

Organization of the Micronuclear Genome of *Oxytricha nova*

Carolyn L. Jahn, Karen E. Prescott and Mark W. Waggener

Laboratory for Cell, Molecular and Developmental Biology, Department of Biological Sciences, University of Illinois, Chicago, Illinois 60680

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ABSTRACT

In the hypotrichous ciliated protozoan *Oxytricha nova*, approximately 95% of the micronuclear genome, including all of the repetitive DNA and most of the unique sequence DNA, is eliminated during the formation of the macronuclear genome. We have examined the interspersal patterns of repetitive and unique and eliminated and retained sequences in the micronuclear genome by characterizing randomly selected clones of micronuclear DNA. Three major classes of clones have been defined: (1) those containing primarily unique, retained sequences; (2) those containing only unique, eliminated sequences; and (3) those containing only repetitive, eliminated sequences. Clones of type one and three document two aspects of organization observed previously: clustering of macronuclear destined sequences and the presence of a prevalent repetitive element. Clones of the second type demonstrate for the first time that eliminated unique sequence DNA occurs in long stretches uninterrupted by repetitive sequences. To further examine repetitive sequence interspersal, we characterized the repetitive sequence family that is present in 50% of the clones (class three above). A consensus map of this element was obtained by mapping approximately 80 phage clones and by hybridization to digests of micronuclear DNA. The repeat element is extremely large (approximately 24 kb) and is interspersed with both macronuclear destined sequences and eliminated unique sequences.

IN ciliated protozoa, "germline" and "somatic" distinctions are made at the level of nuclei within a single cell. During vegetative growth of the organism, the macronucleus (the "somatic" nucleus) is responsible for all or most of the transcriptional activity while the micronucleus (the "germline" nucleus) is transcriptionally inert (RAIKOV 1982). During the sexual phase of the life cycle, the micronucleus participates in meiosis and sexual exchange while the macronucleus is destroyed and replaced by a new copy derived from the newly formed diploid micronucleus. The difference in structure between the macronuclear and micronuclear genomes is greatest in hypotrichous ciliated protozoa such as *Oxytricha*, *Stylonychia* and *Euplotes* (KLOBUTCHER and PRESCOTT 1987). The micronuclei in these species contain typical eukaryotic chromosomes (reviewed in RAIKOV 1982), while their macronuclear genomes consist of small, "gene-sized" linear DNA molecules (SWANTON, GRESLIN and PRESCOTT 1980; SWANTON, HEUMANN and PRESCOTT 1980; KAINE and SPEAR 1982; HELFTENBEIN 1985). Massive DNA rearrangement and elimination occurs during the formation of a macronucleus from a micronucleus (AMMERMANN *et al.* 1974; LAUTH *et al.* 1976; KLOBUTCHER, JAHN and PRESCOTT 1984; RIBAS-APARICIO *et al.* 1987). Reassociation kinetics of the macronuclear and micronuclear DNA of the hypotrichs *Oxytricha sp.* and *Stylonychia lemnae* (formerly

S. mytilis) demonstrated that 90–95% of the sequence complexity of the micronucleus is eliminated during macronuclear development (LAUTH *et al.* 1976; AMMERMANN *et al.* 1974). Because 70% of the *Oxytricha sp.* genome reassociates as a single-copy component the sequence elimination must include large amounts of unique sequence DNA (LAUTH *et al.* 1976).

The nuclear duality of ciliated protozoa presents interesting possibilities for genome organization. The lack of transcriptional activity of the micronucleus and the massive extent of genome rearrangements that generate the macronuclear genes suggests that the micronucleus could be the ultimate haven for "selfish DNA" (DOOLITTLE and SAPIENZA 1980; ORGEL and CRICK 1980), where interruption of genes or addition of new sequences would have minimal consequence. In studying micronuclear sequence organization we not only learn how macronuclear genes are recognized for excision but how a whole genome might evolve if not subject to the usual functional constraints.

Previous studies of *Oxytricha nova* micronuclear genome organization have concentrated on determining how the macronuclear destined sequences are arranged in the micronuclear genome. Thus, either total macronuclear DNA (BOSWELL *et al.* 1983) or individual macronuclear genes (KLOBUTCHER *et al.* 1986) were used to select clones from a micronuclear

genomic phage library. These studies demonstrated that the macronuclear destined sequences are clustered together with very short stretches (<550 bp) of eliminated unique sequence DNA interspersed between them. In addition, short stretches of eliminated DNA were shown to exist internally in the macronuclear destined sequences (termed Internal Eliminated Sequences, or IESs) (KLOBUTCHER, JAHN and PRESCOTT 1984; RIBAS-APARICIO *et al.* 1987). By selecting for clones that hybridized to macronuclear DNA, BOSWELL *et al.* (1983) also identified a repetitive sequence family that is interspersed with macronuclear destined sequences and comprises up to 10% of the micronuclear genome. As discussed by KLOBUTCHER *et al.* (1986), if macronuclear destined sequences are clustered, then large regions of the genome must be devoid of them. In this report, we have taken the approach of randomly selecting micronuclear phage clones in order to characterize the overall organization of the *O. nova* micronuclear genome. Thus, 80% of the clones we have analyzed do not contain macronuclear destined sequences. These clones demonstrate that eliminated repetitive sequences are not highly interspersed with eliminated unique sequences. The only evidence we have obtained for their interspersed nature comes from further characterization of the repetitive element previously identified by BOSWELL *et al.* (1983). By isolating clones containing each end of this large (approximately 24 kb) repetitive element, we have demonstrated its interspersed nature with eliminated unique sequences and clusters of macronuclear destined sequences.

MATERIALS AND METHODS

Enzymes and reagents: Restriction enzymes were purchased from BRL Inc. or Boehringer Mannheim and were used under conditions recommended by the supplier. [³²P]dCTP was purchased from New England Nuclear (NEN). DNA polymerase I and its Klenow fragment were purchased from Boehringer Mannheim. Random hexamer primer, (dN)₆, (see *Labeling reactions* below) was purchased from PL Biochemicals.

Plasmids and phage: The phage clones Ch 8-02, Ch 8-07, and Ch 8-010 are described in BOSWELL *et al.* (1983). Subclones of the 4.5A and 4.5B *EcoRI* fragments were kindly provided by Art Greslin (University of Colorado). The 11L and 11R probes consist of 0.9 and 2.4 kb *EcoRI*-*Bam*HI fragments derived from 11 kb *EcoRI* fragment in Ch 8-02 that were subcloned into pUC12. The micronuclear DNA library used throughout this study consists of *Sau*3A partial digests of micronuclear DNA cloned into λ 47.1 and was described in KLOBUTCHER, JAHN and PRESCOTT (1984). Additional clones were constructed by inserting *EcoRI* partial digests of micronuclear DNA into λ phage EMBL4 (FRISCHAUFF *et al.* 1983). These clones were screened to obtain the phage in group B. Construction of the pMAC and LMAC clones of macronuclear sequences has been described elsewhere (KLOBUTCHER, JAHN and PRESCOTT 1984).

