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Three Different Macronuclear DNAs in *Oxytricha fallax* Share a Common Sequence Block

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The three members of a cross-hybridizing family of macronuclear DNAs (4,890, 2,780, and 1,640 base pairs) from the protozoan *Oxytricha fallax* have in common a conserved sequence block 1,300 to 1,550 base pairs long. Adjacent to the common block in the two larger DNAs are sequences which are unique to them, whereas the smallest DNA contains few if any additional sequences. The family reappears when the macronucleus is replaced after conjugation and can be detected in another *O. fallax* subspecies. In a random collection of cloned macronuclear DNAs, 6 of 15 hybridize to macronuclear DNA families. This high frequency suggests that families sharing common sequence blocks have an important role in macronuclear function.

Oxytricha fallax, a hypotrichous ciliated protozoan, contains two morphologically and functionally distinct nuclei. The macronucleus in hypotrichs is the major site of RNA synthesis in the cell and is capable of sustaining most, if not all, of the vegetative processes of the cell (2, 15). DNA in the macronucleus exists as short (0.5- to 20.0-kilobase) linear chromosomes, each present in about 1,000 copies; there are probably 20,000 different kinds of macronuclear pieces (12, 34), and most or all of these appear to be transcribed (30; J. M. Heumann, Ph.D. dissertation, University of Colorado, Boulder, 1977). Macronuclear DNA pieces are derived, soon after conjugation, from a copy of the diploid micronuclear (germ line) genome. The developmental pathway which leads from micronucleus to macronucleus involves specific DNA amplification, fragmentation, and sequence elimination (1, 3, 25, 33, 36).

Although it is commonly thought that each kind of macronuclear sequence resides on a single size class of macronuclear DNA, relationships between various macronuclear pieces have been demonstrated by hybridization. Kaine and Spear (21, 22) have detected two *O. fallax* macronuclear DNAs homologous to a yeast actin probe. The members of the actin family are different in size and appear to be divergent in sequence, based on experiments designed to examine the thermal stability of heteroduplexes formed between family members. Lawn et al. (26) also detected cross-hybridizing *Oxytricha* macronuclear DNAs of different sizes but did not analyze the relationships between them. Elsevier et al. (14) observed multiple-sized *Stylonychia mytilus* macronuclear DNAs homologous to a histone gene probe. Finally, Boswell et al. (6) suggested that sequence relatedness might exist between macronuclear DNAs in *Oxytricha nova*.

We have been studying randomly chosen clones of entire macronuclear DNA pieces (pMAs) and have discovered that 6 of the 15 tested hybridize to more than one size class of macronuclear DNA. The three cross-hybridizing members in one such family have been cloned and compared. In contrast to the divergent members of the actin family, the members in this family appear to be related by a highly conserved sequence block at least 1,300 base pairs (bp) long. The smallest member of the family consists almost entirely of these sequences, whereas the two larger members have, in addition, sequences outside the common block which are unique to their size classes. DNAs related in this manner have not been demonstrated previously in ciliates, although such an arrangement is implied by the results of Boswell et al. (6) and has been suggested for the histone gene family in *S. mytilus* (14). This arrangement is reminiscent of the alternatively juxtaposed sequences seen in other systems, where certain common blocks can appear adjacent to different DNA or RNA sequences (see below).

Our findings imply that some sequence blocks in macronuclear DNA exist in a variety of contexts. Such alternative arrangements may have important implications for the way in which these sequences are used in the macronucleus and may help us understand how macronuclear DNAs are derived from their micronuclear precursors.

MATERIALS AND METHODS

Cells and growth conditions. *O. fallax* was cultured as described previously (12). Subclone 3.5 (12) was isolated in our laboratory, and clones 9D1 and 9D2 were obtained from R. Hammersmith (Ball State University, Muncie, Ind.), as was *O. fallax* subspecies 4b. Subclones 3.5, 9D1, and 9D2 are derived from different conjugation events.

DNA purification. Macronuclei were isolated from cells by a procedure similar to that described elsewhere (25). The procedure used to purify DNA from nuclei is described by Swanton et al. (37), but the lysis buffer contained 10 mM Tris-hydrochloride, pH 8.8.

Plasmid DNAs were purified from chloramphenicol-treated cultures as described elsewhere (13).

To purify DNA fragments from agarose gels, plasmid DNAs were digested with restriction enzymes (Bethesda Research Laboratories and New England Biolabs) and subjected to electrophoresis in Seakem ME agarose. Fragments of interest were isolated with NA45 membranes (Schleicher & Schuell Co.), and the procedure was supplied by the manufacturer.

Blot hybridizations. The Southern (35) procedure with Schleicher & Schuell BA85 nitrocellulose was employed. For normal stringency hybridizations, the dextran sulfate accelerated-hybridization procedure of Wahl et al. (38) was used, as were their post-hybridization rinse conditions (50°C in 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium

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citrate], 0.1% sodium dodecyl sulfate). In the melt-off experiments, the same wash schedule was used, but the temperature of the final wash was varied. C_4A_4 hybridization was as described by Dawson and Herrick (12). Blots were sometimes reused, after stripping radioactive probe by washing for 1 h at 65°C in prehybridization solution. Nick-translated probes were prepared by the method of Maniatis et al. (28). C_4A_4 probe was prepared as described in Dawson and Herrick (12).

pMA-transformed *Escherichia coli* clones. The original 15 pMAs were provided by W. Morgan and were constructed as described by Kaine and Spear (21).

