequences within several micronuclear DNA clones, which provides information on arrangement of genes in the chromosome. We have also isolated subfragments from micronuclear DNA clones that are expected to contain sequences at the junction between eliminated micronuclear DNA and macronuclear gene sequences.

MATERIALS AND METHODS

A. Preparation of Macronuclear and Micronuclear DNAs

Oxytricha nova were cultured and macronuclear and micronuclear DNAs were prepared as described previously (4, 5).

B. Construction of Macronuclear Recombinant Plasmids and Micronuclear Recombinant Phage

Macronuclear DNA was cloned in pBR325 (the pOH series) as described (5). The procedures for the construction of the micronuclear recombinant phage, designated the Ch8-0 series, were similar to those described by Maniatis *et al.* (6). Micronuclear DNA was digested with 2 units of EcoRI per μg of DNA at 37°C and aliquots were removed at 1, 3, 5, 7, 10, 15, and 30 minutes. Reactions were stopped by addition of an equal volume of 5% SDS,

0.1 M EDTA at 0°C. The 7 samples were assayed for degree of digestion in 0.5% agarose gels. The 7 samples were pooled and size fractionated in a 10-40% sucrose gradient containing 2.0 M NaCl, 20 mM Tris-Cl pH 8.0, 10 mM EDTA centrifuged for 24 hrs at 27K in a SW27 rotor at 18°C. Fractions were collected from the gradient and assayed on a 0.5% agarose gel. Fractions with DNA clearly greater than 15 Kbp were pooled and ethanol precipitated. These pieces of micronuclear DNA were ligated to the EcoRI arms of the λ phage Ch8 as described (6, 7). The ligated concatameric micronuclear-Ch8 DNA was packaged into phage by the procedure of Becker and Gold (8). Purified phage λ A-gene protein was kindly provided by A. Becker. Recombinant phage were stored as a plate lysate.

The micronuclear DNA library was screened with 32 P-labeled macronuclear DNA probes using the Benton and Davis plaque hybridization procedure (9).

C. Subcloning of Micronuclear DNA in Recombinant Phage

Recombinant phage DNA and pBR325 DNA were each digested with EcoRI (10). The cut recombinant phage DNA was ligated to phosphatase-treated, EcoRI cut pBR325 DNA and transformed into *E. coli* HB101 (11, 12, 13). The resulting amp^r tet^r cap^s colonies of pBR325 were screened for inserts by the rapid method for plasmid preparation of Birnboim and Doly (14).

D. Blotting and Hybridization

All DNAs were electrophoresed in 0.5% or 1.0% gels at 100V using the buffer system of Loening (15) and transferred to nitrocellulose by the procedure of Southern (16). DNA sequences used as probes were nick-translated to a specific activity of 5×10^7 to 1×10^8 cpm/ μ g by published procedures (17, 18).

To prepare filters for hybridization, they were wetted in 200 ml of 1.0 M NaCl, 50 mM Tris-Cl pH 8.0, 1 mM EDTA for 30 min. Filters were then incubated in boilable cooking pouches at 68°C in 5-10 ml of 1.0 M NaCl, 50 mM Tris-Cl pH 8.0, 1 mM EDTA, 10X Denhardt's solution (19), 0.1% SDS, 0.1% Na pyrophosphate (NaPPi), 10 μ g/ml polyA, 10 μ g/ml sheared denatured *E. coli* DNA or sheared denatured salmon sperm DNA (Sigma). The filter was incubated in the above mix for 4 to 6 h, after which the solution was removed and replaced with a solution containing the same components plus approximately 2-25 ng/ml of radioactively labeled probe. Hybridizations were carried out for 36 to 40 h at 68°C. The non-hybridized counts were removed by washing one time for 1 h at 68°C in 200 ml of each of the following solutions: (1) 0.15 M NaCl, 50 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.1% SDS, 10X Denhardt's, 10 μ g/ml polyA, 0.1% NaPPi and (2) as #1 minus

Denhardt's and (3) as #2 minus polyA. Filters were air dried and mounted on cardboard and exposed to Kodak XR5 x-ray film without a screen at -70°C for 12-24 h.

RESULTS

Cloning of Micronuclear DNA in Lambda

A partial genomic library of *Oxytricha nova* micronuclear DNA was constructed using the λ cloning vector Charon 8. Partial EcoRI digests of micronuclear DNA were fractionated on sucrose gradients and only those fragments larger than 15 KB were cloned. Since the Charon 8 vector accommodates fragments ranging in size from 10 to 22 KBP, this size fractionation should have minimized the possibility of inserting two or more fragments of DNA that would not be contiguous in the micronuclear genome into a single vector. The initial plating of the recombinant phage yielded 1.5×10^4 clones, which is below the 1.5×10^5 phage needed for complete representation of the micronuclear genome. Hence we refer to this as a partial genomic library.