DNA isolation: Phage DNA was purified by standard

procedures described in MANIATIS, FRITSCH and SAMBROOK (1982). Macronuclear and micronuclear DNA purification has been described elsewhere (KLOBUTCHER, JAHN and PRESCOTT 1984; JAHN 1988).

Gel transfers: The standard Southern blot procedure for denaturation, neutralization and transfer of DNA from agarose gels in 10 \times SSC was followed when transfers were made to nitrocellulose. The nitrocellulose blots were baked at 80°C in a vacuum oven for 2–3 hr. For transfer to Gene Screen Plus (NEN), gels were soaked in 0.4 N NaOH for 10 min and the gel was then blotted using 0.4 N NaOH as the transfer buffer. Following blotting, the membrane was rinsed in 0.2 M Tris-HCl, pH 7.5 and dried. Gels of micronuclear DNA were soaked in 0.25 N HCl for 20 min prior to transfer in NaOH as above.

Labeling reactions: Phage, plasmid and micronuclear DNA were labeled by nick-translation using standard methods. Individual fragments were isolated and labeled by electrophoresing the restriction enzyme digested DNA in low melting agarose, cutting out the appropriate bands and labeling (without elution) using DNA polymerase I Klenow fragment and random hexamer primers as described by FEINBERG and VOGELSTEIN (1983).

Hybridizations: All transfers (nitrocellulose and Gene Screen Plus) were hybridized as follows. The transfer was soaked in 4 \times SSC (1 \times SSC = 0.15 M NaCl, 0.015 M Na₃Citrate), 0.25% powdered milk (JOHNSON *et al.* 1984) for 1–3 hr at 65°C. The blot was then hybridized in 6 \times SSC, 0.25% powdered milk, 0.5% SDS. Aliquots of 100 μ g/ml sonicated herring sperm DNA and polyA were added to the probe, which was then boiled for 10 min and added to the above mixture. The blots were hybridized with probe for 16–24 hr at 65°C after which they were washed in three or more changes of 2 \times SSC, 0.5% SDS followed by three or more changes of 1 \times SSC, 0.5% SDS at 65°C. Transfers made to Gene Screen Plus were kept moist while exposing them to X-ray film and the hybridized probe was subsequently removed by treating the membrane with NaOH as described by the manufacturer. Blots of phage DNA to Gene Screen Plus were reused up to ten times. Blots of micronuclear DNA digests were used up to five times, after which the hybridization was too weak and diffuse to be informative.

RESULTS

Randomly selected clones of micronuclear DNA: To determine the arrangement of eliminated repetitive and unique sequences relative to macronuclear destined sequences, we isolated 30 clones at random from a micronuclear DNA library constructed from *Sau*3A partial digests of micronuclear DNA. These clones were characterized as follows: (1) each clone was labeled by nick-translation and hybridized to Southern blots of macronuclear DNA to detect possible macronuclear destined sequences; (2) nick-translated micronuclear DNA was hybridized to Southern blots of restriction enzyme digests of the clones to determine which fragments in the clones contained repetitive sequences; and (3) the labeled clones were hybridized to Southern blots of restriction enzyme digested micronuclear DNA to determine which family of repetitive sequences, if any, was present. The clones were classified into five groups as shown in

TABLE 1

Characterization of micronuclear DNA clones

Types of sequences in clone	No. of clones	
	Random	Selection for unique sequences
Class I: Unique, macronuclear-destined	5	1
Class II: Unique, eliminated	8	11
Class III: Repetitive, eliminated common family	15	
Class IV: Repetitive, eliminated other families	1	
Class V : Unique, macronuclear-destined with repetitive common family	1	
Total	30	12

Table 1. Three classes of clones (I, II and III, Table 1) are especially prevalent in the micronuclear library and will be discussed individually below.

For simplicity, we refer to the clones as “unique” and “macronuclear destined” according to their behavior in the hybridization experiments described above. The following qualifications should be noted. Clones that do not hybridize to nick-translated micronuclear DNA (classes I and II, Table 1) are referred to as unique. Since this method detects sequences repeated greater than 10–50 times per genome some low copy number repeated sequences would be included in this class. Sequences that hybridized to macronuclear DNA are referred to as macronuclear destined (groups I and V, Table 1). In *O. nova* and *Oxytricha fallax*, DNA sequence analysis of micronuclear clones that contain putative precursors of macronuclear sequences detects different versions of the macronuclear sequence in different clones (KLOBUTCHER, JAHN and PRESCOTT 1984; HERRICK *et al.* 1987a). The versions differ in sequence at approximately 3% of the nucleotides. Only some of the versions are found in the macronucleus; thus it is not always possible to prove that a given sequence is macronuclear destined. To date, in three sets of overlapping micronuclear clones that have been analyzed (C. L. JAHN, unpublished data) all of the different sequence versions are related (by cross-hybridization) for the entire stretch of DNA that has been isolated (20–30 kb). Each version has the same pattern of hybridization to macronuclear DNA and thus appears to contain an allelic version of the same cluster of macronuclear destined sequences. Thus we believe that a micronuclear clone will be representative of its allelic versions and hence has the characteristics of a stretch of micronuclear DNA that is macronuclear destined.

The clones in class I further document the clustering of macronuclear destined sequences in the *O. nova* micronuclear genome. These clones behave like those

TABLE 2

Size classes of macronuclear sequences detected by macronuclear clones

Clone	Size (bp) of insert	Size (bp) of native macronuclear DNA hybridization	Size (bp) of
			<i>Eco</i> RI digested macronuclear DNA hybridization
pMAC1 ^a	1100	1100	
pMAC2	700	700	
pMAC6	3200	3200	
pMAC7	560	560	
pMAC10	7200	7200	
pMAC11	3000	3000	
pMAC12	500	500	
LMAC3	1250	1250	1250
LMAC8	580	580	580
LMAC9	800	800	800
LMAC12	1700	2000	1700, 980, 750, 300
LMAC6	2500	6000	2500, 3200
LMAC11A ^b	1000	1000	1000
LMAC11B	1800	1800, 1900	1800, 1900
LMAC5	1300, 1500	2800, 2500	1300, 1500, 2500
LMAC4 ^c	7400	7400	3500, 3200, 700

^a pMAC and LMAC are plasmid and λ phage clones, respectively. The macronuclear inserts were cloned using *Eco*RI linkers.

^b LMAC11 contains two inserts corresponding to different macronuclear molecules.

