Micronuclear DNA sequences of *Oxytricha fallax* homologous to the macronuclear inverted terminal repeat

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ABSTRACT

The macronucleus of the protozoan *Oxytricha fallax* is generated from a micronucleus following conjugation. While the micronucleus contains high molecular weight DNA, the macronucleus contains only short linear DNA molecules which all end in the same 20 bp inverted terminal repeat (Ma-ITR). The Ma-ITR was radioactively labeled and purified for use as a probe in hybridizations to micronuclear and macronuclear DNA. Sequences homologous to the Ma-ITR were detected in micronuclear DNA. The copy number of the repeat in the micronuclear genome is approximately that required to encode the macronuclear DNA termini. The micronuclear copies are found embedded in repeated long sequence blocks.

INTRODUCTION

Macronuclear DNA fragments in hypotrichs tested to date are terminated with inverted repeats (1-5). Sequence analysis has shown that the *Oxytricha fallax* macronuclear inverted terminal repeat (Ma-ITR) is similar or identical to that present in other hypotrichs (4,5), and consists of the sequence

(A. Pluta and B. Spear, personal communication). Similar sequences have been found at or near the termini of DNAs in several lower eucaryotes. A dC₄A₂ repeat was initially identified on the rDNA termini of the holotrichous ciliate *Tetrahymena* (6) and more recently on the ends of other macronuclear DNA molecules in the holotrichs *Glaucoma* (7), *Tetrahymena* (8), *Paramecium* and *Colpidium* (M.-C. Yao, personal communication). The extrachromosomal rDNA molecules of *Physarum* and *Dictyostelium* bear dCCCTA and dC₁₋₈T repeats, respectively (9,10).

Following conjugation in ciliates, the macronucleus develops from a mitotic sister of the micronucleus. In hypotrichous ciliates, most of the DNA sequence complexity is lost in this process (11,12). Those sequences that are retained in the macronucleus are found in about 2×10^4 different linear

molecules, each amplified several hundred times. These molecules are small and heterogeneous. The number average sized piece in *Oxytricha fallax* is 2.50 kbp (J. Garrett, unpublished results), which is typical of all hypotrich macronuclear DNAs studied (13).

Very little is known about the function of the ubiquitous Ma-ITR sequence. The repeats may have a role in the replication of macronuclear molecules. Recently it was demonstrated that linear episomes can be maintained in yeast, if they are flanked by the terminal dC₄A₂ containing fragments of *Tetrahymena* macronuclear rDNA (14), suggesting that such sequences may indeed play a role in replication. It has also been proposed that copies of this repeat in ciliates may act as the recognition sites for the excision of macronuclear sequences from the precursor genome (2). This proposal predicts that such sequences should be found in the micronuclear genome adjacent to macronuclear destined sequence blocks. The experiments discussed here demonstrate the presence and examine the context of these repeats in the micronuclear genome.

MATERIALS AND METHODS

Biochemicals

Pronase (Calbiochem, grade B), dissolved at 20 mg/ml in 50 mM sodium acetate, pH 4.7, was incubated at 80°C for 10 minutes. RNase A (Worthington RAF) was dissolved at 10 mg/ml in 0.3 M Tris-HCl, pH 8.1, 0.03 M EDTA, 0.3 M NaCl, and incubated at 93°C for 10 minutes. RNase T₁ (CalBiochem, grade B) was dissolved at 10,000 units/ml in 20 mM sodium acetate, pH 4.75, and incubated at 93°C for 5 minutes. Pronase and RNase solutions were then diluted two fold with glycerol (Eastman Spectro grade) and stored at -20°C. Bovine serum albumin (Pentex) at 10 mg/ml in 50% glycerol was incubated at 56°C for 45 minutes and subsequently stored at -20°C. Restriction enzymes were purchased from and used under conditions prescribed by New England Biolabs and Bethesda Research Laboratories. Mung bean nuclease was purchased from P-L Biochemical. T4 DNA polymerase was a gift from Urszula Hibner and Bruce Alberts, and *Neurospora crassa* nuclease S1 was a gift from Larry Gold. Deoxyguanosine 5'-[alpha ³²P] triphosphate (400 Ci/mmol) was purchased from Amersham.

