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Original Article

The processing of macronuclear DNA sequences during macronuclear development of the hypotrichous ciliate *Stylonychia lemnae*

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ABSTRACT The organization of two macronuclear DNA sequences in the polytene chromosomes of the hypotrichous ciliate *Stylonychia lemnae* and their processing from the micronucleus via the polytene chromosome stage up to the macronucleus was analyzed. The overall organization of these sequences in the polytene chromosomes resembles that described for the micronucleus of other hypotrichous ciliates, i.e. they are interrupted by internal eliminated sequences and not associated with telomeric sequences. The spacer region between the genes is bordered by direct repeats and inverted repeats are found at the termini of macronuclear sequences and in the spacer region. The organization of these macronuclear DNA sequences in the micronucleus was analyzed by polymerase chain reactions. The results obtained show that in the sequences analyzed no DNA reorganization occurs during polytene chromosome formation.

KEY WORDS: *macronuclear development, IES, spacer, DNA processing*

Introduction

Ciliated protozoa are characterized by their nuclear dimorphism; each cell contains a diploid micronucleus and a DNA-rich macronucleus. After sexual reproduction of the cells a new macronucleus develops from a micronuclear derivate. In hypotrichous ciliates this macronuclear development is accompanied by DNA- and chromatin rearrangement processes as well as by the selective loss of many DNA sequences in the macronucleus. A first DNA synthesis phase leads to the formation of polytene chromosomes. These chromosomes become degraded and over 90% of the DNA sequences are eliminated during this process. A second DNA synthesis phase then leads to the mature macronucleus. The consequences of this developmental process are the loss of most of the micronuclear DNA sequences in the macronucleus and the specific fragmentation of the macronuclear DNA into small gene-sized DNA molecules. While the morphological events during this differentiation process are well described, very little is known about the molecular mechanisms involved in this process (for review see Klobutcher and Prescott, 1986; Klobutcher and Jahn, 1991; Kraut *et al.*, 1986).

Analysis of macronuclear gene organization in the micronuclear genome revealed that: 1) macronuclear sequences occur in clusters in the micronuclear genome and the different clusters can be separated by long spacer regions (Klobutcher *et al.*, 1986; Klobutcher, 1987; Jahn *et al.*, 1988a,b); 2) in many macronuclear precursor genes intron-like sequences can be found (Klobutcher, 1987; Jahn *et al.*, 1988b); 3) telomeric sequences are not associated with macronuclear precursor genes (Herrick *et al.*, 1985; Stoll *et al.*, 1991, 1993); and 4) the exons of some precursor genes are scrambled within the micronuclear genome (Greslin *et al.*, 1989; Mitcham *et al.*, 1992). Therefore, in order to create a functional macronuclear gene, introns have to be spliced, exons have to be reordered and telomeric sequences have to be added *de novo* to macronuclear gene-sized DNA molecules (for review see Klobutcher and Jahn, 1991).

It has been speculated that some of the genome rearrangements necessary for the processing of macronuclear genes already occur during the formation of polytene chromosomes (Lipps, 1985; Klobutcher and Prescott, 1986) and that the biological function of the polytene chromosome formation would be to allow such rearrangement processes. We therefore determined the organization of some genes in the polytene chromosome stage. Subsequently their organization and structure were analyzed in the micronucleus and the macronucleus. Thus, the processing of macronuclear DNA sequences was followed throughout the whole process of macronuclear differentiation.

NOTE: The EMBL accession numbers of the sequences in this manuscript are: i) for the 1.1 kb macronuclear sequence (SLMAC11): X72955; ii) for the 1.3 kb macronuclear sequence (SLMAC13): X72956.