^c LMAC4 contains a copy of the rDNA gene.

previously described in detail (KLOBUTCHER *et al.* 1986). Each of the clones hybridizes to 3–6 size classes of macronuclear sequences. The sizes of the macronuclear sequences detected by each clone add up to enough DNA to occupy most of each insert and thus each clone appears to contain closely spaced macronuclear destined sequences. Another possible cause of hybridization to multiple size classes of macronuclear DNA molecules is “alternate” or “alternative” processing, which produces several differently sized overlapping sequences from the same region of the micronuclear genome (CARTINHOOR and HERRICK 1984; HERRICK *et al.* 1987a, b). Alternate processing is common in *O. fallax* (6 of 15 randomly selected macronuclear sequences) (CARTINHOOR and HERRICK 1984). However, we have hybridized more than twenty randomly selected macronuclear clones from *O. nova* to macronuclear DNA and observed only two cases of multiple size classes. Data on the macronuclear clone insert size and the sizes of macronuclear DNA molecules detected by hybridization for 15 of the macronuclear clones that we know are clones of intact macronuclear molecules or of an *Eco*RI fragment of an intact molecule are shown in Table 2. The other randomly selected macronuclear clones examined contained inserts unrelated in size to the macronuclear molecule detected by hybridization. The LMAC11b and LMAC5 inserts detect two size classes of macronuclear sequences. Unlike the observations in *O. fallax*, the different molecules detected by these *O. nova* clones are very close in size. Several other

clones detect multiple species upon *Eco*RI digestion of macronuclear DNA. These presumably represent different versions of the same sequence. The data in Table 2 indicates that alternate processing does not occur frequently in *O. nova* and could not explain the high frequency of micronuclear clones that hybridize to multiple size classes of macronuclear DNA.

The reassociation kinetics of the *Oxytricha* sp. micronuclear genome indicate that approximately 70% of the eliminated sequences are unique (LAUTH *et al.* 1976). This contrasts sharply with sequence elimination in *Ascaris* (MORITZ and ROTH 1976) and *Tetrahymena* (YAO and GOROVSKY 1974) where most of the elimination involves repetitive sequences. A C_{ot} analysis has not been done on the *O. nova* micronuclear genome. However, by characterizing the randomly selected micronuclear clones it is possible to determine whether large amounts of eliminated unique sequence DNA exist in *O. nova*. In the random sample of clones, eight (class II, Table 1) did not hybridize to macronuclear DNA and were also negative for hybridization with nick-translated micronuclear DNA. We characterized these eight further to see if they truly represented eliminated unique sequences. Five of the eight clones hybridize to *Eco*RI fragments in digests of micronuclear DNA that correspond in size to *Eco*RI fragments contained in the clone. Since these hybridizations required high specific activity probes and film exposures equivalent to using unique macronuclear sequences as probes, we believe these clones consist entirely of unique sequence DNA. The other three clones detected a high molecular weight smear of fragments in addition to a set of fragments similar in size to those inserted in the clone. The smear was more intense than the discrete bands. These three clones must include some short stretch of DNA that is repetitive and hybridizes to the high molecular weight smear. The majority of the inserted DNA in these clones behaves as unique sequences. Although we consider contamination with other DNA unlikely, as a control we also hybridized all eight of the unique sequence clones to DNA from *Chlorogonium*, the food organism for *O. nova*, and did not detect any hybridization.

To further determine the frequency of macronuclear destined sequences among clones containing unique or low copy number sequences and to see if we could find any evidence for their interspersion, we isolated another set of 12 phage. This group was limited to unique sequence clones by selecting for phage that failed to hybridize to micronuclear DNA labeled by nick translation. Approximately 50% of the phage in the *O. nova* micronuclear DNA library meet this criterion. The 12 clones were classified as described for the 30 random clones (Table 1). Only one of these unique sequence clones hybridizes to macro-

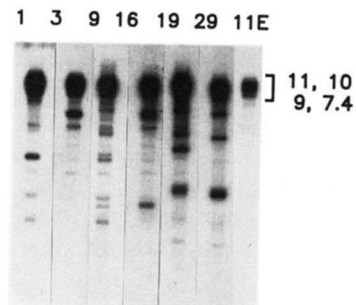


FIGURE 1.—Hybridization of randomly selected clones to micronuclear DNA. Six of the randomly selected clones containing repetitive DNA sequences were nick-translated and hybridized to Southern blotted *Eco*RI digests of micronuclear DNA (lanes 1–6, labeled with the number of the clone). Hybridization of the 11-kb *Eco*RI fragment which is common to all of these clones is shown in lane 7 (labeled 11E). Fifteen out of 16 of the clones containing repetitive sequences hybridized to fragments 11, 10, 9 and 7.4 kb in size (overexposed so that the smaller fragments that vary more from clone to clone are visible).

nuclear DNA. This clone detects four size classes of macronuclear sequences and thus behaves as if it contains a cluster of macronuclear destined sequences. Overall, with a total of 25 clones (all of the class I and II, Table 1) that contain unique sequences, the clones containing eliminated unique sequences are more prevalent (19 of 25) than those containing macronuclear destined sequence clusters (6 of 25). This demonstrates that most of the low copy number or unique sequences that are eliminated are not interspersed with the macronuclear destined sequences and occur on their own in long stretches that are not interrupted by repetitive sequences.

Sixteen of the 30 clones contain repetitive sequences and 15 of these 16 (class III, Table 1) show a pattern of hybridization to micronuclear DNA similar to that seen by BOSWELL *et al.* (1983) and shown in Figure 1. These clones give related patterns of hybridization to *Eco*RI digested micronuclear DNA. All but one of these clones detect large restriction fragments of 11, 10, 9 and 7.4 kb in size in addition to several smaller fragments. BOSWELL *et al.* (1983) showed that a repetitive 11 kb *Eco*RI fragment isolated from a micronuclear phage clone hybridized to fragments 11, 10, 9 and 7.4 kb in size in *Eco*RI digested micronuclear DNA indicating that this repetitive element is found in these four different arrangements. In ethidium bromide stained gels of *Eco*RI digested micronuclear DNA, several intense bands are visible above a background smear, indicating the presence of a high copy number of fragments of certain sizes. As demonstrated previously by BOSWELL *et al.* (1983) these prominent bands are identical in size to those detected by hybridization with the repetitive sequence clones. As seen in Figure 1 the pattern of smaller fragments differs from clone to clone. Similar combinations of these small hybridizing fragments are seen with several of these clones

(compare 3 with 16, and 19 with 29 in Figure 1). These additional hybridizing bands suggested that either the element hybridizing to the 11, 10, 9 and 7.4 kb bands is frequently associated with other repetitive elements that hybridize to the other fragments or that the element is very large and a given phage clone contains only a portion of the element, resulting in variable hybridization patterns from clone to clone. In the latter case the 11, 10, 9 and 7.4 kb hybridizing sequences must occur more frequently in the clones than the other portions of the element. The results described below establish that these repetitive sequences comprise one very large element that is interspersed with both macronuclear destined sequences and eliminated unique sequences.

Isolation of clones containing the repetitive sequence family: To determine the structure of this repetitive element, we isolated and mapped a series of phage clones that encompass the entire element. A consensus map of the element was defined from the restriction maps of the phage clones and from genomic mapping data obtained by hybridization of the phage clones to restriction enzyme digested micronuclear DNA. Since this repetitive element is large and highly abundant, restriction site polymorphisms and deletions/insertions abound and produce variability in the restriction maps. Even with restriction fragments observed in micronuclear DNA (where we are detecting the most abundant versions) several maps could be derived. Our strategy for isolating the clones and all of their restriction maps are illustrated (Figures 2–4 and 6) in relation to the consensus arrangement of the largest possible form of the element. Each of the hybridization probes used to define the internal structure of the element and the fragments these probes hybridize to are identified in the figures by a different shading design (see Figure 2A and subsequent figures). Throughout this report we will refer to hybridization probes used to define a given region of the element by the size of the “consensus” *Eco*RI fragment from that region, as shown at the top of each figure.