Culturing of *Oxytricha fallax*

Oxytricha fallax, originally from the Indiana University collection, was obtained from B. Spear (Northwestern University). Subclone 3.5 was isolated (S. Cartinhour, unpublished results) and grown aseptically in 0.1% Cerophyl

extract (Cerophyl Labs, Inc.), using live Chlamydomonas reinhardtii and autoclaved Escherichia coli as food. Cultures were starved for two days (at 4°C to prevent encystment) prior to harvest.

Nuclear isolation

Micronuclei and macronuclei were purified from cells using a procedure like that described elsewhere (11). Micronuclei were further purified by pelleting through 15 ml of buffered 10% (w/v) sucrose, 1000 x g, 20 minutes, at 4°C. This step eliminates small particles and mitochondria from the micronuclei (unpublished results). The final macronuclear preparation contained less than 1 micronucleus per 25 macronuclei, while the final micronuclear preparation contained less than 1 macronucleus per 1500 micronuclei, as judged by microscopic examinations. Both preparations contained less than 1% Chlamydomonas DNA as judged by dot hybridization.

DNA extraction

DNA was extracted from nuclei essentially as described elsewhere (15) but the pH was 8.8. Micronuclear DNA was subjected to sucrose gradient sedimentation to eliminate small DNA, notably, contaminating macronuclear DNA, which could complicate later experiments. Micronuclear DNA was layered over a 15 ml 5-20% sucrose gradient containing 10 mM Tris-HCl, pH 8.1, 100 mM NaCl and 10 mM EDTA. The gradient was centrifuged at 23,000 rpm, 24 hours at 5°C in a Beckman SW27.1 rotor. Ten 1.5 ml fractions were collected and analyzed by gel electrophoresis (Fig. 1). The bottom six fractions were free of small DNA and were pooled. The pooled fractions were dialyzed, made 50% w/w CsCl, 90 µg/ml ethidium bromide, and centrifuged 40,000 rpm, 49 hours at 20°C in a Beckman Ti65 rotor. The DNA collected from this gradient was butanol extracted, dialyzed, and concentrated by ethanol precipitation.

Figure 1. Removal of small DNAs from micronuclear DNA by sedimentation. Micronuclear DNA (ca. 20 µg) was sedimented through sucrose as described in the text. One hundredth of each of 10 fractions was subjected to electrophoresis in 0.7% agarose, stained with ethidium bromide, and photographed. Lane numbers correspond to fraction numbers, fraction 1 corresponds to the top of the gradient. Fractions 5-10 were pooled for use in following experiments. Symbols at the right indicate the size of linear standards. The arrow indicates the mobility of macronuclear rDNA (16), seen most clearly in lane 4.

Preparation of macronuclear inverted terminal repeat (Ma-ITR) probe

T4 DNA polymerase reactions were modeled after those described by O'Farrell (17). Macronuclear DNA (10 μ g) was incubated with T4 DNA polymerase (0.4 units) in 1.7 x reaction conditions (reaction conditions are 67 mM Tris-HCl, pH 8.5, 6.7 mM MgCl₂, 17.7 mM (NH₄)₂SO₄, 0.167 mg/ml bovine serum albumin, and 0.5 mM dithiothreitol) and 500 μ M dCTP and dATP for 60 minutes at 37°C. Under these conditions the 3' exonuclease activity of the enzyme removed the 3' single-stranded tail as well as several base-paired dG and dTMP residues from the double-stranded portion of the inverted terminal repeats. Both dCTP and dATP were present to prevent extensive digestion of regions beyond the ITR (18), although under these conditions the exonuclease did not ordinarily degrade beyond the dG₄T₄ sequence.

Addition of dTTP (300 μ M) and [α -³²P] dGTP (5 μ M) brought the reaction to 1 x reaction conditions (50 μ l) and allowed regeneration of double-stranded termini. After 15 minutes at 37°C, cold dGTP was added (200 μ M) to assure sufficient substrate for the generation of flush ends. The reaction was quenched with EDTA after another 15 minutes of incubation. Four to six dG residues were generally replaced per 3' end.

DNA was precipitated, dissolved in 16 μ l of 20 mM Tris-HCl, pH 8.1, denatured by heating 15 minutes at 97°C, and cooled 5 minutes on ice. The reaction mixture was brought to 180 mM NaCl and the DNA allowed to renature 10 minutes (C_{ot} = 0.6 M-sec) at 25°C.

