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Rare Internal C_4A_4 Repeats in the Micronuclear Genome of *Oxytricha fallax*

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Approximately 20,000 different short, linear, macronuclear DNA molecules are derived from micronuclear sequences of *Oxytricha fallax* after conjugation. These macronuclear DNAs are terminated at both ends by 20 base pairs of the sequence 5′-dC_4A_4-3′. Sequences homologous to this repeat ($C_4A_4^+$) are also abundant in the micronuclear chromosomes, but most reside at their telomeres. Here we show that nontelomeric C_4A_4 clusters of 20 base pairs or longer exist in only a few hundred copies per micronuclear genome. This demonstrates that nearly none of the 20,000 sequence blocks of micronuclear DNA destined to be macronuclear DNA molecules can be flanked by full-length (20-base pair) C_4A_4 clusters, and therefore C_4A_4 repeats must be added to most, if not all, macronuclear telomeres during macronuclear development. Six internal micronuclear $C_4A_4^+$ loci were cloned, and their structural relationships with macronuclear and micronuclear sequences were examined. The possible origins and functions of these rare, micronuclear internal C_4A_4 loci are discussed.

The macronucleus of *Oxytricha fallax*, a hypotrichous ciliated protozoan, develops from a mitotic sister of the micronucleus. Most of the sequence complexity of the micronuclear genome is lost in macronuclear development (1, 9, 15). Those sequences which are retained in the mature macronucleus are highly amplified and in the form of ca. 20,000 different short, linear DNA molecules. (The macronuclear DNA kinetic complexity is likely to be near 5×10^7 base pairs [bp] [9]; the number average size of native *O. fallax* macronuclear DNA is 2.50 kilobase pairs [kbp] [J. Garrett and G. Herrick, unpublished data].) (For a recent review of macronuclear development, see reference 20).

The macronuclear DNA molecules of *O. fallax* are terminated at their 5′ ends with 20 bases of C_4A_4 repeats, and at their 3′ ends with 36 bases of G_4T_4 repeats, 16 of which are in the form of a single-strand tail (19). Extrapolation from sequence analyses of two specific cloned micronuclear regions suggests that these repeats must be added to all macronuclear DNAs during macronuclear development. Klobutcher et al. (14) compared an *Oxytricha nova* macronuclear sequence with a micronuclear sequence which is almost certainly its precursor. Aside from three small micronuclear insertions, the two sequences are identical throughout the length of the macronuclear DNA; however, the correspondence ends abruptly at the base pair adjacent to the macronuclear C_4A_4 repeats. Similarly, the micronuclear rDNA locus of *Tetrahymena* has only a single C_4A_2 repeat at or near its end, whereas the mature macronuclear rDNA derived from it is terminated with 20 to 70 repeats (3, 13). These two cases must be representative of the development of nearly all macronuclear telomeres in *O. fallax*, since, as our results show, full-sized internal C_4A_4 blocks (i.e., >20 bp) are rare in micronuclear DNA. This leaves to be explained the roles of the rare internal C_4A_4 loci, six of which we cloned and characterized.

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MATERIALS AND METHODS

Culturing of *Oxytricha* and isolation of nuclei. *O. fallax* (subclone 3.5) was grown, and micronuclear and macronuclear DNAs were purified as described elsewhere (9).

Library construction and screening. A library was constructed by inserting size-selected large *Sau*3A fragments of micronuclear DNA into the *Bam*HI site of pBR322 (4). A complete *Sau*3A digest of micronuclear DNA (0.5 μg) was pooled with *Bam*HI-digested pBR322 (4.0 μg) which had been previously treated with bacterial alkaline phosphatase (Worthington BAPF; in 20 mM NaCl and 10 mM Tris-hydrochloride [pH 8.1] at 65°C). The pooled DNAs were size fractionated on a Bio-Rad A-150m column in 0.3 M sodium acetate, 10 mM Tris-hydrochloride (pH 7.5), and 1 mM EDTA. Fractions enriched for pBR322 DNA (and similarily sized micronuclear *Sau*3A fragments) were pooled, isopropanol precipitated, and dissolved. The DNAs were ligated with 800 U of T4 DNA ligase (New England BioLabs) in 0.03 ml at 20°C for 15 min and were then diluted to 0.4 ml and incubated for another 45 min. The ligation mixture was used to transform *Escherichia coli* HB101 (6). Colony hybridizations with a probe of G_4T_4 repeats (9) were performed under conditions essentially like those described elsewhere (12), except that the temperature was 63°C. Wash conditions were as described earlier (9).

DNA sequencing. $C_4A_4^+$ restriction fragments from plasmids pSMi 1, 3, 5, and 6 were 3′ end labeled with avian reverse transcriptase (Life Sciences, Inc.), and sequences were determined (18). The radioactively labeled restriction sites for sequencing pSMis 1 and 3 were *Sau*3A sites, and for pSMis 5 and 6 they were *Hin*fI sites. The direction and extent of sequencing are shown in Fig. 1.