Individual fragments were isolated from clones as shown in Figure 2A for use as hybridization probes specific for different regions of the repetitive element. Since there is variation in restriction maps from clone to clone for this element, we did not necessarily use a fragment as probe that corresponds in size to the “consensus” fragment size. Hybridization of each of these probes to *Eco*RI digested micronuclear DNA is shown in Figure 2B. Each fragment hybridizes to one or more size classes of *Eco*RI fragments. The 4.7 and 11R probes show the greatest heterogeneity with respect to *Eco*RI sites. The size of the most abundant or largest (when more than one band of high abundance is seen) *Eco*RI fragment detected by each probe is

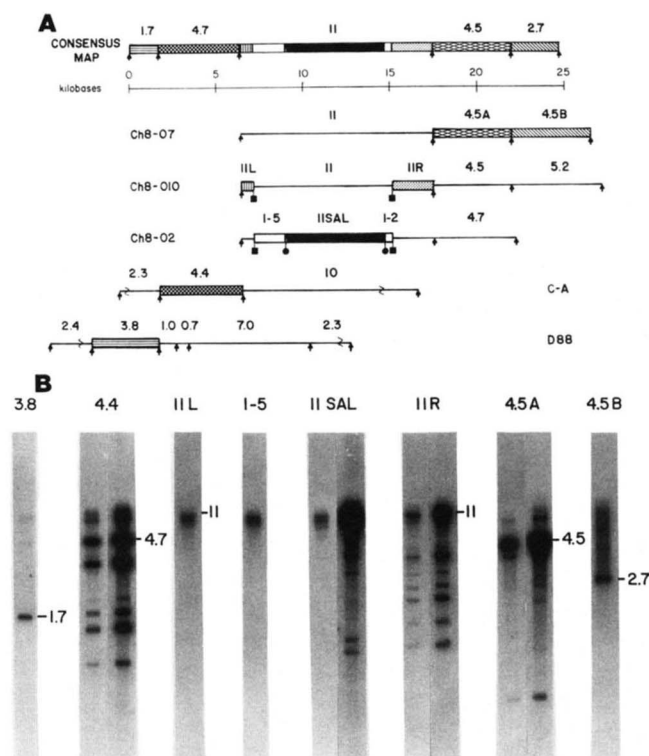


FIGURE 2.—Hybridization probes used to isolate and map the repetitive element. Each fragment that was used as a hybridization probe to characterize the element is shown in the context of the phage clone from which it was isolated (A). Each fragment is given a characteristic design that is used throughout the figures to designate hybridization to that fragment. In B, hybridization of each of the probes to *Eco*RI digested micronuclear DNA is shown. The sizes shown to the right of each strip designate the band that corresponds to the fragment size used in the consensus map. In several cases, two different exposures of the autoradiogram are shown to visualize the variant sized fragments homologous to the probes (*Eco*RI = \blacktriangledown ; *Bam*HI = \blacksquare ; *Sal*I = \bullet).

used in the consensus map. For instance, when the 4.4 kb *Eco*RI fragment from clone C-A (Figure 2A) is hybridized to *Eco*RI digested micronuclear DNA it detects fragments of 4.7, 3.5, 1.9, 1.2 and 0.7 kb in size (Figure 2B). Hybridization to the 4.7 kb is of equal or greater intensity than the other sizes. Hybridizations to genomic digests and mapping of the phage clones demonstrates that all of the smaller fragments homologous to this probe are derived from the 4.7-kb *Eco*RI fragment by deletions or by addition of new *Eco*RI sites (restriction site polymorphisms). Thus, 4.7 is used as the fragment size in this portion of the consensus restriction map. The variability of other regions of this element relative to the consensus map will be discussed below.

The phage isolated to encompass this repeat comprise seven groups that were selected using “chromosome walking” methods as shown in Figure 3. In those cases where we wanted to walk along the element, the phage were isolated by selecting for phage that hybridized to one end of the previously characterized region of the repeat while selecting against hybridization

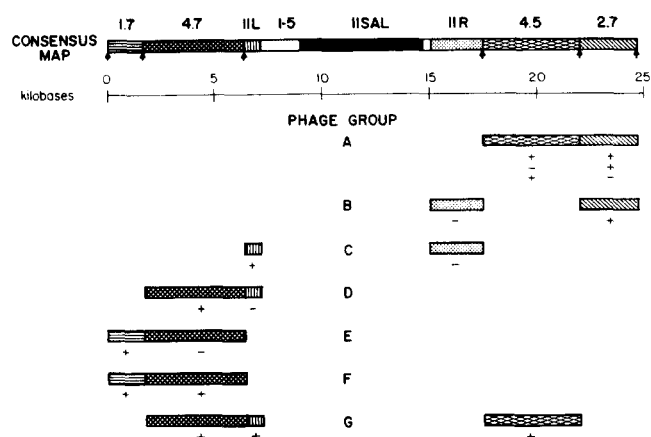


FIGURE 3.—Strategy for the isolation of phage clones encompassing the entire repetitive element. Phage clones were isolated by probing the *O. nova* micronuclear DNA library with the hybridization probes shown. Phage were isolated to “walk” along the repetitive element (groups A, B, C, D, and E) by screening for positive hybridization to one probe (+) with negative hybridization to a second probe (–). In cases where additional phage were isolated to verify our consensus genomic map, phage were selected that hybridized to more than one probe (groups A, F and G). The consensus map of the repetitive element is shown at the top of the figure.

zation to the opposite end (phage groups B, C, D, E). Other clones (groups F and G) were isolated by hybridization with two or more fragments found to be adjacent in previously isolated clones in order to document the generality of the organization of this repeat in the genome and to generate a consensus map. Between 10 and 20 phage were isolated and mapped in each group: representative maps are shown in Figures 4 and 6. The end of the walk was defined as the point where all or most of the clones contained non-repetitive sequences adjacent to the repetitive element (primarily groups B and E).

Establishing linkage of the repetitive sequences as one large element: Evidence that these repetitive sequences comprise one very large repetitive element and are not multiple elements interspersed with each other comes from the following results. First is our ability to “chromosome walk” within the element, which requires a consistent arrangement of the hybridizing sequences oriented right to left. If multiple elements were present they would not always bear the same spatial relationship to each other. For example, walking right to left, all of the phage selected (16 total) by hybridization with the 4.5 and 2.7 probes (group A phage maps, Figure 4) contained 11R-like sequences immediately adjacent to 2.7- or 4.5-like sequences (see discussion of these two versions below). Walking leftward from the 11-kb *EcoRI* fragment, all 20 phage selected by hybridization to the 11L probe (group C phage maps, Figure 4) contained sequences hybridizing to the 4.7 probe immediately adjacent to the 11L-like sequences. Similarly, any clone isolated that extends farther than the 4.7-like sequences con-

tains fragments hybridizing to the 1.7 probe immediately leftward (groups D and F, Figure 4) and any clone extending beyond the 4.5-like sequences has fragments hybridizing to the 2.7 probe immediately rightward (clones A13 and A16 in Figure 4). Except for the presence of two versions, where either 2.7- or 4.5-like sequences can be adjacent to 11R sequences, the internal order of these hybridizing regions is maintained in all of the phage we have isolated and mapped (80 total).