To determine whether cloned fragments of micronuclear DNA had undergone rearrangements during or after cloning, micronuclear DNA clones (the CH8-0 series) were mapped by restriction endonuclease cleavage. Individual recombinant clones were digested with various restriction nucleases singly or in combination, and the sizes of subfragments produced were determined by gel electrophoresis, sometimes using two dimensional separations (20). These subfragments were compared, using hybridization techniques, with subfragments generated from total micronuclear DNA. One possible kind of rearrangement could be the joining of two or more micronuclear DNA EcoRI fragments in a single lambda phage. Therefore, in some of the restriction nuclease mapping, enzymes were used that cut on the two sides of EcoRI sites in the cloned DNA. If the EcoRI site in such a fragment was due to joining of micronuclear DNA pieces during cloning, the RI-containing fragment produced with two other restriction nucleases would not be present in genomic micronuclear DNA cut with the same enzymes. In all cases the sizes of the subfragments produced corresponded to sizes of the homologous fragments obtained by digestion of total genomic micronuclear DNA, which is strong evidence that no rearrangements have occurred in the cloned DNA.

Initially, four DNA lambda clones were chosen randomly. None of these hybridized to macronuclear DNA blotted from gels. These clones, 8-01, 8-02, 8-03, and 8-05 contain repetitive DNA sequences and have some

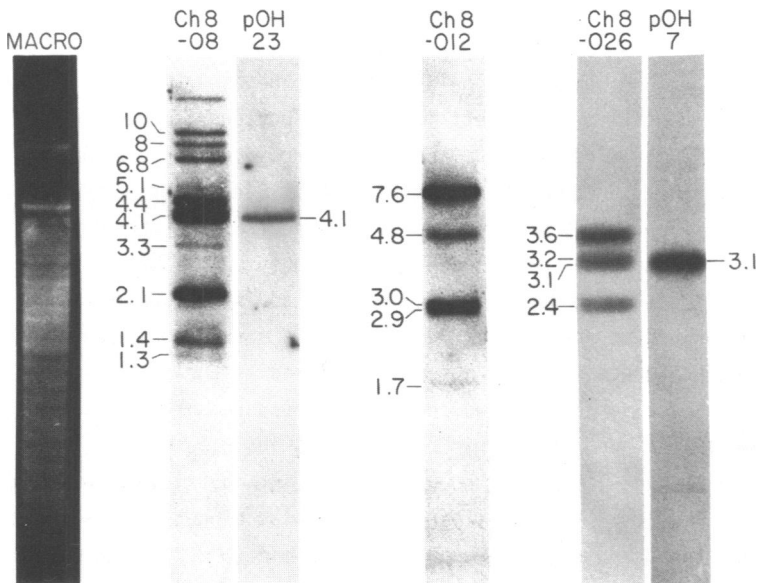


Fig. 1. Hybridization of Micro- and Macronuclear Clones to Macronuclear DNA.

Southern blotted macronuclear DNA was probed with nick-translated clones of micronuclear DNA (CH8-08, CH8-012, and CH8-026) or clones of macronuclear DNA (pOH23 and pOH7), which bear homology to the micronuclear clones (see Fig. 2). Sizes of the hybridizing macronuclear sequences are given in kilobase pairs and were determined from the mobility of Hind III digests of lambda DNA run on each gel. Macronuclear DNA analyzed on a similar gel and stained with ethidium bromide is shown in the first lane.

homology with each other. Twelve additional clones were isolated by probing the library with total macronuclear DNA. These contain sequences homologous to one or more macronuclear DNA sequence(s). About half of these clones contain the repetitious DNA sequences found in CH8-01, 8-02, 8-03 and 8-05 in addition to unique sequences that are found in macronuclear DNA. An additional group of clones was isolated by probing the micronuclear DNA library with a mixture of macronuclear DNA sequences cloned in pBR322. Examples of these different clones will be described in more detail.