The macronuclear DNA library was constructed by using the dG · dC tailing technique of Otsuka (31), as described by Maniatis et al. (27). Native macronuclear DNA was dC tailed and inserted into the dG-tailed *Pst*I site in the beta-lactamase gene of pBR322 to yield chimeric plasmids. *E. coli* χ1976 (9) was then transformed with the plasmid DNA. The cells were allowed to recover from the transformation procedure (27) and then were grown for four generations. Aliquots of the library were frozen in 7% dimethyl sulfoxide (11) at −70°C.

Aliquots of the amplified macronuclear DNA library were plated onto nitrocellulose filters and screened as described by Hanahan and Meselson (18). The probe used to recover pMA83 was a cloned fragment of micronuclear DNA (S. W. Cartinhour and G. A. Herrick, manuscript in preparation). The micronuclear fragment was homologous to all three members of the pMA81 family. pMA82d was recovered by using the nick-translated insert of pMA83 as a probe. After clones had been recovered from the library, plasmids were transfected into *E. coli* HB101 (7).

RESULTS

Detection of macronuclear DNA families. In an analysis of a random collection of 15 pMAs, we discovered that 6 of them hybridized to more than one size class of native macronuclear DNA. This was determined by hybridizing nick-translated pMAs to blots of agarose gels on which had been displayed uncut (native) macronuclear DNA, *Pst*I-digested macronuclear DNA, and *Pst*I-digested homologous plasmid. The pMAs were constructed by inserting whole macronuclear DNA pieces into the *Pst*I site of pBR322 so that the inserts could be liberated by digestion with *Pst*I. The sizes of the macronuclear DNAs detected in these experiments are shown in Table 1.

Nine of the pMAs hybridized, as expected, to only one macronuclear DNA size class. An example of a result like this can be seen in Fig. 1, lane a, in which pMA22 is shown to hybridize to a single band in native macronuclear DNA. The pMA22 insert has the same *Pst*I map as does its homologous macronuclear DNA and is large enough to contain an intact version of it (Fig. 1, lanes b and c). Five of the nine pMAs which hybridize to a single macronuclear DNA size class carry deleted inserts (Table 1); often one or both ends of a macronuclear DNA are deleted when whole macronuclear DNA pieces are cloned.

The remaining six pMAs hybridized to macronuclear DNA families with either two or three members of distinct size (Fig. 1, pMA27 and see Fig. 2, pMA81 and pMA13). The inserts in five of these pMAs comigrate with one of their homologous family members and appear to represent intact versions of them.

The macronuclear DNAs detected by the six family pMAs are in general not the same size (Table 1). To test whether the families detected by pMA11, pMA13, pMA26, pMA27, and pMA81 are mutually exclusive, these plasmids were

TABLE 1. Macronuclear DNA size classes homologous to pMAs

pMA family	Homologous MA DNAs (bp)		
	MA I	MA II	MA III
pMA2[a]	13,500		
pMA6[a]	6,200		
pMA10[a]	6,400		
pMA14[a]	6,300		
pMA15[b]	**7,400**[c]		
pMA18	**1,970**		
pMA19[a]	3,260		
pMA22	**1,770**		
pMA30[b]	**7,400**		
pMA3[a]	2,810	900	
pMA11	6,840	**2,780**[d]	
pMA26	**3,620**	2,660	
pMA27	5,150	**1,310**	
pMA81	**4,890**	2,780[d]	1,640
pMA13	**4,420**	2,880	1,670

[a] pMA insert is smaller than any of its homologous MA DNAs.

[b] pMA15 and pMA30 carry the 7.4-kbp macronuclear rDNA (data not shown).

[c] Boldface, MA DNAs which comigrate with their homologous pMA inserts.

[d] The 2,780-bp members of the pMA11 and pMA81 families have different restriction maps and do not cross-hybridize.