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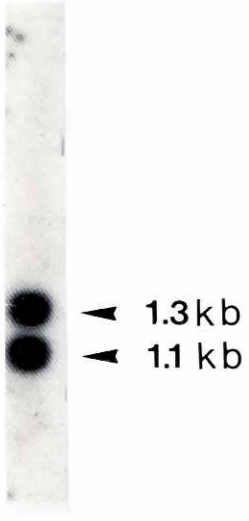

Fig. 1. Hybridization of clone MaA11 to total macronuclear DNA. *Sizes are given in kb.*

Results

Macronuclear homologous sequences in the polytene chromosome stage of the macronuclear anlagen of *Stylonychia lemnae* were isolated from a complete gene library of this nuclear stage (Stoll *et al.*, 1991). $7.5x10^4$ plaques were screened with five randomly chosen macronuclear gene-sized DNA fragments with sizes between 1 and 3 kb. Since the insert size of the polytene chromosome gene library varies between about 10 and 25 kb, $7.5x10^4$ plaques represent about 70% of the haploid polytene chromosome genome. Several positive clones were identified; however one clone showed in a hybridization to total macronuclear DNA homology to the 1.3 kb macronuclear gene used for screening but in addition a homology to a 1.1 kb macronuclear DNA molecule (Fig. 1). This clone was used for further analysis.

The insert size of this macronuclear anlagen clone (clone MaA11) is 13 kb. It can be recovered by SalI digestion yielding two SalI fragments with sizes of 7 and 6 kb. A restriction map of clone MaA11 is shown in Fig. 2a. When the two SalI fragments were hybridized to total macronuclear DNA only the 7 kb SalI fragment showed homology to the two macronuclear gene-sized DNA molecules (Fig. 3). This SalI fragment was subsequently subcloned in PUC12 yielding plasmid pCE7. A detailed restriction map of this clone is shown in Fig. 2b. Individual restriction fragments from this clone were hybridized to macronuclear DNA (Fig. 3). Thus, the region homologous to the two macronuclear genes could be localized on a 3.5 kb EcoRI kb region.

The 1.1 kb macronuclear DNA gene was isolated from a macronuclear λzap gene library using the 1.4 kb EcoRI/BglII fragment from clone pCE7, which shows homology to the 1.1 kb macronuclear gene-sized DNA molecule as a probe. Restriction maps of this gene and the 1.3 kb gene are shown in Fig. 2c and d. Appropriate subclones of these cloned genes were made and a sequence analysis of the 1.1 and 1.3 kb macronuclear genes as well as a 4.1 kb region of the macronuclear anlagen clone pCE7 showing homology to these genes were made. These sequence data are summarized in Fig. 4.

The exact size of the two macronuclear gene-sized DNA molecules is 1186 bp and 1325 bp (including the 36 base telomeric sequence). Under the assumption that TGA is the only stop codon used and that TAA and TAG code for glutamine (Helftenbein, 1985; Harper and Jahn, 1989), a putative open reading frame of 596 bp for the 1.3 kb DNA molecule and two of 248 bp and 777 bp for the 1.1 kb DNA molecule can be found. No homology to other genes was found in the EMBL data bank. 4119 bp of the macronuclear anlagen clone were sequenced. While the complete 1.3 kb macronuclear DNA molecule is found in the anlagen clone, the terminal 100 bp of