Blotting and hybridizations. Blotting to nitrocellulose (23), nick translation of probe DNAs (17), and hybridizations (23) were performed essentially as described elsewhere (9). Hybridizations with nick-translated probes were performed at moderate stringency (24), and hybridizations with the 20-base G_4T_4 probe were performed at a lower stringency (attained by using 30% rather than 50% formamide) to stabilize short duplexes (9).

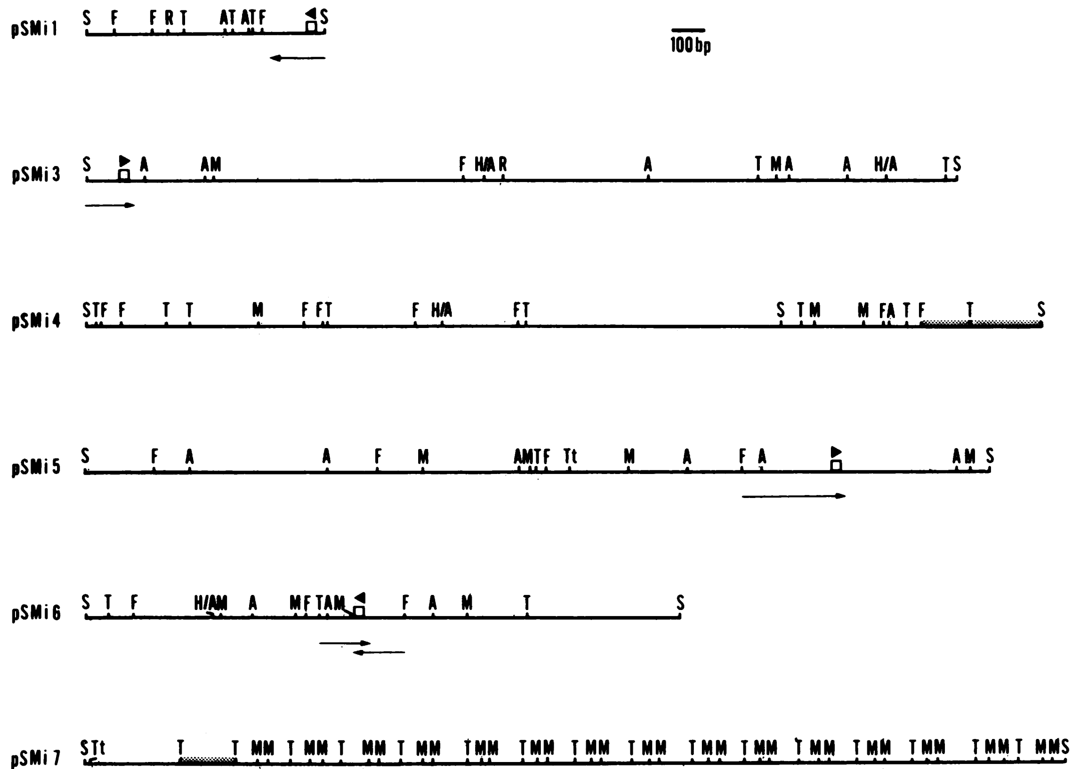

FIG. 1. Restriction maps of $C_4A_4^+$ pSMi inserts. A map of the recognition sites for five restriction enzymes was determined for each insert with the partial digest procedure of Smith and Birnstiel (22). Information derived from complete digests with the same five enzymes, and in some cases with other enzymes, was used to confirm and supplement the partial digest data. Restriction enzymes used were: *AluI* (A), *HinfI* (F), *MnlI* (M), *Sau3A* (S), *TaqI* (T), *HindIII* (H), *EcoRI* (R), and *TthIII I* (Tt). $C_4A_4^+$ subregions were identified by blot hybridization experiments. Stippled bars on the pSMi 4 and 7 maps indicate the smallest restriction fragments to which C_4A_4 homology has been localized. Arrows under maps of pSMis 1, 3, 5, and 6 indicate the direction and extent of sequencing. Open boxes indicate the position of C_4A_4 repeats that were identified by sequencing. Arrowheads over open boxes point toward the 3' side of the C_4A_4 repeats. Note that the pSMi 4 insert is composed of two *Sau3A* fragments; only the rightward one ($C_4A_4^+$) has been studied further.