Second, we have determined the number of phage that hybridize to each probe in the micronuclear DNA library using replica plaque lifts to determine the coincidence of hybridization with the various probes. Linkage of these hybridizing regions as one large element implies that neighboring regions of the repeat would occur at high frequency in the same phage clone. In addition, for internal regions of the element, if a fragment lying in one direction is not in the same phage, then we would expect to find hybridization to the neighboring fragment from the other direction. We therefore determined the frequency that 4.7-like sequences are surrounded by 11L and 1.7 like sequences and the frequency that 4.5-like sequences are surrounded by 11R and 2.7 DNA sequence homology. We also included 11SAL hybridization in our determinations because maps of the clones (groups D and F, Figure 4) and of genomic digests indicate that the 11L region is deleted in some versions of the element leaving 4.7 and 11SAL-like sequences adjacent to each other. Likewise, the presence of two different versions of the element in the group A clones suggested that we should see a high coincidence of 11R and 2.7-like sequences without coincident hybridization to the 4.5 probe.

The chart in Figure 5 demonstrates that the majority of the clones in the micronuclear library that contain this repetitive element show the pattern of hybridization expected from our consensus map of the different hybridizing regions. Most of the phage hybridizing to the 1.7 probe also hybridize to the 4.7 probe (1.7+ 4.7+ in Figure 5). The majority of the phage that hybridize to the 4.7 probe also hybridize to the 11L probe (4.7+ 11L+). Those 4.7 positive phage that fail to hybridize to the 11L probe are positive for either the 11SAL or 1.7 probes (4.7+ 11L– 11SAL+ or 4.7+ 11L– 1.7+). At the right end of the element nearly all of the phage positive for hybridization with the 4.5 probe also hybridize with the 11R probe (4.5+ 11R+). Half of those that are 11R negative hybridize to the 2.7 probe (4.5+ 11R– 2.7+). Hybridization with the 2.7 probe shows that phage containing the 4.5 kb region between 11R and 2.7 hybridizing regions (2.7+ 11R+ 4.5+ and 2.7+ 11R– 4.5+) are equally prevalent to those lacking the 4.5 positive region (2.7+ 11R+ 4.5–). Approximately

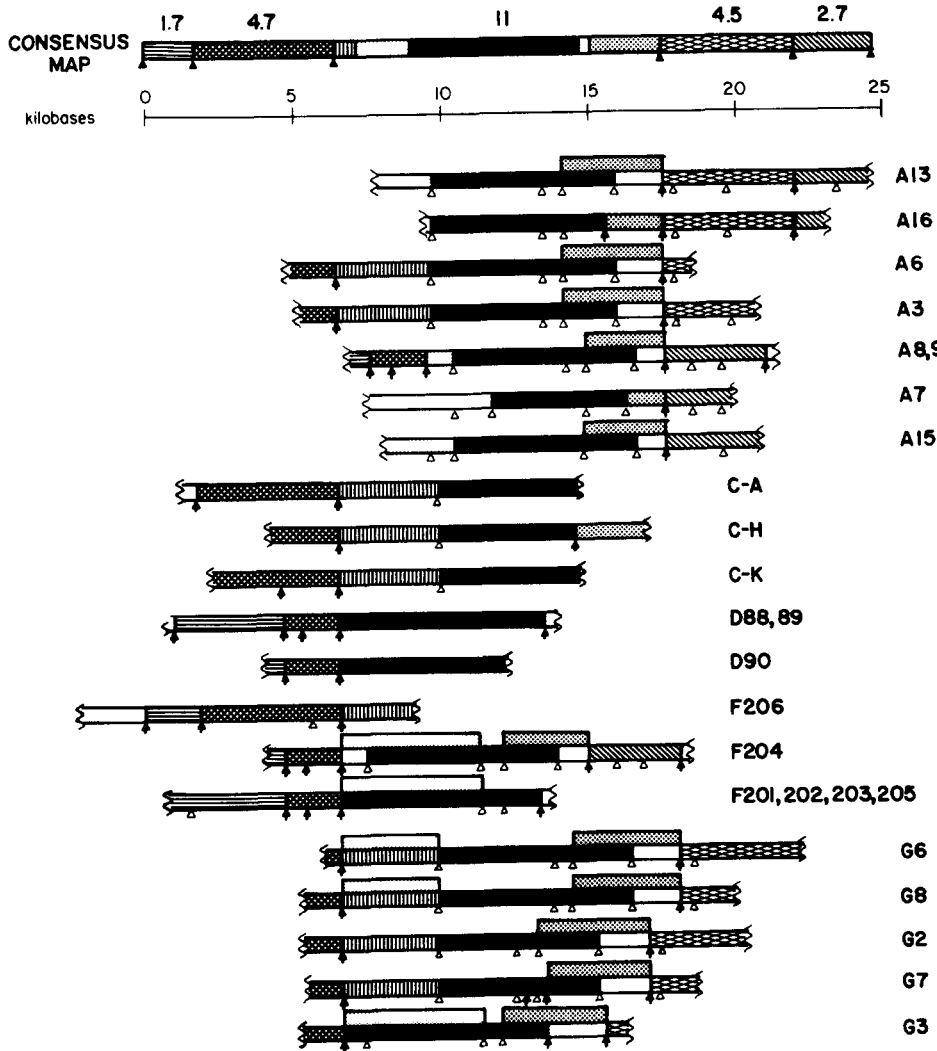


FIGURE 4.—Restriction maps of phage encompassing the repetitive element. Representative maps of clones isolated by screening the micronuclear DNA library are shown. The alphabetical designation of each clone refers to the phage groups shown in the screening strategy (Figure 3). Maps of clones from group A are aligned with the consensus map at the *EcoRI* site at the right end of the 11-kb *EcoRI* fragment. The other maps are aligned with the left *EcoRI* site. Hybridization to the 1-5 and 11R probes are shown above the hybridization to the 11SAL and 11L since these overlap in their hybridization to *XhoI-EcoRI* fragments (*EcoRI* = \blacktriangle ; *XhoI* = \triangle).

half of the phage hybridizing to the 2.7 probe did not hybridize to the other portions of the element and presumably extend into nonrepetitive DNA. This points out that it was much easier to “walk” rightward from the end of the element into adjacent nonrepetitive DNA than it was to move leftward. Many fewer clones in the library (2% *vs* 40%) contain the 1.7 end without the 4.7 and 11 hybridizing regions than contain the 2.7 hybridizing region without the 4.5 or 11.