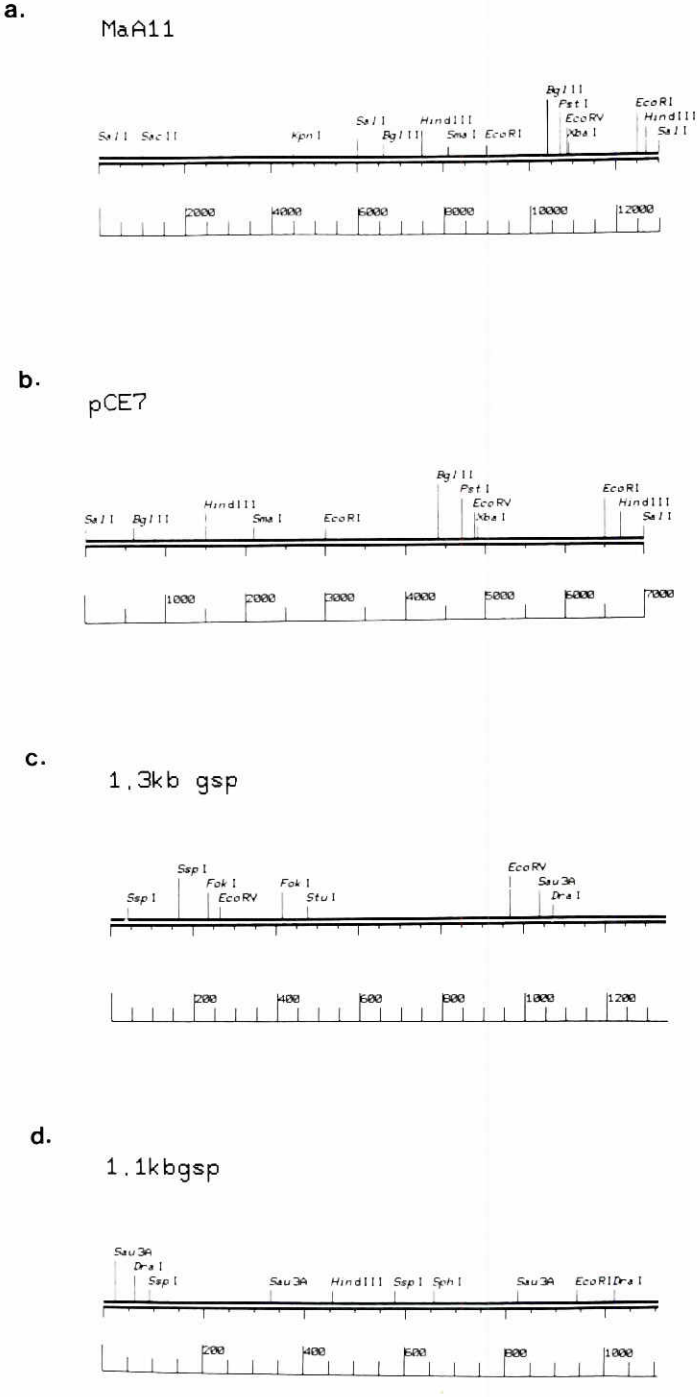

Fig. 2. Restriction maps of (a) clone MaA11, (b) subclone pCE7 derived from MaA11, (c) the 1.3 kb macronuclear DNA molecule, (d) the 1.1 kb macronuclear DNA molecule.

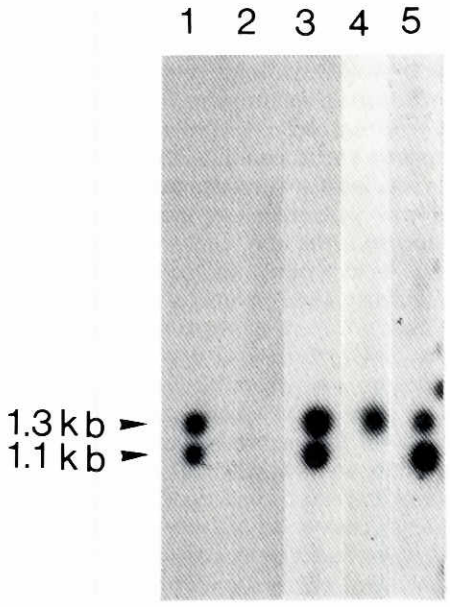

Fig. 3. Hybridization of various subclones derived from MaA11 to total macronuclear DNA. (Lane 1) *7 kb SalI fragment from MaA11,* **(Lane 2)** *6 kb SalI fragment from MaA11,* **(Lane 3)** *3.5 kb EcoRI fragment from pCE7,* **(Lane 4)** *2.2 kb EcoRI/BglII from pCE7, 5. 1.3 kb EcoRI/BglII fragment from pCE7.*