Single-stranded material was digested with S1 nuclease (9.5 units, 0.5 mM ZnCl₂, 180 mM NaCl, 30 mM sodium acetate, pH 4.7) or mung bean nuclease (270 units, 1 mM ZnCl₂, 180 mM NaCl, 30 mM sodium acetate, pH 4.7), at 20°C in 20 μ l.

Hybridizations

Dot hybridization filters were prepared as described by Kafatos et al. (19) using Schleicher and Schuell BA85 nitrocellulose. Blot transfer from agarose gels to Schleicher and Schuell BA83 or BA85 nitrocellulose was accomplished using the Southern procedure (20). Nitrocellulose strips were washed in 2 x SSC (1 x SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7) and dried at 85°C in a vacuum oven for two hours. Hybridizations were performed at 42°C using a dextran sulfate (9% w/w) accelerated procedure, but the formamide concentration was reduced to 30% (21). Washes were in 2 x SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 minutes, and in 1 x SSC, 0.1% SDS, three times at 50°C for 20 minutes each. An excess of Ma-ITR was used in dot hybridizations. The specific activity of the probe was

estimated to range from 0.6 to 2.0×10^8 cpm/ μ g.

RESULTS

Preparation of macronuclear inverted terminal repeat (Ma-ITR) probe

The strategy for preparing radioactively labeled macronuclear inverted terminal repeat sequences was as described (3) but modified to take advantage of the recently determined sequence of the inverted terminal repeat. The protruding 3' single strand and then a portion of the base-paired dG₄T₄ sequence was removed by the 3' exonuclease activity of T4 DNA polymerase. Addition of dTTP and [α -³²P] dGTP allowed fill-in of the resulting gaps and yielded flush-ended macronuclear DNA with its ITR sequence labeled.

When the end-labeled macronuclear DNA was subjected to electrophoresis in denaturing conditions, two populations of single strands were observed. The vast majority of the label is incorporated into molecules larger than 500 bases (Fig. 2B, lane 1). However, a population of smaller fragments is reproducibly seen in these preparations. These migrate as two clusters of bands ranging approximately from 22 to 18 bases and from 14 to 11 bases (Fig. 2B, lane 1). The structure of these small fragments is unknown, but because of the specific labeling procedure employed, their sequence may be similar to that of the Ma-ITR and they may be associated with the 3' single-stranded ends of macronuclear DNA molecules. Their abundance in macronuclear DNA may be

Figure 2. Analysis of nuclease treated snapback macronuclear DNA by gel electrophoresis. End-labeled snapback macronuclear DNA was prepared as described in the text. Samples were taken prior to addition of and during digestion with single strand specific nuclease, quenched (in 50 mM Na₄P₂O₇, 20 mM Na₃EDTA, 200 mM NaCl, 100 mM Tris-HCl, pH 8.1), denatured in formamide and subjected to electrophoresis through 20% polyacrylamide, 7 M urea gels (22). A. Time course of S1 nuclease digestion. Lanes 1 to 5: 0, 5, 30, 75, and 120 minutes, respectively. B. Time course of mung bean nuclease digestion. Lanes 1 to 5: 0, 5, 15, 30, and 120 minutes, respectively. A long exposure autoradiogram is shown to permit minor bands to be seen.

much greater than indicated here, since such small pieces would likely be selectively lost in our macronuclear DNA preparation procedure.

Radioactive inverted terminal repeats were prepared from the end-labeled macronuclear DNA in two steps. First, the DNA was denatured and allowed to renature as intramolecular single-stranded circles held together by duplex "necks" consisting of the Ma-ITR sequence (1,3). Second, the single-stranded portions of the circles were digested with either S1 or mung bean nuclease. The two nucleases give essentially identical results in all respects. The progress of an S1 nuclease digestion, monitored by electrophoresis in denaturing conditions, is shown in Figure 2A. By 30 minutes (lane 3) the high molecular weight material has been reduced to a series of small fragments. Because this pattern is unchanged after 75 minutes of digestion (lane 4), it probably represents a heterogeneous population of S1 resistant duplexes rather than incomplete digestion products (eventually "nibbling" does occur, 120 minutes, lane 5). The major fragment has been estimated to be 21 bases, and the larger fragments increase in size by one base increments. The sizes of these fragments have been difficult to determine because they do not co-migrate exactly with any 5'P, 3'OH terminated size standards tested. The anomalous mobility of the ITR fragments may be due to their unusual sequence.