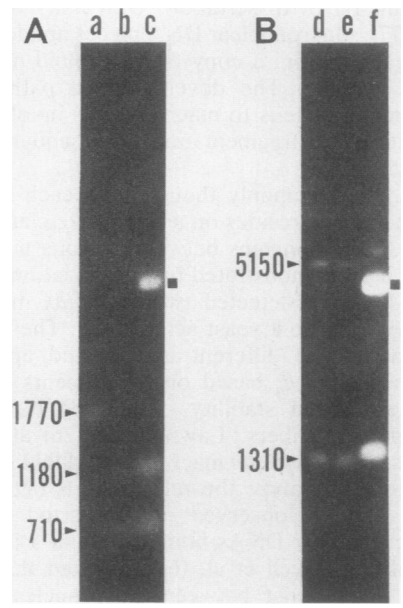

FIG. 1. Hybridization of pMA22 and pMA27 to macronuclear DNA. Each panel represents a separate hybridization experiment. Lanes a and d contain 2 μg of native macronuclear DNA, lanes b and e contain 2 μg of *Pst*I-digested macronuclear DNA, and lanes c and f contain ca. 200 pg of *Pst*I-digested pMA22 or pMA27, respectively. The DNAs were displayed on 0.7% agarose gels, blotted to nitrocellulose, and hybridized with nick-translated pMA22 (A) or pMA27 (B). To the left of the autoradiograms are the sizes in bp of the native and *Pst*I-digested macronuclear DNAs detected in each hybridization. The squares on the right side of the autoradiograms indicate the linear pBR322 DNA liberated by *Pst*I digestion of the pMA. Note that the pMA22 insert and its homologous macronuclear DNA each contain a single *Pst*I site. The uppermost band in lane f is partially digested pMA27.

nick-translated and hybridized sequentially to a single blot of macronuclear DNA (data not shown). We found that the macronuclear DNAs hybridized by a given probe were not hybridized by the other four probes. The frequency with which such families are detected, and the fact that the families are not overlapping, suggests that macronuclear DNA may contain a large variety of unrelated families.

One possible explanation for the ease with which we demonstrate cross-hybridization among macronuclear DNAs is that our hybridization conditions are permissive enough to allow divergent sequences to form stable heteroduplexes. To test whether hybridization to uncloned family members could be eliminated by more stringent washes, pMA13 and pMA81 were hybridized to panels of identical blots of macronuclear DNA, and after hybridization, the blots were washed at various temperatures (Fig. 2). The temperature range used brackets the melting temperature for whole macronuclear DNA from *O. fallax* (34). We were unable to detect obvious preferential loss of probe from any of the family members across a 13°C range of wash temperatures. If a 1% mismatch results in a 1°C decrease in melting temperature (4), we probably could have detected a 5% mismatch in this experiment. We conclude that portions of the pMA81 and pMA13 probes are closely related to the uncloned members of their families.

pMA81 family members are related by a common sequence block. To determine how the members of one macronuclear sequence family are related, we studied the structure of the pMA81 family. First, the structure of each pMA81 family member was determined by analyzing cloned versions. The three members are designated MA I (4,890 bp), MA II (2,780 bp) and MA III (1,640 bp). Second, we compared the structures of the three family members, which allowed us to identify a common sequence block and demonstrate that it is juxtaposed to unique sequences on the two larger family members.

Structures of the pMA81 family members. A macronuclear DNA library was constructed by using the dG · dC tailing technique and screened to obtain additional representatives of the family (see above). Among the plasmids recovered were several with properties that allowed us to identify them as clones of MA II and MA III. pMA83 and pMA83s contain inserts which comigrate with the smallest member of the family. pMA82d is a deleted version of MA II. Restriction maps of the pMA81, pMA82d, and pMA83 inserts, generated with single and double restriction digests, are shown in Fig. 3A. The maps of the inserts were demonstrated to be colinear with the maps of their respective uncloned macronuclear DNAs by a series of comigration tests (Fig. 3B). The regions of MA II not carried on pMA82d were mapped as described in Fig. 4.

Although pMA inserts and their respective MA DNAs in general showed identical restriction maps, several exceptions were noted. First, some MA II molecules were not cut at the leftward *Eco*RV site, whereas all molecules were cut at the rightward site. We do not know the cause of this apparent polymorphism of the leftward site. Second, we were unable to detect any cutting at one of the predicted *Cla*I sites common to MA I, MA II, and MA III (Fig. 3C). Since digestion by *Cla*I is inhibited by methylation at the N^6 position of adenine (29), and this modification is known to occur in *O. fallax* macronuclear DNA (34), we suspect that this particular set of *Cla*I sites has been modified. Finally, an *Sph*I polymorphism common to MA I, MA II, and MA III was detected. The evidence for polymorphism at this site is as follows. MA III: a minority of MA III molecules are cut at this site and pMA83s contains an *Sph*I site at the expected position, but pMA83 does not. MA II: a minority of MA II molecules are cut by *Sph*I at this site even though pMA82d is not (see below and Fig. 3). MA I: a minority of MA I molecules are cut by *Sph*I at this site, whereas the leftward *Sph*I site of MA II is quantitatively cut in the same digest (data not shown). Although the resistance of these MA I molecules to *Sph*I could be conferred by base modification rather than an actual sequence difference, the polymorphisms in MA II and MA III at this position strongly suggest the latter interpretation.