the 1.1 kb molecule are missing. Since no hybridization to this macronuclear DNA molecule was found on adjacent restriction fragments in the original MaA11 anlagen clone, it has to be assumed that this 100 bp sequence is separated from the rest of the macronuclear homologous sequences by at least 3 kb. The region of homology to the two macronuclear DNA molecules in the macronuclear anlage runs over 2416 bp. The two macronuclear homologous sequences are separated by an 11 bp spacer and the homology terminates with the last base before the macronuclear telomeric C_4A_4-repeat starts. Thus, as already described for several macronuclear precursor genes in the micronucleus of other hypotrichous ciliates (Klobutcher *et al.*, 1986; Klobutcher, 1987; Jahn *et al.*, 1988a,b), and also in the polytene chromosomes of *Stylonychia,* these genes are not associated with a telomeric sequence. The macronuclear precursor genes in the polytene chromosomes are interrupted by intron-like sequences (IES, internal eliminated sequences; Klobutcher and Jahn, 1991). Three of these IES with sizes of 68 bp, 59 bp and 10 bp are found in the 1.3 kb gene and two IES with sizes of 41 bp and 31 bp in the 1.1 kb gene. These IES show an organization similar to that described in the micronucleus of other hypotrichous ciliates (Ribas-Aparicio *et al.*, 1987; Bierbaum *et al.*, 1991; Klobutcher and Jahn, 1991). They are

Fig. 4. Partial sequence of the 3.5 kb EcoRI fragment from clone pCE7 aligned with the sequences from the 1.1 kb and 1.3 kb macronuclear DNA molecule. *Direct repeats bordering the IES are underlined, direct repeats bordering the spacer region are underlined by a dotted line, inverted repeats at the termini of the macronuclear precursor DNA molecules and in the spacer region are indicated by arrows, gsp: gene-sized DNA molecule.*

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247
CGACATAATC CAGCCATCGC TGGATTTCCC CCCCAGAAGT GCCGGATCTT  pCE7

297
ATAAATTAAT TGCATGAATT TGGAAAGCCA ATTTATAATG AATCTATATT  pCE7
347
GAATTCCACT TCCAGGCTAC ACTGGCCACG TTCCAGGCAA ..........  pCE7
GAATTCCACT TCCAGGCTAC ACTGGCCACG TTCCAGGCAA ..........  1,0 kb gsp
123

595                                      IES 1
TTATAAATTA ATCAGCTACT AAGATATTTG TTGATTAGTC TATAAATACT  pCE7
TTAAATACTT ATCA______ __________ __________ __________  1,0 kb gsp
373

645
AATCACTATT CAAGGCTGAG TTTATCCAGC ATGCTCATAA ..........  pCE7
_____CTATT CAAGGCTGAG TTTATCCAGC ATGCTCATAA ..........  1,0 kb gsp

1142
GCAATTGTGC CAGAATATAA GAGACCAAAA CAAGACGATC T  GGTATTT  pCE7
GCAATTATAC CAGAATATAA GAGACCAAAA CAAGAGCATC TCTGG_____  1,0 kb gsp
882

1190          IES 2
CAAATATAGT ATATATTTTT GAAAGGTATT GAAATCAGAT ..........  pCE7
__________ __________ ______TATT GAAATCAGAT ..........  1,0 kb gsp

1289                                          ccccaaaac  1,3 kb gsp
GTATTTAAAG TTCAAATTTC TTCTAAAATC TTTGAATT A TAC GATCAT  pCE7
GTATTTAAAG TTCAAATTTC TTCTAAAATC TTTGAATTTA TAAAGATCAT  1,0 kb gsp
1001