RESULTS

Frequency of internal micronuclear $C_4A_4^+$ loci. A plasmid library carrying large *Sau3A* fragments of micronuclear DNA was constructed (see above) and screened by hybridization to a radioactive probe of G_4T_4 repeats (9). Six $C_4A_4^+$ clones (pSMis) were identified among ca. 5,500 recombinant plasmids. These clones gave clear, unambiguous hybridization signals, and weaker signals were not observed. Given the frequency of $C_4A_4^+$ clones among the pSMis tested, the average pSMi insert size (1,600 bp [unpublished data]), and the estimated size of the micronuclear genome (5×10^8 bp [A. Pluta and B. Spear, personal communication; see also reference 8]), we estimate that there are ca. 400 such $C_4A_4^+$ loci per genome. A similar estimate of 500 internal $C_4A_4^+$ loci per genome was deduced from a screen of a bacteriophage lambda library carrying partial digest *Sau3A* micronuclear DNA inserts (constructed by S. Cartinhour; unpublished).

$C_4A_4^+$ subregions of pSMis 1, 3, 4, 5, 6, and 7. Restriction maps of the inserts of the six pSMis were prepared (Fig. 1), and $C_4A_4^+$ subregions were localized by Southern blot hybridization with the G_4T_4 probe. In each case, the homology could be localized to a single restriction fragment 100 to 600 bp long (Fig. 1).

The nucleotide sequences of $C_4A_4^+$ regions of four of the pSMis were determined. pSMis 1, 3, 5, and 6 were found to contain 36, 20, 20, and 40 bp of C_4A_4 repeats, or, more exactly, $(C_4A_4)_4C_4$, $(C_4A_4)_2C_4$, $(C_4A_4)_2C_4$, and $A_4(C_4A_4)_4C_4$, respectively. Although pSMis 4 and 7 were not analyzed, each must also contain a cluster of C_4A_4 repeats or some very closely related sequence.

Homology of pSMi inserts to micronuclear and macronuclear DNA. Hybridizations of pSMi insert probes to micronuclear and macronuclear DNA were performed for determinations of the approximate micronuclear copy number of the cloned sequences and the relationship of each to macronuclear DNA. pSMis 4 and 7 failed to hybridize detectably to macronuclear DNA (Fig. 2A and B, lanes 3 and 4) and carry members of moderately and highly repetitious micronuclear sequence families (Fig. 2, lanes 2). Other experiments (not shown) have localized the most repetitious portion of pSMi 4 to the $C_4A_4^+$ *HinfI-Sau3A* fragment. In pSMi 7, sequences to the left of the $C_4A_4^+$ *TaqI* fragment hybridize to *Sau3A* fragments of a variety of sizes (Fig. 2B), and sequences to the right of the $C_4A_4^+$ *TaqI* fragment hybridize to a micronucleus-limited family of highly repetitious, tandemly repeated sequences whose *Sau3A* fragment sizes are integral multiples of 180 bp (D. Dawson, B. Buckley, S. Cartinhour, R. Myers, and G. Herrick, Chromosoma, in press). Because internal C_4A_4 repeats associated with members of micronucleus-limited repetitious families have been described in *Tetrahymena* (7, 27), the nature of the pSMi 4 and 7 loci was not surprising, and they were not characterized further.

pSMis 1, 3, 5, and 6 are different from pSMis 4 and 7 in two respects. First, each shows strong homology to a single