After longer exposure, minor bands become visible (Fig. 2B). Nuclease resistant molecules larger than 21 bases are evident in Figure 2B, lane 5. These have been assigned sizes 9, 18, 27, and 36 bases longer than the main fragment (i.e., 30, 39, 48, 56 bases). A similar fragment of 30 bases has also been observed in preparations of *Oxytricha* sp. Ma-ITR (unpublished results). Another major fragment is regularly seen in our Ma-ITR preparations; however, its apparent size varies from 23 to 24 bases depending on the preparation (Fig. 2A). The S1 resistant material in our Ma-ITR preparations should yield the pyrimidine tract 5' pTpTpTpTp* 3'. Pyrimidine tract analysis (23,24) shows ³²P only in inorganic phosphate and a single homogeneous oligonucleotide of the appropriate size (data not shown).

Quantitation of the Ma-ITR homologous sequence in micronuclear DNA

The Ma-ITR probe was used to determine the copy number of the Ma-ITR sequence in micronuclear DNA relative to its copy number in macronuclear DNA. Various quantities of denatured micronuclear and macronuclear DNA were dotted in triplicate on nitrocellulose (19), then hybridized with the Ma-ITR probe. Dots were cut out and the radioactivity of each determined by scintillation counting.

A given mass of macronuclear DNA hybridized to approximately 6 times as

much probe as did an equal mass of micronuclear DNA, as is shown graphically in Figure 3. By expanding the macronuclear DNA abscissa 6.3 times relative to the micronuclear abscissa, a single line can be drawn through the data points.

While a simple interpretation of these results is that Ma-ITR sequences are 6.3 times more abundant per ng in macronuclear than in micronuclear DNA, quantitation of our dot hybridization results should be viewed with caution. First, while the Ma-ITR is highly conserved in the macronucleus, the homologous micronuclear sequences might be heterogeneous. Second, we have no evidence which proves that the micronuclear and macronuclear sequences are identical, although they must be very similar. [Note that they are not detected in *Chlamydomonas* DNA (Fig. 3)]. Third, micronuclear and macronuclear sequences homologous to the probe are probably found in different contexts. While all or most macronuclear copies are on the ends of molecules

Figure 3. Dot hybridization of Ma-ITR probe to micronuclear and macronuclear DNA. Micronuclear DNA and macronuclear DNA were pipetted (10 μ l) in 0.5 cm diameter dots on nitrocellulose in triplicate. Samples were adjusted with the addition of *Chlamydomonas* DNA prior to application, such that all dots contained 50 ng of total DNA. *Chlamydomonas* DNA alone was also applied to the filter (50 ng/dot). Following hybridization with Ma-ITR probe and autoradiography, dots were cut out and analyzed by liquid scintillation counting. Note that the micronuclear and macronuclear points are plotted using separate abscissas differing in scale by a factor of 6.3, calculated using an averaging procedure in which the micronuclear data points were fit to the macronuclear curve. Also shown is cpm/50 ng of *Chlamydomonas* DNA (arrow).

(unpublished results of Bal 31 digestions), micronuclear copies probably are internal, and may be longer or shorter than a single Ma-ITR. Also, the macronuclear copies form snapback structures under some conditions and could, therefore, be unavailable to the Ma-ITR probe in our dot hybridization experiments. Whether this is true of micronuclear copies is unknown and difficult to test, given our present knowledge of their structure and organization. However, we have determined that snapback formation does not interfere with the ability of filter-bound macronuclear DNA to hybridize to the Ma-ITR, using our standard conditions. In a control experiment, equal amounts of filter-bound untreated macronuclear DNA, and macronuclear DNAs in which the single-stranded circle-forming ability had been destroyed, or destroyed and subsequently restored (by the nuclease and fill-in activities of T4 DNA polymerase), hybridized to equal amounts of the Ma-ITR probe (data not shown). Fourth, it is possible that native macronuclear DNA termini include short MA-ITR-like sequences base-paired to the single-stranded tails. If this is the case, these may have been lost in our macronuclear DNA preparation, or if not lost, these might not bind to nitrocellulose.