Micronuclear Clones Containing only Unique Sequence DNA which Hybridizes to Multiple Macronuclear Sequences

Clone CH8-08 hybridized to 10 macronuclear DNA molecules ranging in size from 1.3 to 10 KBP (Fig. 1). These 10 molecules represent a

total of 46.5 KBP, far in excess of the 19.2 KBP in the micronuclear insert. CH8-08, however, shows strong hybridization to only three sizes of macronuclear DNA and weak hybridization of the other 7 sizes of molecules. Our tentative interpretation of this pattern is that the insert in CH8-08 contains macronuclear genes corresponding to molecules of 2.1, 4.1, and 4.4 KBP, a total of 10.6 KBP. Since weak homology due to the inclusion of only a portion of a macronuclear sequence in a micronuclear clone, that is at each end, can only occur for two macronuclear sequences per micronuclear clone, the additional homologies seen with CH8-08 (at least five) must represent sequence relatedness of macronuclear genes. One or more of the three strongly hybridizing molecules could also reflect sequence relatedness rather than presence of the definitive gene in the micronuclear DNA insert.

When CH8-08 was labeled with ^{32}P and used as a hybridization probe to screen a library of macronuclear DNA molecules cloned in the plasmid pBR322 (5) it hybridized to a recombinant plasmid (pOH-23) containing a 4.1 KBP molecule of macronuclear DNA. This 4.1 KBP insert is an intact macronuclear DNA molecule, as shown by hybridization. When this plasmid (pOH-23) was hybridized to an EcoRI digest of CH8-08, it hybridized to a single EcoRI fragment of 4.6 KBP (Fig. 1 and 2). This 4.6 KBP fragment, as expected, hybridized to a 4.1 KBP molecule in total macronuclear DNA. Both pOH-23 and the 4.6 KBP fragment of CH8-08 hybridize to a 4.1 KBP molecule in EcoRI-digested, total macronuclear DNA. Since pOH-23 lacks an EcoRI site, all of these hybridizations indicate that the two clones contain the same 4.1 KBP macronuclear DNA sequence. Since the 4.6 KBP fragment in CH8-08 and the 4.1 KBP molecule in pOH-23 hybridize only to a 4.1 KBP molecule in total macronuclear DNA, this macronuclear DNA sequence does not account for any of the 7 weaker homologies between CH8-08 and macronuclear DNA.

CH8-012, containing a 22.0 KBP insert of micronuclear DNA, hybridized to 5 sizes of macronuclear DNA (Fig. 1). CH8-012 is large enough to contain all 5 macronuclear DNA molecules (aggregate size = 20.0 KBP), but two of the macronuclear sequences (4.8 and 1.7 KB) hybridize to a lesser extent and may represent partial homology due to incomplete inclusion of these sequences in the micronuclear clone.

Another micronuclear clone, CH8-026, has been studied in some detail. It was one of the clones isolated by probing the micronuclear library with clones of macronuclear DNA. CH8-026 is homologous to the macronuclear

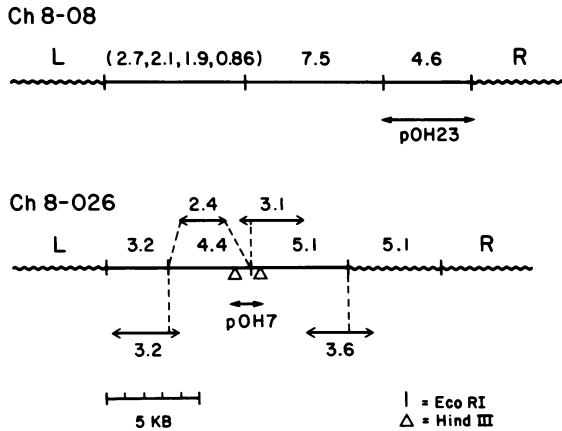


Fig. 2. Regions of Micronuclear Clones Bearing Homology to Cloned Macro-nuclear Genes.

Restriction endonuclease maps of CH8-08 and CH8-026 are shown with the regions determined to be homologous to isolated clones of macronuclear DNA indicated with arrows. The four smaller EcoRI fragments in Ch8-08 (2.7, 2.1, 1.9 and 0.86 KB) were not mapped completely in the region left of the 7.5 and 4.6 KB fragments and are shown as one contiguous region. Homology between CH8-08 and pOH23 was mapped to a 4.6 KB EcoRI fragment; both hybridize to a 4.1 KB sequence in macronuclear DNA (see Fig. 1 and text). The homology between CH8-026 and pOH7 was mapped to a region between two Hind III sites as shown. Since the macronuclear clone (pOH7) is truncated, the entire macronuclear sequence must extend further into the 5.1 KB EcoRI fragment of CH8-026. Both pOH7 and this region of the CH8-026 clone hybridize to a 3.1 KB sequence in macronuclear DNA (see Fig. 1). The other macronuclear sequences homologous to the CH8-026 clone were mapped by hybridizing the 3.2, 5.1 and 4.4 KB fragments individually to Southern-blotted native and EcoRI digested macronuclear DNA. The 3.1, 3.2 and 3.6 KB macronuclear sequences contain EcoRI sites and can be aligned with the map of CH8-026 as shown. The 2.4 KB macronuclear sequence does not contain an EcoRI site and hybridizes only to the 4.4 KB EcoRI fragment from CH8-026. Regions of the clones shown as wavy lines are vector sequences.