Finally, we have hybridized each of the probes to Southern blots of single and double restriction enzyme digests of micronuclear DNA. Although the fragments detected are large and multiple fragments are detected with each probe, the 1.7, 4.7 and 11L probes all hybridize to similar patterns of *Bam*HI, *Sal*I and *Xho*I fragments (data not shown) indicating that these large fragments span from within the 11 kb *Eco*RI fragment leftward to conserved sites within the repetitive element. Similarly the 11R, 4.5 and 2.7 probes show related patterns of hybridization to *Bam*HI, *Bgl*II, *Sal*I and *Xho*I digested micronuclear DNA. Unfortunately, the complexity of the patterns of

bands seen with each probe did not allow the construction of a consensus map for these other restriction enzymes.

Multiple versions of the repetitive element: As mentioned previously, both the restriction maps of the clones and the genomic hybridizations demonstrate heterogeneity in this repetitive element. Some of the alternate forms are equally prevalent in the genome. Variability in the middle of the element results in the presence of 11, 10, 9 and 7.4 kb *Eco*RI fragments in micronuclear DNA. This variation is seen in the restriction maps of the clones as well. For example, comparison of the *XhoI-EcoRI* maps of clones G6 and G8 to that of G2 and G7 (Figure 4) shows a loss of sequences lying in the 11SAL hybridizing regions of G2 and G7. In both G7 and G3, new *Eco*RI sites are present toward the right end of the 11 kb hybridizing regions. The small *Eco*RI fragments hybridize to 11R and correspond to fragments hybridizing in 11R in digests of micronuclear DNA (see the 11R lane in Figure 2B). At the left end of the element, in all cases where smaller fragments hybridizing to the

PROBES			1.7	4.7	11L	11SAL	11R	4.5	2.7	
CONSENSUS MAP			[Diagram showing probe positions on a consensus map]							
COMBINATION OF PROBES			PERCENT OF PHAGE HYBRIDIZING TO COMBINATIONS OF PROBES							
1	2	3								
1.7+	4.7+		[Diagram]							92%
4.7+	11L+		[Diagram]							58%
	11L-	11SAL+	[Diagram]							30%
		1.7+	[Diagram]							8%
4.5+	11R+		[Diagram]							90%
	11R-	2.7+	[Diagram]							5%
2.7+	11R+	4.5-	[Diagram]							29%
		4.5+	[Diagram]							13%
	11R-	4.5+	[Diagram]							13%
PERCENT OF PHAGE HYBRIDIZING TO EACH PROBE			1.7	4.7	11L	11SAL	11R	4.5	2.7	
			11%	18%	12%	25%	20%	10%	8%	

FIGURE 5.—Determination of linkage of different portions of the repetitive element by hybridization to replica plaque lifts. Four replica transfers of 1000 to 1200 phage from the *O. nova* micronuclear library plated on 150-mm petri plates were hybridized with either the 1.7, 4.7, 11L and 11SAL probes or the 2.7, 4.5, 11R and 11SAL probes. The replica lifts were then compared to determine the number of phage that hybridized to the combinations of probes shown. Data was averaged for replica transfers from three different plates. The combinations of probes used and the percentages of hybridizing phage are displayed relative to the consensus map. The bottom of the chart includes the percentages of phage that hybridized to each probe by itself.

4.7 probe occur (clones D88–90, F201–205 and A8, 9, 11, 12), the DNA sequence homology to 11L is missing. We have not mapped the internal regions of the 4.7-kb fragment in enough detail to know what parts are missing, but it seems possible that a large deletion could remove the rightward half of the 4.7 fragment and the leftmost portion of the 11-kb region. A similar sized deletion could account for the two versions of the element found at the right end (with either 4.5 or 2.7 hybridization adjacent to the 11-kb hybridization). In this case, comparison of *XhoI-EcoRI* maps of the clones (A13, 16, 6, 3 vs A8, 9, 11, 12, 7, 15, Figure 4) indicates that a small region at the rightmost end of the 11-kb fragment and all of the 4.5-kb fragment could have been deleted to bring the 2.7 hybridizing region adjacent to the 11-kb fragment.

Preliminary characterization of the repeat element end points: As mentioned earlier, the ends of the repeat element were defined by continuing the “walk” until inserts in the isolated phage contained unique sequence DNA adjacent to the repetitive sequences. Digests of all of the clones were probed with nick-translated micronuclear DNA to determine the arrangement of repetitive vs “unique” sequences in each clone. At the right end, ten clones that were isolated by their hybridization to the 2.7 fragment all contained unique sequence DNA adjacent to the 2.7 hybridizing region (Figure 6, group B clones). Thus, the *EcoRI* site at the right end of the 2.7 hybridizing

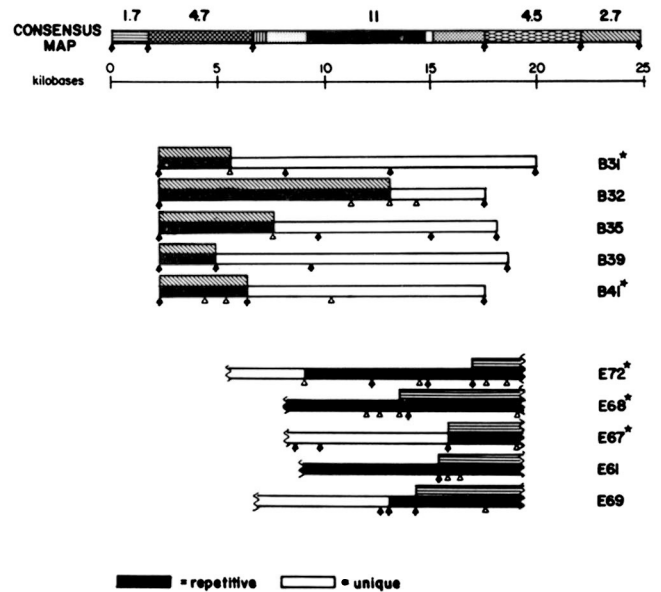


FIGURE 6.—Phage containing the ends of the repetitive element. These two groups of phage represent the ends of the repetitive element. Most of the phage isolated by walking “leftward” from the 1.7 hybridizing region (phage group E) and “rightward” from the 2.7 hybridizing region (phage group B) contain unique sequence DNA (fragments designated without any design). The black areas indicate hybridization to nick-translated micronuclear DNA which detects additional repetitive sequences, with the region hybridizing to the 1.7 or 2.7 probes shown above the micronuclear DNA hybridization. The clones that have stars next to the number contain sequences that hybridize to macronuclear DNA (*EcoRI* = †; *XhoI* = Δ).

region must be very close to the terminus of the element. At the left end, 6 of 10 of the clones isolated by hybridization to the 1.7 probe contain adjacent unique sequences (Figure 6, group E clones). In all of the clones selected with the 1.7 probe, the repetitive sequences extend beyond the 1.7 hybridizing region. When these clones are hybridized to *EcoRI* digested micronuclear DNA, no conserved *EcoRI* fragment is detected by this adjacent DNA. We do, however, detect an increased background smear of hybridization with these clones. Currently, at this end, the terminus of the element is not defined relative to any conserved restriction sites. We found that 5 of 10 and 4 of 10 of the clones hybridizing to the 2.7 and 1.7 ends, respectively, hybridized to macronuclear DNA sequences. Thus, this element is frequently associated with macronuclear destined sequences. Three of these clones show evidence of clustered macronuclear destined sequences: two hybridize to three size classes and one hybridizes to five size classes of macronuclear DNA. It should be noted that most of the clones isolated in groups B and E do not contain an *EcoRI* fragment of the consensus 2.7- or 1.7-kb size. It seems possible that our selection scheme for these groups of phage yields primarily nonconsensus arrangements because of a bias in cloning. Nevertheless, clones in groups B (hybridizing to 2.7 but not 11R) and E

(hybridizing to 1.7 but not 4.7) are present as 0.5% and 1%, respectively, of the phage in the library. Thus we do not believe that these examples of junctions between the element and unique sequence DNA are exceptional. By examining more examples of clones hybridizing to the 1.7 and 2.7 probes without selecting against hybridization to the 11R or 4.7 probes, we may find more examples of clones containing the consensus size *Eco*RI fragments.