A trivial explanation for the presence of Ma-ITR homologous sequences in micronuclear DNA is contamination of our micronuclear DNA with substantial amounts of macronuclear DNA. Three facts eliminate this possibility. First, nuclei counts of our micronuclear preparation suggested that less than 5% of the total mass of DNA prepared from these nuclei was macronuclear in origin. Second, because the macronuclear DNA is so small, most of it (especially the smaller, ITR-rich pieces) was removed from the micronuclear DNA by sucrose sedimentation (Fig. 1). Finally, the Ma-ITR homologous sequences in our micronuclear DNA preparation proved to be in a very different context from those in macronuclear DNA (see below).

Context of the Ma-ITR homologous sequence in micronuclear DNA

Native and restriction endonuclease digested micronuclear and macronuclear DNAs were subjected to agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with the Ma-ITR probe (Fig. 4). The hybridization patterns of undigested micronuclear and macronuclear DNA are substantially different. The micronuclear homology is largely in high molecular weight material, although a signal corresponding to contaminating macronuclear rDNA is also evident (indicated by an arrow). The macronuclear DNA homology centers around 2.5 kbp as expected, and very little signal is discernible above the rDNA band.

Results of hybridization to various restriction digests of micronuclear

Figure 4. Hybridization of Ma-ITR probe to restriction digests of micronuclear and macronuclear DNAs. A and B. Restriction enzyme digests of DNAs were subjected to electrophoresis in 0.7% agarose, ethidium bromide stained, and photographed (panel A). Gels were blotted to nitrocellulose and hybridized with Ma-ITR probe. The autoradiogram is shown in panel B. Lanes 1-4 show micronuclear DNA (400 ng/lane): 1, uncut, 2, EcoRI, 3, HindIII, 4, Sau3A. Lanes 5-8 show macronuclear DNA (100 ng/lane): 5, uncut, 6, EcoRI; 7, HindIII, 8, Sau3A. While EcoRI failed to cut the micronuclear DNA, most other digestions can be judged complete by appearance of appropriately sized fragments of rDNA (16). C. Sau3A digested micronuclear DNA (240ng) was subjected to electrophoresis through 1% agarose and then treated as described above. Lane 1, photograph of ethidium bromide stained DNA. Lane 2, autoradiograph following hybridization.

DNA suggest that sequences homologous to the Ma-ITR probe are embedded in large sequence blocks repeated many times in the genome. As an example, results of a HindIII digested micronuclear DNA blot hybridization are shown in Figure 4B, lane 3. Two classes of probe-homologous micronuclear fragments are seen. (Two weak bands at 1.24 and 0.84 kbp represent the terminal HindIII fragments of contaminating macronuclear rDNA - ref. 16.) A minor class of probe-homologous sequences is represented by the light smear centered at 5 kbp. The majority of homologous micronuclear sequences are found in a 9.1 kbp band. The width of the band (ca. 2 kbp) suggests it represents a heterogenous population of similarly sized fragments.

Probed blots of SstI and EcoRI digested micronuclear DNA also display their major homologies as broad bands (8.8 and 6.5 kbp, respectively, data not shown). Nearly all BamHI and PstI fragments homologous to the probe are very large (>20 kbp), despite extensive (presumably complete) digestion as judged by the size distribution of the bulk of the DNA, stained with ethidium bromide (data not shown).

As is the case with HindIII, digestion with Sau3A generates two size classes of fragments homologous to the probe. The larger fragments are in a smear centered at 3.3 kbp (Fig. 4B, lane 4), much larger than expected for random sequence fragments generated by a restriction enzyme with a four base recognition site. The intensity of this hybridization signal is relatively weak when compared with signals observed in hybridizations to uncut and HindIII digested micronuclear DNA (Fig. 4B, lanes 1-3). A second size class of homologous sequences is composed of Sau3A fragments probably too small to be efficiently retained on nitrocellulose. Hybridization to a different blot of Sau3A digested micronuclear DNA demonstrates this small size class more clearly (Fig. 4C, lane 2). We suspect in both cases that most of the small fragments, which may comprise the major class of probe-homologous Sau3A fragments, were lost during blotting and are still not detected.