clone pOH7, which contains a truncated version of a macronuclear gene, that is, it hybridizes to a 3.1 KB macronuclear gene but contains only a 0.7 KB insert. One end of this 3.1 KB macronuclear gene is included in the pOH7 clone (see 28 for details). CH8-026 contains a 12.75 KB insert and hybridizes to macronuclear DNA of 2, 4, 3.1, 3.2, and 3.6 KB (aggregate size of 12.3 KB) (Fig. 1). pOH7 hybridizes to two of the EcoRI fragments in CH8-026 (Fig. 2). The pOH7 clone contains an EcoRI site and can be aligned with CH8-026 at this site. Homology of pOH7 to CH8-026 was fur-

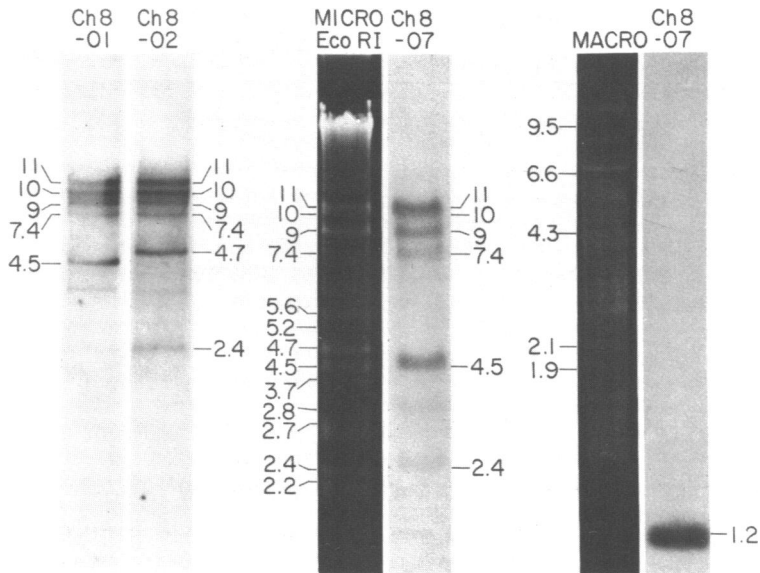


Fig. 3. Hybridization of Repetitive Sequence-Containing Micronuclear Clones to Micronuclear and Macronuclear DNA.

The three micronuclear clones CH8-01, CH8-02 and CH8-07 were nick translated and hybridized to Southern blots of EcoRI digested micronuclear DNA. The patterns of hybridization observed are similar and correspond to bands that are visible in a ethidium bromide-stained EcoRI digest of micronuclear DNA (shown in lane 3). CH8-07 contains a region of unique sequence DNA in addition to this repetitive sequence family and hybridizes to a 1.2 KB sequence in macronuclear DNA (lane 6). Macronuclear DNA stained with ethidium bromide is shown in lane 5; sizes correspond to a Hind III digest of lambda DNA, run in parallel.

ther mapped to a Hind III fragment spanning this EcoRI site (Fig. 2).

Clones CH8-015, CH8-021, CH8-030, CH8-031, and CH8-032 showed hybridization patterns with macronuclear DNA that were similar to those described above. Each clone hybridized to three to five macronuclear molecules. The size of the insert in each of these clones was large enough to include all of the macronuclear genes identified by the Southern blotting.

Overall, these clones of micronuclear DNA show that at least some macronuclear genes occur in small clusters (2 to 5 genes) and that sequences flanking a cluster and, in at least some instances, sequences between the genes within a cluster are eliminated during macronuclear development. Further, weak hybridizations between these micronuclear DNA clones and macronuclear DNA point to the presence of sequence homologies among

different macronuclear genes.