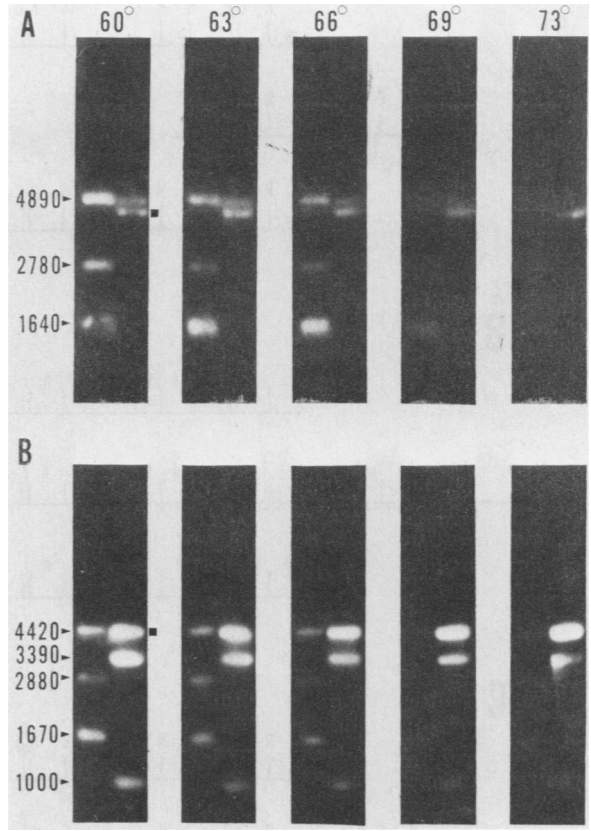

FIG. 2. Thermal stability of cross-homologies within the pMA81 and pMA13 families. A and B show hybridization to the pMA81 and pMA13 families, respectively. In both families the largest member has been cloned intact in the pMA (Table 1). In each experiment, five identical blots were prepared from 0.7% agarose gels on which had been displayed 2 μg of native macronuclear DNA (left lanes) and ca. 200 pg of *Pst*I-digested pMA81 (A, right lanes) or pMA13 (B, right lanes). The blots were hybridized with nick-translated pMA81 (A) or pMA13 (B) and then rinsed at the indicated temperatures (degrees Celsius) (see the text). At the left are the sizes in bp of the macronuclear DNAs and pMA insert fragments detected by the probes; linear pBR322 is indicated by squares on the right. Hybridization to pBR322 persists at 73°C because this DNA has a relatively high dG+dC content. Note that the pMA13 insert contains a *Pst*I site. This same site is present in the largest member (4,420 bp) of the pMA13 family (data not shown).

Relationships between pMA81 family members. Restriction maps of the inserts in pMA81, pMA82d, and pMA83 and their corresponding MA DNAs are aligned in Fig. 3 to demonstrate that the three family members apparently share a common restriction map across a large region. The fact that each pMA hybridizes to all three members of the pMA81 family (see Fig. 2 and 6; data not shown for pMA82d)