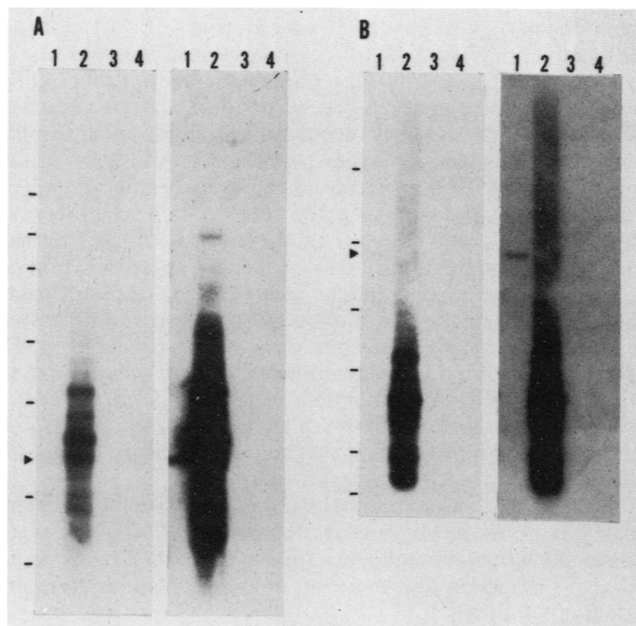

FIG. 2. Hybridization of micronuclear and macronuclear DNA with pSMis 4 and 7. pSMi DNA (with 2 μg of salmon sperm carrier DNA), micronuclear DNA, and macronuclear DNA were digested with *Sau*3A, subjected to gel electrophoresis, blotted to nitrocellulose, and hybridized with radioactively labeled portions of pSMi 4 (rightward *Sau*3A fragment) or pSMi 7 (leftward *Sau*3A-*Taq*I fragment; see Fig. 1). Lightly and heavily exposed autoradiograms for each experiment are shown. The arrow to the left of lane 1 in each panel indicates the *Sau*3A insert fragment. Dashes to the left of each panel indicate the mobilities of size standards: from top to bottom, 6.6, 3.3, 2.0, 1.3, 0.62, and 0.31 kbp. Panel A shows hybridization of the pSMi 4 fragment to the following, by lanes: 1, *Sau*3A pSMi 4 (11 pg); 2, *Sau*3A micronuclear DNA (2 μg); 3, *Sau*3A macronuclear DNA (0.4 μg); 4, native macronuclear DNA (0.4 μg). Panel B shows hybridization of the pSMi 7 fragment to the following, by lanes: 1, *Sau*3A pSMi 7 (5 pg); 2, *Sau*3A micronuclear DNA (2 μg); 3, native macronuclear DNA (1.1 μg); 4, *Sau*3A macronuclear DNA (1.1 μg).

size class of native macronuclear DNA (Fig. 3A to D, lanes 3 and 4). In three cases, the homology resides on a single size of macronuclear DNA *Sau*3A fragments. However, pSMi 5 hybridizes to two different *Sau*3A fragments (990 and 470 bp; Fig. 3C, lane 3) and to two different *Pvu*II fragments (see Fig. 6). Second, unlike pSMis 4 and 7, pSMis 1, 3, 5, and 6 are free of moderately and highly repetitious sequences (with the exception of C_4A_4 repeats). Instead, each hybridization shows one or a few bands in *Sau*3A-digested micronuclear DNA (Fig. 3, lanes 2).

Relationship between macronuclear-homologous sequences and C_4A_4 repeats on the pSMi 1, 3, 5, and 6 inserts. Because macronuclear DNAs are terminated with C_4A_4 repeats, it was of interest to determine whether the micronuclear sequences cloned in the pSMis resembled the terminal portions of their respective homologous macronuclear DNAs. The pSMi sequences homologous to macronuclear DNA might be expected to begin at, and extend in the 3′ direction from, the C_4A_4 repeats, as is the case in macronuclear DNA.

With pSMis 1 and 3, it was not possible to determine whether sequences from the "wrong" (5′) side of the C_4A_4 repeats hybridized to macronuclear DNA, since in both plasmids nearly the entire insert lies 3′ to the C_4A_4 repeats (Fig. 1). However, in pSMis 5 and 6 the repeats are more internal, so there are hundreds of base pairs on each side of the repeats. In the case of pSMi 6, the *Hin*fI-*Sau*3A fragment to the 5′ side of the C_4A_4 repeats displays no homology to macronuclear DNA, but all tested restriction fragments 3′ to the C_4A_4 repeats do hybridize to the macronuclear DNA (data not shown). Results obtained with pSMi 5 are also consistent with the model that the macronuclear homology is 3′ to the C_4A_4 repeat: the (5′) *Sau*3A-*Tth*III I fragment exhibits no homology to macronuclear DNA, but the C_4A_4-containing *Hin*fI-*Sau*3A fragment does (data not shown).

Restriction mapping analyses comparing pSMis 1, 3, 5, and 6 and their homologous macronuclear DNAs. Two further tests of the relationship of pSMi sequences and homologous macronuclear sequences were performed with restriction mapping experiments. One test shows whether the pSMi and its homologous macronuclear DNA share a region which is colinear by comparing the sizes of nonterminal macronuclear DNA restriction fragments with their homologous pSMi restriction fragments. The second test shows whether terminal restriction fragments from the homologous macronuclear DNA are of the size predicted if the macronuclear DNA and the pSMi are colinear, beginning at the C_4A_4 repeats and extending in the 3′ direction. Both tests were performed with pSMis 1 and 6 and their homologous macronuclear DNAs, and the pSMi 6 experiment is shown in Fig. 4. pSMi 6 restriction fragments comigrate with homologous macronuclear DNA restriction fragments, suggesting that pSMi6 and its homologous macronuclear DNA are colinear. In the second kind of experiment, it was shown that *Hin*dIII (Fig. 4) and *Sau*3A (Fig. 3D) digests of macronuclear DNA yield fragments which are homologous to pSMi 6, and of the size expected if they extend from the macronuclear DNA terminus to internal *Hin*dIII or *Sau*3A sites predicted by the pSMi 6 map. The probable relationship between the pSMi 6 insert and the homologous macronuclear sequences is shown in Fig. 5A.

Similar results (not shown) were obtained in a comparison of pSMi 1 and its homologous macronuclear DNA, suggesting that the DNAs are colinear beginning at the C_4A_4 repeats, although the pSMi insert appears to contain ca. 20 bp that are not found in the macronuclear DNA (Fig. 5B). These additional base pairs must be in the leftward *Sau*3A-*Hin*fI fragment, since the pSMi 1 and macronuclear *Hin*fI fragments comigrate whereas the pSMi 1 *Sau*3A-*Alu*I fragment appears slightly larger than that from the macronuclear DNA. This extra 20-bp region may or may not be analogous to micronucleus-limited sequences interrupting macronuclear DNA-homologous blocks observed by others (8, 14, 28).