DISCUSSION

The inverted terminal repeat in macronuclear DNA

In several hypotrichs all macronuclear pieces share the same 20 base pair inverted terminal repeat. Our results indicate that many individual macronuclear pieces in *Oxytricha fallax* actually have repeats longer than the common 20 base pair repeat. Previously it was shown that most macronuclear fragments in *Oxytricha sp.* have 23 bp repeats (3). Presumably, the 20 bp common repeats are extended on most macronuclear DNA molecules by one or a few internal base pairs which are unique to a given fragment or population of fragments. Consistent with our results, of those macronuclear fragments which have been sequenced, one (an actin gene in *O. fallax*) has a 22 bp ITR (26), while two others in *O. nova* have repeats of 20 and 23 base pairs (5).

A number of larger molecules (30, 39, 48, 57 bases) were seen reproducibly in our Ma-ITR preparations. The fact that these formed duplexes in the snapback renaturation procedure suggests that they are found as inverted repeats in the genome, although highly repeated sequences might form interstrand duplexes under these conditions.

The presence of Ma-ITR homologous sequences in the micronuclear DNA

Our results demonstrate that the Ma-ITR, or a sequence quite similar to it, can be found in the micronuclear genome. Dot hybridization has shown that macronuclear DNA hybridizes to about 6 times as much Ma-ITR probe as does an equivalent mass of micronuclear DNA. This number would be consistent with there being an equivalent copy number of the repeat in each genome if the micronuclear genome were about 6 times larger than the macronuclear genome. Available data indicate the micronuclear genome size is about 9 times that of the macronucleus, suggesting that about 60,000 Ma-ITR equivalents exist per micronuclear genome (27). However, we would like to reemphasize that caution should be exercised in any quantitative interpretation of the dot hybridization data. We feel the major significance of these results is that Ma-ITR homologous sequences do exist in high copy number in the micronuclear genome.

What is the function of the micronuclear sequences homologous to the Ma-ITR probe? While these sequences are identical or similar to the Ma-ITR sequences, there is no evidence that they play a role in the generation of the macronuclear termini. The macronuclear inverted terminal repeats could, for example, be synthesized de novo during generation of the macronuclear molecules from the precursor genome. There could also be a small number of master copies of the Ma-ITR in the micronuclear genome among many homologous sequences which play no part in macronuclear development. In either case the micronuclear sequences may serve a role in the function of micronuclear chromosomes per se. Perhaps the two populations of HindIII and Sau3A Ma-ITR homologous restriction fragments reflect functionally different populations of probe-homologous sequences.

Previously, the Ma-ITR sequences have been assumed to exist in micronuclear DNA and serve the role of recognition sites for the simple excision of the macronuclear pieces, complete with their ITRs, from the micronuclear DNA. Our dot hybridization values are roughly consistent with this view. However, we are aware of three instances in which Ma-ITR probes have failed to hybridize to micronuclear DNA fragments carrying putative junctions between macronuclear destined sequence blocks and adjacent non-retained sequences; that is, by this test there do not appear to be full length Ma-ITR sequences in place at the ends of these macronuclear sequences in their micronuclear form. In 0. fallax we have probed a micronuclear fragment which appears to carry the ends of two adjacently-encoded macronuclear fragments and a short non-retained stretch between (we could have

detected one complete Ma-ITR copy per 20 copies of the fragment, D.D., G.H., and R. Myers, unpublished results). Similar results were obtained in another hypotrich, *O. nova* (27), and in the holotrich *Tetrahymena* (M.-C. Yao, personal communication). It therefore appears that the Ma-ITR homologous sequences we detect in micronuclear DNA might not act as sites for the excision of macronuclear destined blocks. If these Ma-ITR homologous sequences are involved in the generation of the Ma-ITRs, a rearrangement mechanism might be required to place them adjacent to the "bodies" of macronuclear pieces.

The context of Ma-ITR homologous sequences in micronuclear DNA

Blot hybridization results have led us to conclude that the majority of micronuclear Ma-ITR homologous sequences are embedded in copies of a long sequence block which is repeated in the micronuclear genome. We will devote the remainder of the Discussion to the possible structure of these repeated long sequence blocks. That the repeating units are quite large we deduce from the fact that most probe-homologous sequences are found in 20 kbp or larger fragments refractory to BamHI or PstI digestion. These large fragments must contain all of, or at least the portions of, the major size classes of HindIII, SstI, EcoRI, and Sau3A fragments that are homologous to the Ma-ITR probe. It should be noted that the probe-homologous BamHI and PstI fragments are large enough to contain multiple copies of the HindIII, SstI and EcoRI fragments, which in turn are large enough to contain multiple Sau3A fragments.