10
ccccaaaacccc TCAGTCCAG GATTCTTTAG AATAATATTT AATGAAAATG  1,3 kb gsp
AAACTGATTAC TCAGTCCAG GATTCTTTAG AATAATATTT AATGAAAATA  pCE7
ggggttttgggg ttttgggg                                   1,0 kb gsp
1051

61
ATTACAAAAA TTAATTACTT ATATCCCAGT TATTTTTAAT ..........  1,3 kb gsp
ATTACAAAAA TTAATTACTT ATATCCCAGT TATTTTTAAT ..........  pCE7
1387

557
TGCCATTTA  CATTT_____ _____ACAAT GATTTGAG T ..........  1,3 kb gsp
TGCCATTTAA CATTTGGAGT TTGAGACAAT GATTTGAGTT ..........  pCE7
1878              IES 3

787
CAAATGT GC CATGTGCAAA TTGACATTT  _________ _________  1,3 kb gsp
CAAA GTTGC CATGTGCAAA TTGACATTTT AATTGCTTCA ATAATTATTG  pCE7
2126

_________ _________ _________ _________GT GGCCATATCT  1,3 kb gsp
TGCTTATATG GGATTATAAT AAGAATGAGT AGACATTTGT GGCCATATCT  pCE7
2175          IES 4

827
GCATTATCCG AACTCTATCC AGTTTTTACA CATTTCAGTC ..........  1,3 kb gsp
GCATTA CC  AACTCTATCC AGTTT  ACA CATT CAGTC ..........  pCE7
2225

1075
TTGAATTGAT GAGTGATATC  T_________ _________ _________  1,3 kb gsp
TTGAATTGAT GAGTGATATC CTGCAAATTT ATAAATCTAT TTAAATGCTA  pCE7
2468

_________ _________ _________ _________ _GCAAAGGGG  1,3 kb gsp
TAAATATATA CCATCTAGAT AAAAAGTCAT TAAAATGTAT GGCAAAGGGG  pCE7
2518              IES 5

1105
TTCTTAGATA ATGCTGTTGA GAGAC TTGT GACTGAAATA ..........  1,3 kb gsp
TTCTTAGATA AGTTTGTTGA GGGACGTTGT ATCTGAAATA ..........  pCE7
2568

1251
A TAAA TCA TACTT CTTA TTTCAATCAA CTAT ATTTC  GACCGTggg  1,3 kb gsp
AATAAAATCA TACTTTCTTA TTTCAATCAA CTATTATTTC TGACCGCTGT  pCE7
2717

1297
gttttggggt tttgggg                                      1,3 kb gsp
ATATTAAGTA GTAATATATC ATCTAGTTTA AATGAAGATA AATTAATCCT  pCE7
2767
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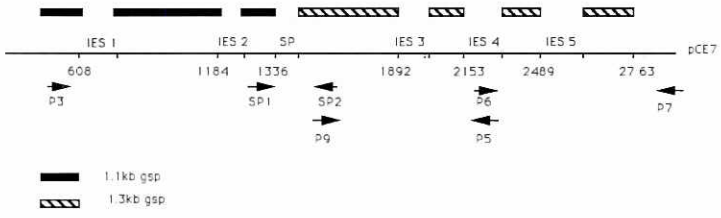

Fig. 5. Schematic diagram showing the position of macronuclear homologous sequences within clone pCE7 and the positions of the primers used in the polymerase chain reactions. *sp: spacer region.*

bordered by 4-7 bp direct repeats, with one copy of the repeat retained in the macronuclear DNA molecule. The imperfect repeats often present in the IES are only detected in one IES (IES2). Also neither direct nor inverted repeats are found in the 10 bp IES of the 1.3 kb gene. Interesting sequence features not described so far are also found in the 11 bp spacer region between the two genes. First, it is bounded by a 3 bp direct repeat and second, 5 bp inverted repeats are found at the termini of both genes as well as in the spacer region. One more remarkable sequence feature in the macronuclear anlagen clone is a stretch of seven cytosines 66 bp upstream of the 1.1 kb DNA molecule (Fig. 4).