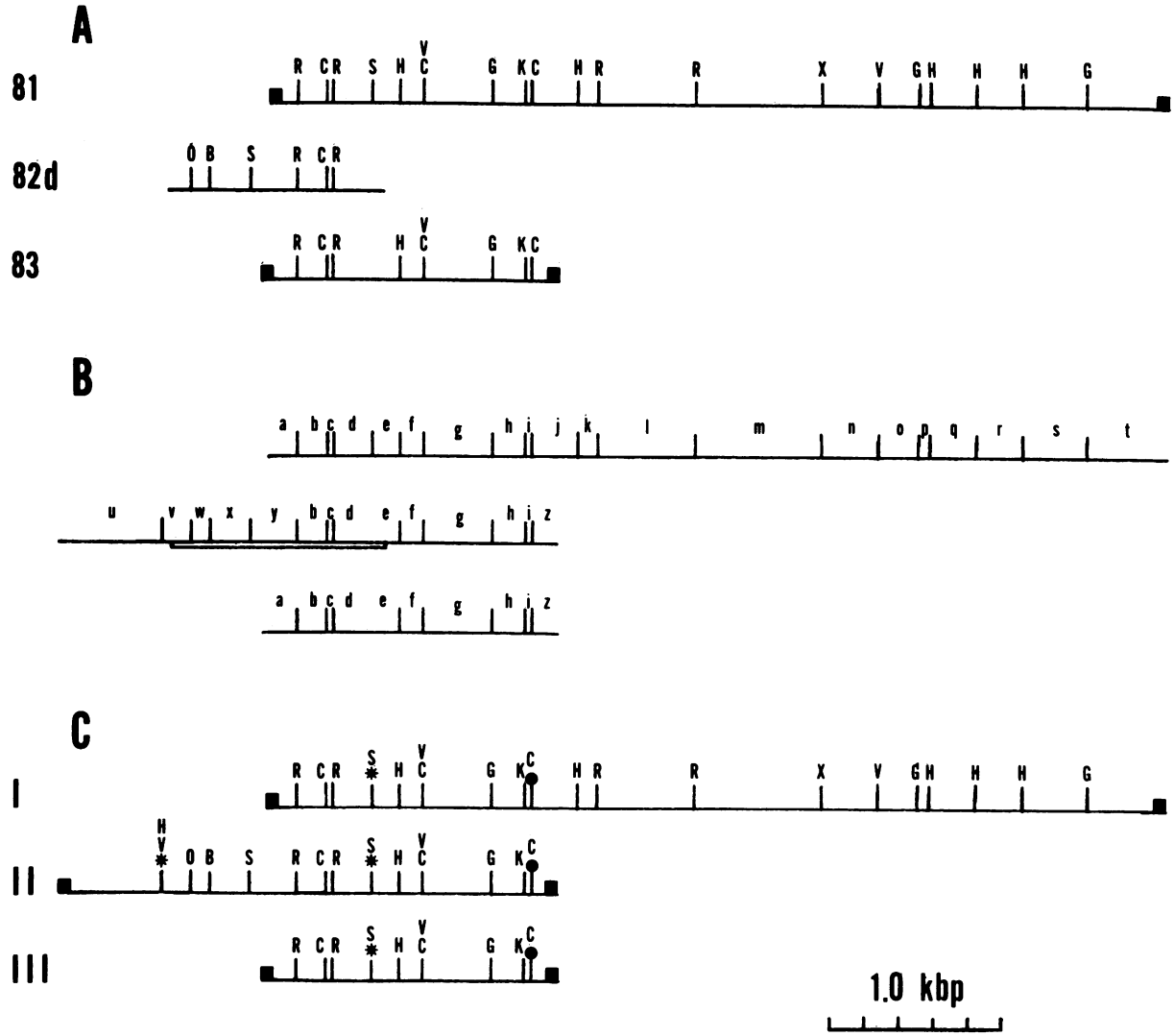

FIG. 3. Restriction maps of pMA81 family members. (A) Restriction maps of pMA81, pMA82d, and pMA83 inserts were prepared with complete restriction enzyme digests analyzed on 0.7 and 1.5% agarose gels. The restriction enzyme sites shown are: B, *Bam*HI; C, *Cla*I; G, *Bgl*II; H; *Hin*dIII; K, *Kpn*I; O, *Xho*I; R, *Eco*RI; S, *Sph*I; V, *Eco*RV; and X, *Xba*I. The squares at the ends of the pMA81 and pMA83 maps represent C_4A_4 repeats detected by hybridization (Fig. 5) or sequencing or both. Note that no C_4A_4 repeats have been detected on pMA82d (see text). When two restriction enzyme sites are indicated by a single mark, they are less than 20 bp apart and have not been ordered. In pMA81 and pMA83, the *Eco*RI site of pBR322 lies 750 bp to the right of the insert; the pMA82d insert is oriented in the opposite direction. (B) pMA-MA, pMA-pMA, and MA-MA DNA map correspondences. The intervals between restriction enzyme sites on MA I, MA II, and MA III have been named to facilitate a summary of the data concerning relationships between different pMA inserts and MA DNAs. MA II sequences which are cloned in pMA82d are represented by the thickened portion of the MA II map. Each fragment is named by listing only its terminal intervals; for example, the intact MA I molecule is a–t. In these experiments, pMA restriction fragments comigrated with MA DNA fragments, detected by blot hybridization with pMA probes. For pMA81 versus MA I, comigration was demonstrated for fragments t, s–t, o–t, n–t, m–t, i–t, e–t, a–t, a–m, d–k, f–j, and g–n. In one case where comigration was expected (fragment j–t), it was not observed because the rightmost *Cla*I site in MA I is refractory to digestion. However, all other *Cla*I sites on MA I can be cut. Note also that most MA I molecules are not cut by *Sph*I. For pMA82d versus MA II, comigration was demonstrated for fragment w–b. Other MA II sites were assigned as described in the legend to Fig. 4. For pMA83 versus MA III, comigration was demonstrated for fragments h–z, g–z, f–z, d–z, and a–z. pMA81 and pMA83 fragments which together comigrate with MA DNA fragments include a–e, a–f, a–g, a–h, and c–f; as explained below, these fragments are derived from the common region and are expected to be the same size in MA I and MA III. Correspondence of pMA81, pMA82d, and pMA83 maps in the common region was tested by comparing restriction enzyme fragments from pMA81, pMA82d, and pMA83 on 0.7 and 1.5% agarose gels (data not shown). The following sets of fragments were observed to comigrate: pMA81, pMA82d, and pMA83, b–c; pMA81 and pMA83, c–f, g–i (for *Cla*I and *Cla*I + *Eco*RV), f–g, and g–h. Comigration was not observed for fragment a, pMA81, and pMA83. The left-terminal *Eco*RI fragment of the pMA83 insert is slightly larger than the corresponding fragment from pMA81, which may reflect the number of dG · dC base pairs acquired during cloning rather than additional MA DNA sequences. MA DNA-MA DNA correspondences within the common region were tested in several hybridization experiments (data not shown). Various restriction enzyme digests of macronuclear DNAs were displayed on 0.7 or 1.5% agarose gels, blotted to nitrocellulose, and then hybridized with nick-translated pMA fragments that were derived from the common sequence block. In these experiments, all bands observed could be accounted for by our pMA and MA DNA maps; we infer from the maps that many of these bands represent fragments from more than one family member. Bands attributed to both MA I and MA III represent fragments a–b, a–d, a–e, a–f, a–g, and a–h. Bands attributed to both MA II and MA III represent fragments h–z, g–z, f–z, e–z, and d–z. One band is attributed to MA I, MA II, and MA III (c–f). Note that band g–i is not seen, and g–z results because the rightmost *Cla*I sites on MA II and MA III are refractory to digestion; all other *Cla*I sites on MA II and MA III can be cut. (C) Restriction maps of MA I, MA II, and MA III. Restriction enzyme sites and C_4A_4 repeats are designated as in (A). In addition, we show *Sph*I and *Eco*RV polymorphisms (*) and the *Cla*I- refractory sites (●).