Micronuclear Clones Containing a Prevalent Interspersed Repeat Sequence

The four clones, CH8-01, CH8-02, CH8-03 and CH8-05 hybridize to numerous fragments in EcoRI digested micronuclear DNA but fail to hybridize to any sequences in macronuclear DNA. All four contain inserts possessing an 11 KB EcoRI fragment contiguous to an EcoRI fragment that is either 5.2, 4.7, or 4.5 KB in size. These clones give similar but non-identical patterns of hybridization to EcoRI digested micronuclear DNA. For instance, CH8-01 hybridized to 11, 10, 9, 7.4 and 4.5 KB EcoRI fragments of micronuclear DNA while CH8-02 hybridized to 11, 10, 9, 7.4, 4.7 and 2.4 KB size classes (Fig. 3). The sizes of the micronuclear fragments homologous to these clones correspond to those of prominent bands visible in ethidium bromide stained EcoRI digested micronuclear DNA (Fig. 3), indicating that these fragments comprise a major repetitive sequence family of the micronuclear genome. The repetitive nature of these cloned fragments is further demonstrable by hybridization of nick-translated total micronuclear DNA to Southern blotted digests of the clones: this probe is capable of detecting sequences repeated 50 times or more in the micronuclear genome. The 11 KB and associated 4.5, 4.7 or 5.2 KB EcoRI fragments in these clones all hybridize with the micronuclear DNA probe.

Four additional clones containing the 11 and 4.5 KB EcoRI repetitive sequence fragments were isolated (CH8-07, CH8-09, CH8-010, CH8-011). These contained as well unique sequence DNA that hybridizes to sequences in macronuclear DNA. CH8-07 is described here as an example of this group.

Digestion of CH8-07 with EcoRI yields three subfragments of micronuclear DNA, one of 11 KBP and two of 4.5 KBP each (Fig. 4). These EcoRI fragments were subcloned in pBR322. The 11 KB and one of the 4.5 KB EcoRI fragments (4.5A) did not hybridize to macronuclear DNA. The other 4.5 KB fragment (4.5B) hybridized to a 1.2 KB molecule in native macronuclear DNA. These various observations allow us to construct the map of CH8-07 and the relationship it bears to macronuclear DNA (Fig. 4).

The region bearing homology to the 1.2 KB macronuclear sequence was further mapped by digesting the subclone of the second 4.5 KB fragment (4.5B) with Sal I and EcoRI (Fig. 4) and using the two subfragments to probe macronuclear DNA. Only the 2.75 KB Sal I-EcoRI fragment hybridized to the 1.2 KB macronuclear sequence (Fig. 4). The smaller fragment (1.75 KB) was demonstrated to contain a portion of the micronuclear repet-

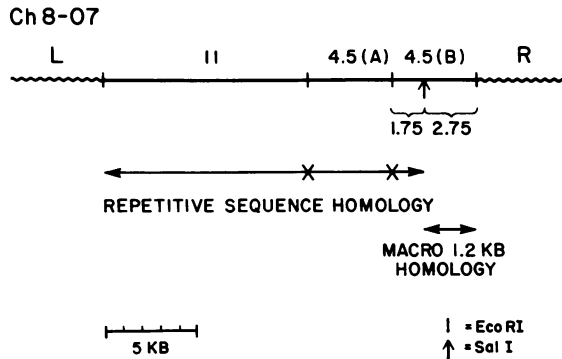


Fig. 4. Mapping of Repetitive Sequence Homology and Macronuclear Sequence Homology in CH8-07.

A restriction map of CH8-07 is shown with the regions homologous to the 11 KB-EcoRI-micronuclear repetitive sequence family and the 1.2 KB macronuclear sequence as shown. In this case, subclones of the EcoRI subfragments of the 4.5 (B) subclone were used to probe Southern blots of digests of micronuclear DNA and native macronuclear DNA (see text).

itive DNA sequence.

Clones CH8-09, CH8-010, CH8-011 show hybridization patterns similar to CH8-07 and hybridize to one, four, and two macronuclear sequences respectively. Thus, in at least one of these cases (CH8-010) the repetitive sequence appears to be adjacent to a cluster of macronuclear sequences similar to that observed in the clones described in the previous section of this paper.

Further Characterization of the Interspersed Repeat Sequence

The 11 KB EcoRI fragment of CH8-07 has been used to probe the other 11 KB EcoRI fragment-containing clones and was found to hybridize to the 11 KB EcoRI fragments but not to any smaller fragments. Thus, the 11 KBP fragment must contain similar sequences in the clones tested. However, the 11 KBP fragments are not identical in sequence since they differ with respect to a number of restriction endonuclease sites (data not shown).