A comparison of nonterminal restriction fragment sizes has not been possible for pSMi 5 and its homologous macronuclear DNA, due to the limited number of known restriction sites 3′ from the C_4A_4 repeats. However, the second kind of test was performed. The length of the shorter of the two pSMi 5 homologous *Sau*5A fragments (470 bp) is similar to the estimated distance from the pSMi 5 C_4A_4 repeats to the *Sau*3A site in the 3′ direction (450 bp). It is possible that pSMi 5 carries a region colinear with the terminal portion of at least one of its homologous macronuclear DNAs.

No colinear region has been observed in the examination of pSMi 3 and its homologous macronuclear DNA. For instance, the pSMi 3 insert hybridizes to a 500-bp *Sau*3A macronuclear DNA fragment (a very weak signal in Fig. 3B, lane 3). This is much shorter than the distance, on pSMi 3, from the C_4A_4 repeats to the *Sau*3A site in the 3′ direction (2,800 bp). Restriction fragments 1,500 bp apart on the pSMi

3 insert hybridize to the same size *Sau*3A macronuclear fragment (data not shown), so it appears that this homology is scattered across the insert in at least two places rather than being confined to one place.

BAL 31 nuclease analyses of the relationship between the pSMi 1, 3, 5, and 6 inserts and their homologous macronuclear DNAs. The results described above suggest that the inserts of pSMis 1, 5, and 6 may each have a region, beginning at the C_4A_4 repeats, which is colinear with a region at the end of a macronuclear DNA. This was tested by determining whether macronuclear sequences homologous to these pSMis were sensitive to digestion with BAL 31 nuclease (which acts as if it were a double-strand exonuclease [11]). Macronuclear DNA was digested with BAL 31, cut with an appropriate restriction enzyme, subjected to agarose gel electrophoresis and blotted to nitrocellulose, and then hybridized with a portion of pSMi 1, 5, or 6 (Fig. 6). The ethidium bromide-stained gel (Fig. 6A) shows that the macronuclear DNA molecules became progressively smaller with increasing BAL 31 digestion and that most of the sequences were not removed by the digestion. The rate of BAL 31 digestion was ca. 20 bp per end per min on rDNA (Fig. 6A) and 30 to 50 bp per end per min on DNAs homologous to pSMis 5, 1, and 6 (Fig. 6B, C, and D, respectively). Two kinds of evidence from these BAL 31 experiments suggest that the pSMi 5, 1, and 6 probes (Fig. 6B, C, and D, respectively) each hybridize to the terminus of a macronuclear DNA molecule. First, in each case, the amount of material removed from the probed region of the macronuclear DNA molecule in the 0- to 10-min interval is approximately the same as in the 10- to 20-min interval (very weak signals could be seen in the 20-min samples on the original autoradiograph), which suggests that the probed sequences were immediately accessible to the nuclease. Second, there was a dramatic decrease in hybridization intensity after the first 10 min of digestion (Fig. 6B, C, D, lanes 2). In each case, the amount of DNA which must be removed to eliminate homology to the pSMi probe corresponds to the distance between the pSMi C_4A_4 repeats and the end of the probe farthest from the repeats (in the 3′ direction).

Similar BAL 31 experiments have been performed with portions of the pSMi 3 insert used as probe. The macronuclear sequences homologous to the probe are resistant to BAL 31 digestion and therefore must be located internally (data not shown).