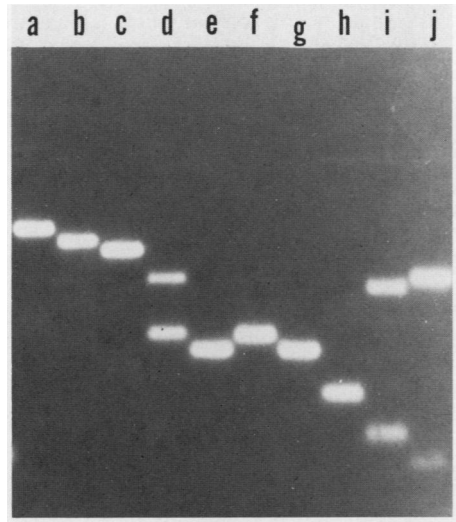

FIG. 4. Restriction mapping of MA II by indirect end-labeling. Macronuclear DNA was digested separately with a variety of restriction enzymes, subjected to electrophoresis in 1.5% agarose, and blotted onto nitrocellulose. The blot was then hybridized with a radioactive MA II-specific fragment from pMA82d (fragment v–x, Fig. 3B). This experiment allows us to detect restriction enzyme sites which are proximal to the probed region (39). In the following list we identify the restriction enzyme used to cut the macronuclear DNA in each lane and name the fragments that are detected by the probe. Fragments are named according to the scheme in Fig. 3B. Lane a, Native macronuclear DNA (u–z); lane b, *Kpn*I (u–h); lane c, *Bgl*III (u–g); lane d, *Eco*RV (v–f and u–f); lane e, *Hin*dIII (v–e); lane f, *Cla*I (u–b); lane g, *Eco*RI (u–y); lane h, *Sph*I (u–x); lane i, *Bam*HI (u–w and x–z); and lane j, *Xho*I (u–v and w–z). The positions of the leftmost *Hin*dIII and *Eco*RV sites were confirmed in another hybridization experiment which is not shown. Note that only a fraction of the MA II molecules are cut at the leftward *Eco*RV site, whereas cutting in the common region is quantitative (lane d); the leftward site is specially marked in Fig. 3C. The *Sph*I polymorphism in MA II was detected in another experiment (data not shown). Two bands appear in the *Bam*HI and *Xho*I lanes (i and j) because the probe spans the sites for these enzymes.

and that those cross-homologies tested are strong (Fig. 2), led us to test whether the three members might indeed carry identical copies of a common sequence block.

First, in six of six cases examined (see Fig. 3 legend), sets of pMA81 and pMA83 restriction fragments expected to be the same size were observed to comigrate, establishing that a large internal section of the pMA83 insert is duplicated in pMA81. The common region extends nearly the entire length of the pMA83 insert from its leftmost *Eco*RI site to its rightmost *Cla*I site (b–i in Fig. 3B), a distance of about 1,300 bp. The results are less conclusive for pMA82d, since only one *Eco*RI fragment in the common region was tested in the experiment (b–c in Fig. 3B), but this fragment comigrates with the corresponding fragments from the other clones.

Second, and analogously, certain sets of MA I, MA II, and MA III restriction fragments are expected to be the same size. To test these expectations, restriction enzyme-digested macronuclear DNAs were displayed on 0.7 or 1.5% agarose gels, blotted to nitrocellulose, and then hybridized with nick-translated pMA fragments that were derived from the common sequence block. We infer that many of the bands detected in these experiments represent fragments from either two or three family members. For example, the c–f fragments of MA I, MA II, and MA III are deduced to be the same size because only a single band representing all three fragments was observed in such an experiment (Fig. 3B legend).

Third, homology between pairs of pMAs was also tested by hybridization (data not shown). In one experiment, pMA83 was hybridized to blotted restriction digests of pMA81, and in another experiment, pMA81 was hybridized to blotted restriction digests of pMA82d. The results are completely consistent with the existence of the common block revealed by the maps, and also indicate that the common block must extend at least slightly beyond the 1,300 bp between the *Eco*RI and *Cla*I sites and into the terminal regions of pMA83 (intervals a and z in Fig. 3B).

The experiments we have described thus far establish that MA I, MA II, and MA III are related by a highly conserved block of sequences at least 1,300 bp long. As described earlier, an *Sph*I polymorphism appears at identical locations on MA I, MA II, and MA III, and thus the common region occurs in two versions.