To verify that the described organization of the two macronuclear precursor genes in the macronuclear anlagen clone is indeed the real configuration in the polytene chromosomes polymerase chain reactions were made using total polytene chromosome DNA. To keep the synthesis of fragments from contaminating macronuclear DNA, primers used in these reactions were either derived from the regions homologous to the two macronuclear DNA molecules or from an IES and a region homologous to the same gene-sized DNA molecule. In fact, when a polymerase chain reaction with total macronuclear DNA was made using these primers, no fragment was synthesized. The primer localization and the primer combinations are shown in Figs. 5 and 6. The polymerase chain reaction experiments with total polytene chromosome DNA are summarized in Fig. 6. In all reactions a fragment of the expected size was synthesized.

Finally, the question was raised whether the same gene organization can be found in the micronuclear genome. Therefore, polymerase chain reactions with total micronuclear DNA were performed, using the same primer combinations as in the reactions with polytene chromosome DNA. As shown in Fig. 6, in all primer combinations fragments identical to those obtained with polytene chromosome DNA were synthesized.

Discussion

In this report the organization of macronuclear precursor DNA sequences in the polytene chromosome stage of the macronuclear anlage is described. Moreover, the processing of these sequences from the micronucleus in the polytene chromosome stage up to the macronucleus is analyzed. This analysis seemed especially interesting since so far the biological function of polytene chromosome formation during macronuclear development is unknown and it has been speculated that already during polytene chromosome formation DNA reorganization processes may take place.

From a gene library of the polytene chromosomes a clone was isolated showing homology to two macronuclear gene-sized DNA molecules. The two corresponding macronuclear DNA molecules were also cloned and a sequence comparison between these macronuclear DNA molecules and their precursor sequences in the polytene chromosomes were made. These precursor sequences are arranged in a linear order. However they are interrupted by short internal eliminated sequences (IES, Klobutcher and Jahn, 1991) and are not associated with macronuclear telomeric sequences. Thus, this overall organization of macronuclear precursor DNA sequences seems to be very similar to that described in the micronucleus of several other hypotrichous ciliates (Klobutcher *et al.*, 1986; Klobutcher, 1987; Jahn *et al.*, 1988a,b; Bierbaum *et al.*, 1991; Jahn, 1991). The IES show the typical 4-7 bp direct repeats (Ribas-Aparicio, 1987; Klobutcher and Jahn, 1991), although the imperfect inverted repeats typical for many IES are found only in one case. Recently evidence has been presented that the IES are eliminated in form of DNA rings during polytene chromosome stage and prior to chromosome fragmentation (Tausta and Klobutcher, 1989). The facts that we still find the IES in our anlagen clone and that in polymerase chain reactions only one sequence version was synthesized suggest that IES elimination must occur very late in the polytene chromosome stage. A stretch of seven cytosines is found 66 bp upstream of the 1.1 kb gene. Nothing can be said about the biological significance of this sequence. Possibly it could be a cis-acting sequence necessary for correct excision of macronuclear genes similar to an external cis-acting sequence described in *Tetrahymena thermophila* (Godiska and Yao, 1990). In *Bacillus subtilis* a stretch of seven cytosines is part of an inverted repeat which represents a recognition element for recombination. Two