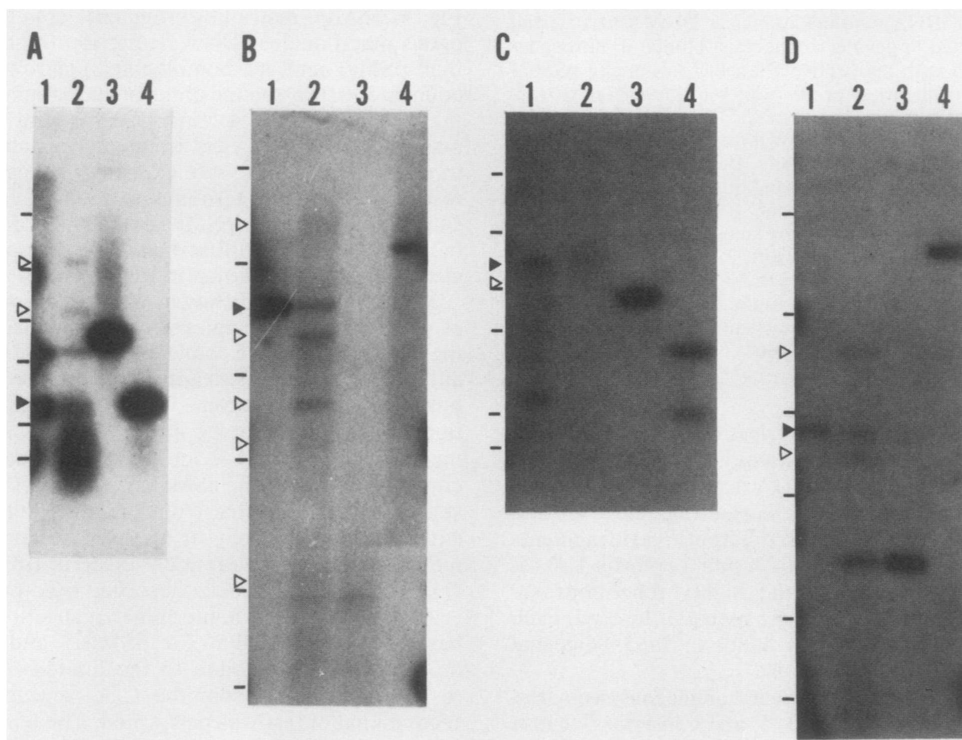

FIG. 3. Hybridization of micronuclear and macronuclear DNA with pSMis 1, 3, 5, and 6. Blots were prepared, hybridized, and displayed as described in the legend to Fig. 2. Arrows to the left of each panel mark positions of *Sau*3A micronuclear DNA bands, with solid arrows marking cloned fragments and open arrows marking uncloned fragments seen in lanes 2 (other bands in lanes 2 are attributable to contaminating macronuclear DNA or pBR322). (A) Lanes: 1, *Sau*3A pSMi 1 (9.1 pg); 2, *Sau*3A micronuclear DNA (0.8 μg); 3, native macronuclear DNA (2.0 μg); 4, *Sau*3A macronuclear DNA (2.0 μg). The probe was the pSMi 1 insert from which 100 bp had been removed at each end with BAL 31 nuclease (this eliminates the G_4T_4 repeats which hybridize to all macronuclear DNA molecules and obscure hybridization to specific macronuclear fragments by the rest of the insert). The smear at the bottom of lane 2 is probably due to pBR322 sequences in the probe which hybridize to the degraded *E. coli* DNA which contaminates the micronuclear DNA; autoclaved *E. coli* is used as food for *O. fallax*. (B) Lanes: 1, *Sau*3A pSMi 3 (30.0 pg); 2, *Sau*3A micronuclear DNA (2.0 μg); 3, *Sau*3A macronuclear DNA (0.4 μg); 4, native macronuclear DNA (0.4 μg). The probe was the entire pSMi 3 insert except for the rightward *Hin*dIII-*Sau*3A fragment (see Fig. 1). (C) Lanes: 1, *Sau*3A pSMi 5 (23.5 pg); 2, *Sau*3A micronuclear DNA (0.8 μg); 3, native macronuclear DNA (2.0 μg); 4, *Sau*3A macronuclear DNA (2.0 μg). The probe was the rightward pSMi 5 insert *Hin*fI-*Sau*3A fragment (see Fig. 1). (D) Lanes: 1, *Sau*3A pSMi 6 (15.0 pg); 2, *Sau*3A micronuclear DNA (2.0 μg); 3, *Sau*3A macronuclear DNA (1.1 μg); 4, native macronuclear DNA (1.1 μg). The probe was the leftward pSMi 6 *Sau*3A-*Hin*dIII fragment (see Fig. 1).

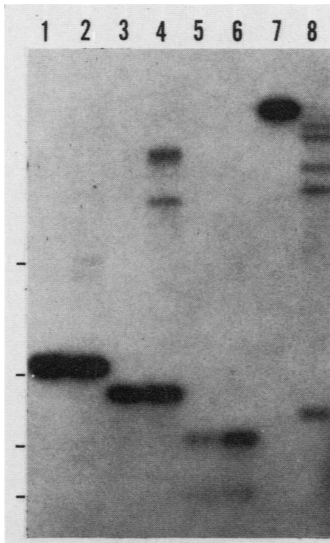

FIG. 4. Size comparison of pSMi 6 and homologous macronuclear DNA restriction fragments. Macronuclear (2.2 μg) and pSMi 6 (0.14 ng plus 2 μg of salmon sperm DNA) DNAs were digested with restriction enzymes, subjected to agarose gel (1.5%) electrophoresis, blotted to nitrocellulose, and hybridized with the leftward radioactively labeled *Taq*I fragment of pSMi 6. Dashes to the left indicate mobilities of fragments of 1.4, 0.67, 0.36, and 0.21 kbp. Lanes 1 to 6 show pairs of macronuclear (lanes 1, 3, and 5) or pSMi 6 (lanes 2, 4, and 6) DNA digested with the same enzyme or enzymes: lanes 1 and 2 with *Taq*I (T), lanes 3 and 4 with *Hin*fI (F), and lanes 5 and 6 with *Sau*3A (S) and Alu (A). Minor bands in lanes 2 and 4 are due to pBR322 fragments contaminating the gel-purified probe. Lanes 7 and 8 show hybridization to native and (partially) *Hin*dIII (H)-digested macronuclear DNA, respectively.