The common block exists in different sequence environments. On MA I and MA II, but not on MA III, it is juxtaposed to large sequence blocks which extend either to the right (MA I) or to the left (MA II) of the common block. Experiments in which pMA probes were hybridized to either blotted pMA digests (mentioned above) or blotted macronuclear DNA digests (Fig. 3 and 4) demonstrate two facts. First, the right block of MA I and the left block of MA II do not share sequences with each other (or the common block). Second, where we have been able to test (i.e., with those sequences which are cloned), the sequences of these blocks are unique and do not exist elsewhere in macronuclear DNA (nor does the common block).

Limits of the common block. The inverted terminal repeat sequence 5′-$C_4A_4C_4A_4C_4$-3′ is present at the ends of all macronuclear DNA molecules (32). As expected, a C_4A_4 probe hybridized to the termini of the pMA81 and pMA83 inserts (Fig. 5). Chemical sequencing confirmed that the expected number of C_4A_4 repeats on pMA81 are present precisely at the ends of the insert (D. Dawson and S. W. Cartinhour, unpublished data). C_4A_4 sequences are represented by squares on the maps in Fig. 3.

Failure to detect C_4A_4 sequences at two locations shows that the common block does not contain the full-size C_4A_4 runs present at the ends of MA III. First, the pMA81 restriction fragment corresponding to the right end of pMA83 fails to hybridize to the C_4A_4 probe (fragment h–o, Fig. 5). Second, C_4A_4 sequences were not detected by hybridization of pMA82d (not shown). Although the common block does not contain the full-size C_4A_4 runs, it could still include a few C_4A_4 repeats because a short C_4A_4 run would not have been detected in this experiment (23). If the common block terminates at the C_4A_4 repeats, it would be about 1,550 bp long. Therefore, the common block could be no longer than about 1,550 bp, and, as discussed earlier, must be somewhat longer than 1,300 bp.

Stoichiometry in the pMA81 family. Because the pMA83 insert is virtually identical to the common region shared by MA I, MA II, and MA III, we were able to use it as a probe to determine the relative copy number of the three family members. Macronuclear DNA from our standard laboratory stock of *O. fallax* was displayed on a 0.7% agarose gel, blotted to nitrocellulose, and then hybridized with nick-translated pMA83 insert (Fig. 6, lane c). Densitometric analysis showed that the molar distribution for MA I, MA II, and MA III is ca. 1:1:3.

Inheritance of the pMA81 family. The macronucleus is

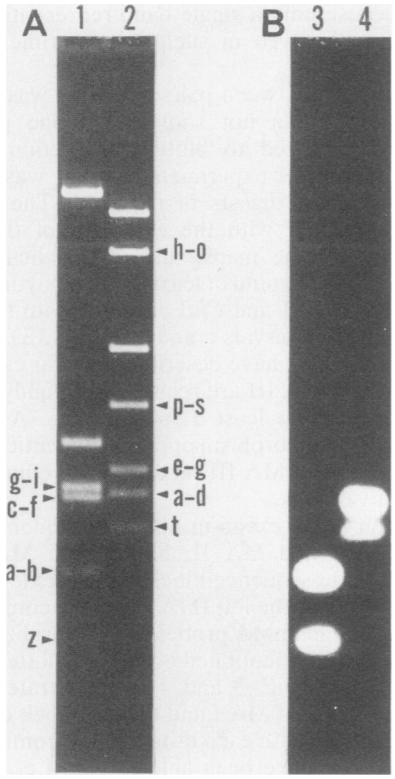

FIG. 5. Detection of $(C_4A_4)_n$ sequences in pMA81 and pMA83. pMA81 and pMA83 were digested with various restriction enzymes and subjected to electrophoresis on a 1.5% agarose gel. The DNAs were blotted onto nitrocellulose and hybridized with C_4A_4 probe. (A) Photograph of the ethidium bromide-stained gel, and (B) the autoradiograph. Lanes 1 and 3 contain 300 ng of *Pst*I + *Cla*I-digested pMA83; lanes 2 and 4 contain 300 ng of *Bgl*II + *Pst*I + *Sph*I-digested pMA81. Letters to the left and right of lanes 1 and 2 relate the insert fragments to the restriction enzyme maps in Fig. 3B. Fragments without addresses are derived from pBR322.

generated from a mitotic daughter of the micronucleus after conjugation. To test whether the pMA81 family is generated in a reproducible fashion in new macronuclei, we compared the stoichiometry of the family in our standard clonal line

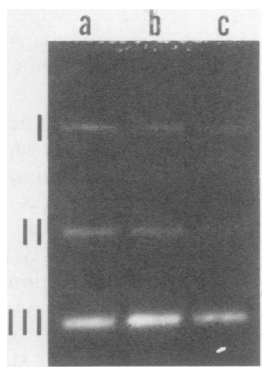

FIG. 6. Stoichiometry of the pMA81 family in macronuclear DNA from three different exconjugant clones. Macronuclear DNAs (2.0 μg) from three different exconjugant clones were displayed on a 0.7% agarose gel, blotted to nitrocellulose, and hybridized with nick-translated pMA83 insert DNA. Lane a, Clone 9D2; lane b, clone 9D1; and lane c, clone 3.5.