We have examined two other possible characteristics of the termini of this element. To look for end to end DNA sequence homology as possible direct or inverted repeats, we labeled each of the fragments mapping rightward from the 2.7 hybridizing region in the group B clones shown in Figure 6 and determined whether they hybridized to phage plaques that hybridized to the 1.7 probe ("left end") on a replica transfer of the micronuclear library. None of these "right end" fragments hybridized to plaques that hybridize with the "left end" probe. (There is also no cross-hybridization between the 1.7 and 2.7 *Eco*RI fragments). As a second possibility, we looked for hybridization to G₄T₄ or C₄A₄ repeats at the ends of the element. In both *Tetrahymena thermophila* and *O. fallax*, macronuclear telomeric repeat sequences have been found at the ends of repetitive elements and are proposed to have arisen during transposition of the elements (CHERRY and BLACKBURN 1985; HERRICK *et al.* 1985). In *O. fallax*, the small size of these telomeric repeats allowed hybridization only with a (G₄T₄)₂ oligonucleotide and not a (C₄A₄)₂ oligonucleotide (HERRICK *et al.* 1985). We used the same oligonucleotides and hybridization conditions to determine whether telomeric repeats were present at the ends of this repetitive element. We probed replica plaque lifts of the *O. nova* micronuclear DNA library and looked for hybridization of either oligonucleotide to a plaque that hybridized to either the 1.7 or 2.7 probes. Although the frequencies of G₄T₄-positive, C₄A₄-negative and G₄T₄-positive, C₄A₄-positive plaques are very similar to that observed in *O. fallax* (HERRICK *et al.* 1985), none of these positively hybridizing plaques corresponded to those with DNA sequence homology to the left or right end probes.

Determining the copy number of the repetitive element: We have quantitated the amount of the repetitive element in the genome by hybridizing different phage clones containing the element to dilutions of *Eco*RI digested micronuclear DNA electrophoresed and blotted side by side with dilutions of digests of each of the page (Figure 7). We used three different clones (A11, A13 and F206, restriction maps shown in Figure 4) in order to include all regions of the element. Comparison of the hybridization of phage insert fragments to the *Eco*RI fragments in micronuclear DNA indicates that the different regions

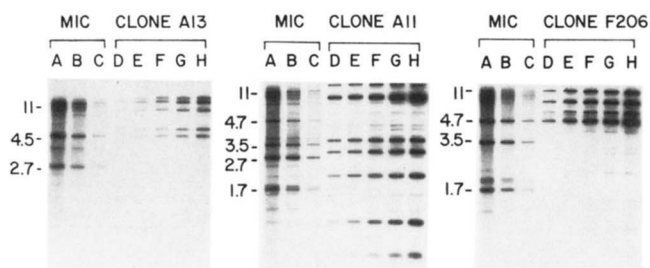


FIGURE 7.—Determining the copy number of the repetitive element. Known quantities of micronuclear DNA and a dilution series of each of three different clones containing the element were digested with *Eco*RI and electrophoresed and Southern blotted together. Each of the three phage clones was nick-translated and hybridized to their respective blots. Lanes A, B and C contain 200, 130 and 50 nanograms (ng), respectively, of micronuclear DNA. Lanes D, E, F, G and H contain 5, 12.5, 25, 50 and 100 ng, respectively of phage clone A13, A11 or F206. Sizes shown to the left of each autoradiogram refer to the prominent bands visible in the micronuclear *Eco*RI digest.

of the element are present in similar amounts. Comparing the intensity of hybridizations of different dilutions yielded values of 9–12% as the amount of the micronuclear genome comprising the element, or a range of 2250–3000 copies per genome (assuming 6×10^8 bp as the genome size, based on the published value for *Oxytricha* sp.) (LAUTH *et al.* 1976).

DISCUSSION

Characterization of randomly chosen genomic clones provides an alternative to reassociation kinetics as a method of determining repetitive sequence content and interspersions in the genome. The random clone method was recently used to characterize the *Arabidopsis thaliana* genome and yielded interspersions data that was not available from reassociation kinetic data (PRUITT and MEYEROWITZ 1986). In ciliates, this is a particularly useful method for determining the interspersions of eliminated and retained (macronuclear) sequences in addition to repetitive and unique sequences. PRUITT and MEYEROWITZ (1986) have discussed the importance of complete genomic representation in the library and of adequate sample size in using this method to study sequence organization. The micronuclear DNA library used in this study was sufficiently large to be representative and in addition has been successfully screened for at least eight single copy genes. Under-representation of only one class of sequences has been noted: clones of sequences homologous to C₄A₄ repeats are present at one-tenth the number expected. However, evidence suggests that these repeats are telomeric in *O. nova* (JAHN 1988) as observed previously for *O. fallax* (DAWSON and HERRICK 1984) and therefore would not be clonable by the usual methods used for producing genomic libraries. We thus believe that the interspersions trends visible in this random sample should be representative

of the genome. On the other hand, our sample of clones is not large enough to make estimates of the amounts of repetitive or unique sequences or the number of macronuclear-destined sequence clusters: 30 clones of 15-kb average size account for 0.075% of the micronuclear genome. We therefore interpret our results as demonstrating three major aspects of micronuclear genome organization, corresponding to the three major classes of clones obtained: (1) a high prevalence of a large (approximately 24 kb) repetitive element, (2) clustering of macronuclear destined sequences, and (3) the occurrence of long stretches of eliminated unique sequence DNA.

The isolation of clones encompassing all of the repetitive element allowed us to determine its copy number and interspersions in the micronuclear genome. At the resolution of the hybridization probes we used to define the element, there is no internal redundancy. Thus, it does not appear to be derived from a block of tandemly repeating units. The element is much larger than any interspersed middle repetitive elements we have seen described (reviewed by BOUCHARD 1982). Because of its size, it would be very interesting to know how this element has become dispersed throughout the *O. nova* genome. In an attempt to determine whether it has any transposable element like properties, we tried to find inverted or direct repeats at the ends of the element. By hybridization with terminal fragments, we were unable to detect any DNA sequence homology between the ends of the element. However, the terminal repeats of transposable elements or retrotransposons can be very short sequences. These would go undetected in our hybridizations. It is also possible that many copies of the element have lost their termini. The fact that most of the terminal clones we isolated are lacking the consensus arrangement of *Eco*RI sites may mean they are lacking the termini. Knowing the structure of the termini will require more detailed mapping and DNA sequence analysis of the element endpoints. As a further investigation into the dispersal of this element, we also plan to determine whether all or portions of it are found in the micronuclear genome of other *Oxytricha* sp.