DISCUSSION

One notion about the source of macronuclear telomeres is that each of the ca. 20,000 micronuclear sequence blocks destined to be a macronuclear DNA molecule is bounded by a full complement of C_4A_4 repeats at each end—a full complement being at least 20 bp, and as many as 36 bp, of C_4A_4. Two types of information show that the number of internal C_4A_4 repeats 20 bp or longer is much lower than the number of kinds of macronuclear DNA ends encoded in micronuclear DNA (ca. 40,000). First, although total $C_4A_4^+$ sequences are about as abundant in the micronuclear genome as in a haploid complement of macronuclear DNA (probably fortuitously), most are at the micronuclear telomeres and are rapidly digested from native micronuclear DNA by BAL 31 nuclease (9, 10). Only a minor fraction (<5%) of the micronuclear $C_4A_4^+$ sequences are resistant to BAL 31, and these are probably located internally (10). Second, C_4A_4 clusters of the size found on macronuclear termini (20 bp) were readily detectable in our hybridization conditions (note that pSMis 3 and 5 have 20-bp C_4A_4 clusters) but were found only in a few hundred loci per genome by library screens. These results confirm earlier proposals (5, 9) that most of the ca. 20,000 sequence blocks destined to be macronuclear DNA molecules are not flanked by full-length C_4A_4 clusters (many could be flanked by very short C_4A_4 clusters and not be detected in hybridizations to micronuclear DNA and genomic libraries). This conclusion is consistent with the fact that the two well-characterized micronuclear loci known to give rise to a macronuclear DNA carry only one or zero copies of the macronuclear terminal sequence (13, 14). The implication is that nearly all macronuclear DNAs gain C_4A_4 repeats during macronuclear development. Although the source of the macronuclear repeats is unknown, they might be added by de novo synthesis, or, alternatively, they might come from the micronuclear telomeres or be derived from the few internal C_4A_4 repeats and be added to the macronuclear DNA ends by a rearrangement mechanism.

There are a number of possible explanations of the few internal C_4A_4 repeats we did detect. For example, the structural similarities between the pSMi 1, 5, and 6 inserts and their homologous macronuclear DNAs are consistent with the model that these inserts contain macronuclear progenitor sequences that are only unusual in that they carry large C_4A_4 clusters. The sequences cloned in pSMis 3, 4, and 7 do not exhibit such straightforward relationships to macronuclear DNAs, but we have not eliminated the possibility that they might also be involved in the genesis of macronuclear DNAs. However, it is important to note that although pSMis 1, 3, 5, and 6 contain micronuclear sequences that are homologous to macronuclear DNAs, there is no evidence that any of these is actually macronuclear-destined. This is especially apparent for pSMis 1, 3, and 6, since each hybridizes clearly to multiple micronuclear loci. In each case, the homologous macronuclear DNA might just as well prove to arise from any of the other (noncloned) micronuclear loci.

Since it is apparently unnecessary for macronuclear-destined sequences to be flanked by C_4A_4 repeats, the rare internal repeats may have nothing to do with the generation of macronuclear sequences. Perhaps the simplest explanation for the internal $C_4A_4^+$ sequences is that they are displaced copies of functional repeats. There are two potential sources of $C_4A_4^+$ sequences, the micronuclear and macronuclear telomeres. The macronuclear-homologous sequences embedded in the pSMi 1, 3, 5, and 6 loci may have arisen by the insertion of macronuclear sequences back into the micronuclear genome. Interorganelle exchanges of genetic material are not without precedent (16). Such a model could explain most of what is known about the structure of the pSMi 1, 5, and 6 inserts, since in all three cases the C_4A_4 repeats are adjacent to sequences that are similar to the end of a macronuclear DNA. The short insertion into pSMi 1 might be viewed as a more recent evolutionary event. It is

FIG. 5. Colinearity of the pSMi 1 and 6 inserts and their homologous macronuclear DNAs. Only restriction sites tested for both the pSMis and their homologous macronuclear DNAs are indicated. (A) Restriction map of the left side of the pSMi 6 insert, and the proposed map of its homologous macronuclear (Ma) DNA. (B) Restriction map of the pSMi 1 insert, and the proposed map of its homologous macronuclear DNA. The dotted lines connecting the pSMi 1 and macronuclear maps indicate that the leftward pSMi 1 *Sau*3A-*Hin*fI fragment is ca. 20 bp larger than the homologous macronuclear *Sau*3A-*Hin*fI fragment.