(lane c) with that in two clonal lines which arose from different matings (lanes a and b). The mass distribution between the members of the pMA81 family is nearly identical in the three MA DNAs (Fig. 6). In a second part of this experiment (not shown), we compared the three *Sph*I-cut macronuclear DNAs to test whether the polymorphisms in the common region are also regenerated. The macronuclear DNAs yielded identical *Sph*I products with the same stoichiometry. We conclude from these experiments that the stoichiometry and structure of the pMA81 family are reproduced each time a new macronucleus is formed. We also note that the pMA81 family has been detected in *O. fallax* subspecies 4b (16, 17; data not shown). Although respective pMA81 family members in the two subspecies are identical in size, the members in the heterologous subspecies have diverged somewhat in sequence or are present in lower copy number, based on their reduced hybridization to nick-translated pMA83 insert.

DISCUSSION

When *O. fallax* pMAs are hybridized to blots of native macronuclear DNA, 6 of 15 hybridize to families composed of two or three members of different sizes. One of these families (pMA81) has been analyzed in detail, and provides a paradigm for understanding the relationship between the members of the other five families. In the pMA81 family, MA I, MA II, and MA III are related by a common block of sequences 1,300 to 1,550 bp long. A feature of the common block is an *Sph*I polymorphic site which is shared by all three members of the family. The common block is responsible for the thermal stability of the duplexes formed between pMA81 and MA II and MA III (melt-off experiment, Fig. 2). The pMA13 family members exhibit similar strong cross-homology (Fig. 2). This is consistent with pMA13 family members sharing a highly conserved common region as well. The presence of a common block also leads to the prediction that the restriction enzyme maps of family members can be arranged in an overlapping manner. Evidence consistent with this has been obtained for the *Pst*I maps of pMA13 and pMA11 family members (data not shown). Although we have no evidence that the remaining three families are related by family-specific common sequence blocks, we feel this model is reasonable.

The macronucleus of *O. fallax* probably contains 20,000 different kinds of macronuclear DNAs (12). The high frequency with which we detect sequence families in macronuclear DNA implies that a large fraction of these macronuclear DNAs are involved in such families. Note that our estimate of family frequency may be too low if some of the pMAs which hybridize to only one macronuclear DNA size class have lost their common block in a deletion event: five of the eight pMAs which detect a single macronuclear DNA size class have suffered deletions (Table 1). The macronuclear DNA families we detected appear to be unrelated to each other. Each family consists of a distinct set of macronuclear DNAs (Table 1). Family independence is also suggested by the fact that the unique portions of pMA81 and pMA82d fail to hybridize to any macronuclear DNA size class but MA I and MA II, respectively. The independence of the families and their frequency indicate that many different sequences are distributed as common blocks in the macronucleus of *O. fallax* and suggest that this pattern of genome organization has functional significance.

It is intriguing that the common sequence block in the pMA81 family exists in different contexts on three distinct

macronuclear DNAs. Whereas MA III consists almost entirely of common block material, in MA I and MA II additional sequences are adjacent to the common block. The additional sequences are unique in that they appear on only one macronuclear DNA size class, and they are placed either to the left (MA II) or to the right (MA I) of the common block (Fig. 3). Alternate sequence juxtapositions, in which common sequence blocks can appear in different DNA or RNA contexts, have been described in a variety of organisms. In the mammalian immune system, diversity is at least partially accounted for by alternate DNA joining programs which juxtapose variable, J, D, and successive constant regions for immunoglobulin genes (20). Expression of variant surface glycoprotein genes in trypanosomes is often associated with DNA rearrangements (5). Possibly the examples of alternate juxtaposition most analogous to the pMA81 family are those in which RNA sequence blocks exist in different sequence contexts in the same cell or tissue. Such alternate RNA processing is involved in the expression of the genes for γ-fibrinogen (8), α-crystallin (24), and glycinamide ribotide transformylase (19). Other cases of alternative RNA processing have been reviewed recently (10), and in some of these the alternate juxtapositions can be correlated with alternate gene product function. We have not investigated whether members of the pMA81 family contain expressed genes (although most macronuclear DNAs are transcribed) (30; Heumann, Ph.D. dissertation), but as noted above, the abundance of macronuclear DNA families suggests that they represent a general strategy for genetic regulation in the macronucleus.

The pMA81 family is stably inherited through many generations of vegetative growth and also reappears, with the same structure and stoichiometry, when a new macronucleus is generated after conjugation. We conclude that the pMA81 family is generated in a precise, reproducible fashion each time a macronucleus is formed. However, the manner in which the pMA81 family is produced from its progenitor sequences in micronuclear DNA is not understood. The three members of the pMA81 family might arise from three different micronuclear loci; in this view the existence of the common block is not intimately related to the genesis of the family. Alternatively, the pMA81 family could be generated from a single micronuclear locus if the components of the pMA81 family (i.e., the leftward sequences unique to MA II, the common block, and the rightward sequences unique to MA I) are assembled during macronuclear development in alternate ways to produce three related MA DNAs.

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