In each case examined, probe-homologous restriction fragments were not distributed as the bulk of ethidium bromide stained DNA. Two types of size distributions of Ma-ITR homologous restriction fragments were observed: a cluster of similarly sized but heterogeneous restriction fragments (HindIII, SstI, EcoRI), or a population of large fragments refractory to restriction enzyme digestion (PstI, BamHI). The dearth of the latter type of restriction sites could reflect a number of different possible characteristics of the repeated long sequence block: restriction site methylation, eccentric base composition (e.g., AT-rich), asymmetric base composition of the two strands, or a sequence structure composed predominantly or exclusively of a large number of copies of a simple sequence. Note that a long block of tandem repeats of the Ma-ITR sequence $-(dC_4A_4)_n$ - would have both the latter characteristics.

The repeated long sequence block might consist of tandem $(dC_4A_4)_n$, bounded by PstI and BamHI sites in (non-repeated?) flanking sequences. However, this seems unlikely because such a $(dC_4A_4)_n$ block does not itself contain recognition sites for any known restriction enzyme, and the repeated long sequence block must have a number of internal restriction sites (HindIII,

etc.). At the other extreme, the repeated long sequence block might contain the equivalent of only one copy of the Ma-ITR sequence. However, the following argument suggests that there are at least a few and perhaps many copies of the Ma-ITR homologous sequence on each 9.1 kbp HindIII fragment. We have calculated that there are of the order of 60,000 copies of the Ma-ITR homologous sequence per micronuclear genome (27). If these are distributed, only one per each repeated long sequence block, or even only one per each Ma-ITR homologous 9.1 kbp HindIII fragment, then we calculate that the total micronuclear DNA should reside in these 9.1 kbp fragments. This is clearly not the case (Fig. 4A, lane 3). The above argument and a preliminary analysis of cloned Ma-ITR homologous HindIII fragments lead us to feel that the repeated long sequence block consists of an intermediate number of copies of Ma-ITR-like sequence in addition to other sequences of unknown character.

It is interesting to note that the HindIII, SstI and EcoRI Ma-ITR homologous populations of fragments share in common the feature of limited size heterogeneity. This heterogeneity could be due to variability in the number of repeats of the Ma-ITR homologous sequence on the various copies of the repeated long sequence block. Size heterogeneity is a feature of the terminal fragments of the *Tetrahymena* macronuclear rDNA (6) and occurs at the rDNA termini in other lower eukaryotes (9,10).

We would like to close by speculating that Ma-ITR homologous repeated long sequence blocks are located at the telomeres of micronuclear chromosomes. One notion arising from the recent work of Szostak and Blackburn (14) on the *Tetrahymena* macronuclear rDNA termini is that macronuclear pieces might be viewed as miniature chromosomes, their ITRs serving as telomeres. It seems reasonable, therefore, that Ma-ITR homologous sequences might be found at micronuclear telomeres.

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27. The *O. fallax* G₁ micronucleus contains 1.1 pg of DNA (A. Pluta and B. Spear, personal communication). Hypotrich micronuclei are generally assumed to be diploid (11), making the *O. fallax* micronuclear genome 5.0×10^8 bp. While the *O. fallax* macronuclear genome size has not been measured, values for 5 other hypotrichs fall within a tight distribution around 5.6×10^7 bp (standard deviation, 1.6×10^7 bp) as determined by kinetic complexity (11, 25). If the *O. fallax* macronuclear genome is 5.6×10^7 bp, its micronuclear genome is 9 times as large ($5.0 \times 10^8 / 5.5 \times 10^7$). Thus, our dot hybridization data suggest that there are roughly equivalent numbers of the repeats in each genome. To calculate the apparent copy number of Ma-ITR equivalents per micronuclear genome, we assume the above micronuclear and macronuclear genome sizes, a number average macronuclear piece size of 2500bp, each piece with 2 Ma-ITR copies, and the relative weight ratios of Ma-ITR in macronuclear vs. micronuclear DNA of 6.3 (Fig. 3). The number of kinds of macronuclear pieces per macronuclear genome is thus $5.6 \times 10^7 / 2500 = 22,000$, and the number of Ma-ITRs per macronuclear genome is 44,000. The number of Ma-ITR equivalents per micronuclear genome is thus $(9/6.3) 44,000 = 63,000$.
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