Hybridization of the 11 KB and 4.5 KB (4.5A in Fig. 4) fragments from CH8-07 to EcoRI digested micronuclear DNA demonstrated that the 11 KB hybridizes to 11, 10, 9, and 7.4 KB size classes while the 4.5 KB hybridizes only to a 4.5 KB size class. This suggests that the variability in hybridization of these 11 KB fragment-containing clones to EcoRI digested micronuclear DNA is primarily due to the fragments associated with the

11 KB fragments since all of these clones hybridize to the same large EcoRI fragments in micronuclear DNA (11, 10, 9 and 7.4) but different smaller fragments. Presumably the 4.5, 4.7 and 5.2 KB EcoRI fragments do not share extensive homology.

To obtain an estimate of the repetitiousness of this sequence, the intensity of blot hybridizations of 11 KBP DNA to a graded series of different amounts of EcoRI digested DNA of CH8-07 was compared with the intensity of hybridization of the same 11 KBP DNA to a known amount of total micronuclear DNA. From this comparison, we estimate that the 11 KBP sequences represent at least 2% of the micronuclear genome. Since the 11 KB sequences are homologous to the 10, 9, and 7.4 KB sequences in EcoRI digested micronuclear DNA, this entire family of sequences could comprise as much as 10% of the micronuclear genome.

DISCUSSION AND CONCLUSIONS

Previously we proposed that the genes in the micronucleus were highly dispersed throughout the chromosomes with long stretches of spacer DNA separating individual genes (21, 22). In that model and in the discussion here each of the small molecules of DNA in the macronucleus is defined as a gene. This definition has been validated for the several such molecules that have been examined so far (reviewed in 2).

In the original model the spacers between genes were assumed to be the 95% of the unique sequences that are eliminated from the micronuclear genome during macronuclear development, an assumption based on a comparison of reassociation kinetics of macronuclear and micronuclear DNAs (4). The idea of spacers between genes was based on the observed transection of the polytene chromosomes through all interbands during macronuclear development (23) and the destruction of most of the DNA of each band, assumed to be the spacer DNA (see 24 for review). We further assumed that each band of a polytene chromosome contained a single gene species, drawing on the one band-one gene hypothesis for Drosophila (25).

In this paper we have described a direct analysis of the organization of genes in micronuclear chromosomes of O. nova. This new information requires revision of the original model: Some, and perhaps many genes do not occur singly but, rather, are closely grouped in clusters of two to five genes. A similar situation is found in Drosophila where cloned genetic loci cytologically corresponding to single bands in a polytene chromosome are found to contain a cluster of genes (for example, 26). The

clusters could represent some functional relatedness of the member genes, as is found for gene families in many other organisms.

Approximately one-quarter of the micronuclear clones that were isolated, either by selection at random or by hybridization with macronuclear DNA, contained the 11 KB EcoRI sequences and one of its associated repetitive sequence-containing fragments (5.2, 4.7, or 4.5 KB). This indicates the high abundance of this sequence in the micronuclear genome. Since these clones were constructed from EcoRI partial digests of micronuclear DNA, we may have selected for clones containing this repetitive sequence. The size of the repeat in the clones that we examined (11 KB plus 4.5, 4.7 or 5.2 KB) is perfect for inclusion in a 15-20 KB size fraction. Hybridization of the 11 KB sequence to micronuclear DNA indicated that it comprised 2% of the genome and that its related sequences could account for up to 10% of the genome.

The high frequency with which this repetitive sequence is found in association with unique sequence DNA that hybridizes to one or more macronuclear sequences indicates that this repeat is interspersed with macronuclear sequences in the micronuclear genome. Its prevalence and high degree of interspersion suggests that this repeat sequence is a major component of the DNA spanning between different clusters of macronuclear genes. It remains to be seen what the actual spacing between macronuclear sequence clusters is and whether this relates to the bands seen in Oxytricha polytene chromosomes.

Finally, these micronuclear DNA sequences are being used to study problems of chromosome processing in O. nova. For example, all gene-sized molecules in the macronucleus have the same termini consisting of an inverted repeat sequence composed of multiple repeats of the sequence CCCCCAAA (C₄A₄) (27). Utilizing the clones described here, we have shown that these termini are not present on copies of macronuclear genes as they exist in micronuclear chromosomes, but are added during creation of the individual, free genes (28). In addition, we have isolated a micronuclear DNA clone that contains copies of the terminal inverted repeat sequence that lacks any macronuclear gene sequences. This clone will be described elsewhere.

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