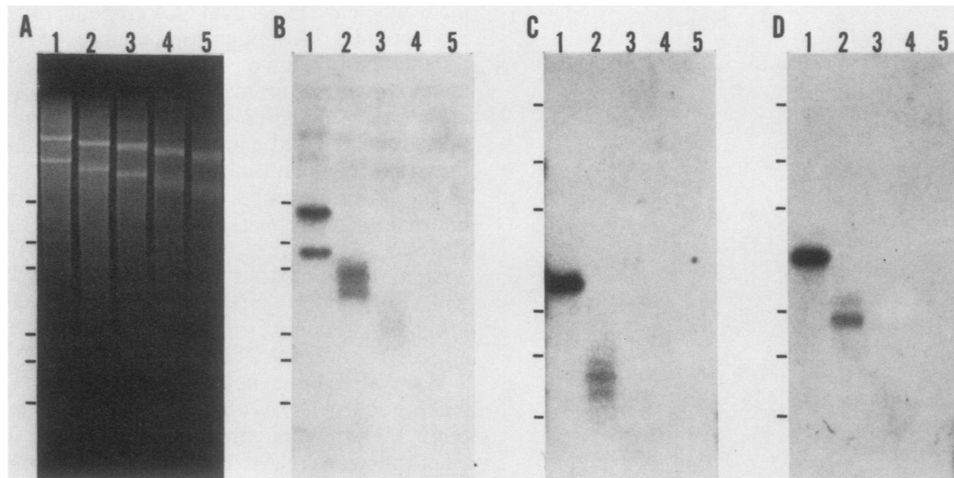

FIG. 6. Effect of BAL 31 nuclease digestion of macronuclear DNA species homologous to pSMis 1, 5, and 6. Macronuclear DNA (60 μg) was digested with 2.5 U of BAL 31 (Bethesda Research Laboratories) in 0.6 ml of the manufacturer's recommended buffer, at 30°C. Samples were removed at 0, 10, 20, 30, or 40 min, quenched with EDTA, phenol extracted, ethanol precipitated, and dissolved. DNA from each time point was then digested with an appropriate restriction enzyme, subjected to electrophoresis through a 2% agarose gel, blotted to nitrocellulose, and hybridized with a nick-translated portion of a pSMi insert. In each panel, lanes 1 to 5 contain macronuclear DNA (3 μg) from the 0-, 10-, 20-, 30-, and 40-min BAL 31 time points, respectively. Dashes to the left of each panel indicate the mobilities of (from top to bottom) 2.0-, 1.4-, 1.1-, 0.6-, 0.5-, and 0.3-kbp size standards. (A) Ethidium bromide-stained gel of *Pvu*II-digested BAL 31 timepoints. (B) Autoradiograph of the blotted gel shown in A, hybridized with the rightward *Hin*fI-*Sau*3A fragment of the pSMi 5 insert (see Fig. 1). The top band represents full-length macronuclear DNA molecules that were not cut by *Pvu*II, and the other band represents the larger fragment of a variant population of molecules that were cut by *Pvu*II. As expected, the full-length molecules, which were degraded from both ends, decrease in size at twice the rate of the smaller fragments, which were degraded from one end (80 versus 40 bp per min). (C and D) *Sau*3A-digested BAL 31 time points hybridized with the pSMi 1 leftward *Sau*3A-*Eco*RI fragment (C) or the pSMi 6 *Hin*dIII-*Taq*I fragment (D) located just to the left of the C_4A_4 cluster (Fig. 1).

more difficult to explain the pSMi 3 locus by this model, since here the C_4A_4 repeats are adjacent to sequences that are homologous to an internal portion of a macronuclear DNA. One possible explanation for the structure of the pSMi 3 locus is that it was created by the insertion of a macronuclear DNA into the micronuclear genome and has subsequently undergone extensive alterations.

This insertion model applies even less readily to the pSMi 4 and 7 loci, which exhibit no homology with macronuclear DNA and carry repetitious micronucleus-limited elements. If the pSMi 4 and 7 loci were created by the insertion of macronuclear sequences into the micronuclear genome, then any homology to macronuclear sequences has since been lost. An alternate explanation for the origin of the $C_4A_4^+$ sequences in the pSMi 4 and 7 loci is that they are displaced copies of micronuclear telomeric $C_4A_4^+$ sequences. Internal C_nA_m tandem repeats have been detected in both *Tetrahymena* (2, 7, 27) and yeasts (25, 26), and it has been suggested that the nicking and amplification processes that involve telomeric copies of such sequences may mediate their dispersal to internal locations (21).

In summary, because the frequency of internal micronuclear $C_4A_4^+$ loci is low, at least most macronuclear telomeres must gain C_4A_4 repeats during macronuclear development. One simple view is that no macronuclear-destined blocks are bounded by hybridization-detectable C_4A_4 blocks, and that those few blocks we do detect are better explained in other ways.

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