The frequency of clones containing this repetitive element indicates that it comprises a majority of the interspersed repetitive DNA in the *O. nova* micronuclear genome. This does not mean that this is the only repetitive sequence element in the *O. nova* genome: there could be families of smaller elements present in fewer copies (less than 1000 copies per genome) that would go undetected by our random sample. Two other families have been identified in clones that were selected by hybridization to C₄A₄ repeats (C. L. JAHN, unpublished data). These are both present in lower copy number (100–1000/genome) and have not been

characterized further. We have also identified three families of tandemly repeating sequences that comprise approximately 10% of the genome (C. L. JAHN, unpublished data). These families occur in large tandem arrays and therefore cannot be dispersed to very many locations.

We expected to define an interspersions pattern of eliminated and retained sequences that reflects the band by band breakdown of the micronuclear chromosomes during macronuclear development (PRESCOTT and MURTI 1973). If the clusters of macronuclear sequences were evenly distributed throughout the chromosomal bands and vesicles that are visible in the developing macronucleus, we would expect approximately seven macronuclear sequences per cluster [see KLOBUTCHER *et al.* (1986) for calculations]. The clusters would then occupy stretches of at least 20–30 kb and the eliminated sequences would occur in 200–300 kb stretches between the clusters. To date, macronuclear sequence clusters have not been observed adjacent to long stretches of eliminated unique sequences. We would expect clones that spanned such a region to contain a large stretch of eliminated unique sequence DNA contiguous with a region that hybridizes to a macronuclear sequence at one end of the clone. These would appear different from the other clones by virtue of their hybridization to single macronuclear sequences. It seems quite likely that we have not screened enough clones to find this type of interspersions (only one clone demonstrating interspersions of the repetitive element with macronuclear sequences was obtained in the random selection).

The only evidence we have for a genome-wide interspersions pattern comes from the characterization of the repetitive element. The isolation of clones containing junctions of the element with neighboring unique sequence DNA demonstrates that this element is interspersed with both eliminated unique sequences and macronuclear destined sequences. Our current picture of its interspersions includes the two possibilities shown in Figure 8. Since we have isolated clones containing continuous blocks of up to 25 kb of either clustered macronuclear destined sequences or eliminated unique sequences we assume that either can be adjacent to the element. With this assumption, we can define the sequence organization of regions approximately 45–50 kb in length (Figure 8). Several questions need to be answered to determine how these two patterns are arranged relative to each other: (1) how large are the clusters of macronuclear destined sequences and the stretches of eliminated unique sequence DNA, (2) are long stretches of eliminated unique sequence DNA ever adjacent to macronuclear sequence clusters, and (3) does the repetitive element interrupt clusters or does it border clusters? With respect to this last question, the repetitive element

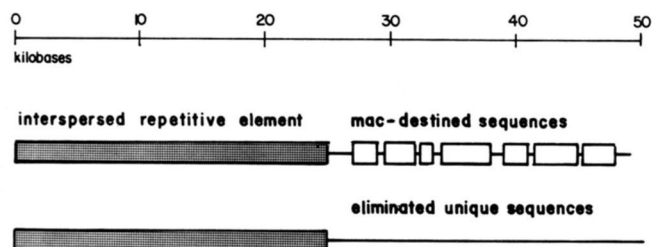


FIGURE 8.—Interspersion pattern of the repetitive element. The clones shown in the previous figure demonstrated that the element is interspersed with both macronuclear destined sequence clusters and with eliminated unique sequence DNA. These two patterns of interspersion are shown. At present we do not know the average size of the clusters or stretches of eliminated unique sequences, although both must be longer than the typical insert contained in λ phage in the micronuclear library.

from *O. fallax* that contains C_4A_4 repeats at its termini (TBE1) has been shown to interrupt a possible macronuclear destined sequence (HERRICK *et al.* 1985, 1987a). In addition, we have identified a repetitive element in *Euplotes crassus* that interrupts the macronuclear sequence clusters (C. L. JAHN, L. NILLES and M. KRIKAU, unpublished data). Thus it seems possible that the *O. nova* element is occurring within macronuclear sequence clusters. One surprising feature of the interspersion of this repetitive element is that it is approximately equally distributed between the two types of arrangements shown in Figure 8. Since the macronuclear sequences represent 10% or less of the micronuclear genome the repetitive element must be preferentially associated with macronuclear destined sequences. We have observed a similar interspersion pattern for the highly prevalent interspersed repetitive element family identified in *E. crassus* (C. L. JAHN, L. NILLES and M. KRIKAU, unpublished data).

The characterization of a random sample of micronuclear clones presented here and the previous studies of clones hybridizing to macronuclear sequences (BOSWELL *et al.* 1983; KLOBUTCHER *et al.* 1986) indicate a segregation of sequences to be eliminated from those that are retained in addition to a low level of interspersion of the eliminated repetitive and unique sequences. These eliminated sequences each occur in very long stretches with similar stretches of DNA carrying clusters of macronuclear destined sequences. Only a small amount of eliminated DNA occurs within these clusters. The organization of the *O. nova* genome differs significantly from what is observed in random samples of micronuclear clones from *T. thermophila*. Analyses similar to those reported here demonstrated that eliminated sequences 1–10 kb in size are dispersed throughout the *T. thermophila* genome, occurring on the average, once every 30–40 kb (KARRER 1983; YAO *et al.* 1984; HOWARD and BLACKBURN 1985). These eliminated sequences have been compared to IESs in hypotrichous ciliates be-

cause they require breakage and rejoining of the chromosome for their removal (YAO *et al.* 1984; AUSTERBERRY and YAO 1987). The end result of the elimination processes in Tetrahymena and Oxytricha are very different. In Tetrahymena, 10–20% of the genome is eliminated (YAO and GOROVSKY 1974), producing approximately 100 molecules averaging 600 kb in size (ALTSCHULER and YAO 1985; CONOVER and BRUNK 1986). Most of the elimination is internal to the resulting molecules. In Oxytricha, 90% or more of the genome is eliminated producing approximately 20,000 molecules averaging 2.2 kb in size (LAUTH *et al.* 1976; SWANTON, HEUMANN and PRESCOTT 1980). The IESs account for less than 1% of the eliminated sequences (RIBAS-APARICIO *et al.* 1987). Thus, most of the eliminated sequences are external to the resulting molecules. The segregation of the eliminated and retained sequences in Oxytricha suggests there are two kinds of eliminated sequences in the micronuclear genome; (1) IESs which require breakage and rejoining, and (2) sequences occurring outside the macronuclear sequence clusters, which would not require rejoining of DNA. The different kinds of sequences could be eliminated by different mechanisms.

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