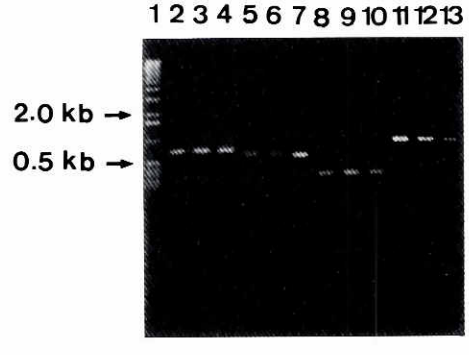

Fig. 6. Fragments synthesized in polymerase chain reactions from either clone pCE7, total polytene chromosome or micronuclear DNA. *Primers used are indicated in Fig. 5. They all were between 15 and 20 b long. Experimental conditions were as follows: annealing temperature 52°C, extension temperature 72°C. denaturation temperature 95°C, annealing time 120 sec.; extension time varied between 60 and 150 sec. and the denaturation time was 120 sec. DNA synthesis was performed in the case of pCE7 in 30 cycles, with polytene chromosome or micronuclear DNA in 60 cycles.* **(Lane 1)** *Molecular weight marker (75 bp-12.2 kb).* **(Lanes 2-4)** *Polymerase chain reactions using the primer combination p9 and p5. Lane 2: pCE7; Lane 3: polytene chromosome DNA; Lane 4: micronuclear DNA.* **(Lanes 5-7)** *Polymerase chain reactions using the primer combinations p6 and p7. Lane 5: pCE7; Lane 6: polytene chromosome DNA; Lane 7: micronuclear DNA.* **(Lanes 8-10)** *Polymerase chain reactions using the primer combinations sp1 and sp2. Lane 8: pCE7; Lane 9: polytene chromosome DNA; Lane 10: micronuclear DNA.* **(Lanes 11-13)** *Polymerase chain reactions using the primer combinations p3 and sp2. Lane 11: pCE7; Lane 12: polytene chromosome DNA; Lane 13: micronuclear DNA. Sizes are given in bp.*

regions of two different genes are brought together by homologous recombination (Stragier *et al.*, 1989). A most interesting sequence organization not described so far is found in the spacer region between the two macronuclear DNA sequences. The spacer is bordered by direct repeats localized in the macronuclear sequences and inverted repeats are found at the termini of both macronuclear DNA molecules and in the spacer region. If this were the general organization of spacer regions it might explain the correct excision of spacers in a mechanism somehow similar to that described for IES (Klobutcher and Jahn, 1991).

To see whether the two macronuclear DNA sequences occur in the same configuration in the micronucleus, a number of polymerase chain reactions were performed. The primers used did not amplify macronuclear sequences eventually contaminating the polytene chromosome or micronuclear DNA preparations, thus making sure that the fragments obtained come from the polytene chromosomes or the micronucleus. In all cases fragments of a similar size to that obtained from polytene chromosomes were synthesized from micronuclear DNA. This strongly suggests that within the sequences analyzed, i.e. macronuclear precursor DNA sequences, spacer regions between these sequences and the sequences bordering macronuclear precursor sequences in the micronuclear DNA, no DNA reorganization takes place during polytene chromosome formation. These results do not exclude DNA rearrangement processes in other regions of the polytene chromosomes. It would be especially interesting to compare the sequence organization in the polytene chromosome DNA of those genes, where the different exons are scrambled within the micronuclear genome (Greslin *et al.*, 1989; Mitcham *et al.*, 1992). However, the experimental strategy used in this study may prove useful to answer these questions.

Materials and Methods

Growth of *Stylonychia*, isolation of macronuclei, micronuclei and polytene chromosomes and the isolation of DNA were performed as described earlier (Ammermann *et al.*, 1974). The polytene chromosome gene library used was described by Stoll *et al.* (1991). A macronuclear DNA library was constructed using the λzap vector system as described by Sambrook *et al.* (1989). Individual macronuclear DNA molecules were subcloned in PUC12. Isolated DNA or restriction digests were separated on 0.5-1% agarose gels and transferred to nitrocellulose filters as described by Southern (1975). DNA was radioactively labeled by oligolabeling (Feinberg and Vogelstein, 1983). Hybridization was done in 4x SSC, 10x Denhardt's medium, 0.1% SDS at 65°C. Sequencing of DNA fragments was done as described by Sanger *et al.* (1977). Restriction digestions and procedures used for subcloning of DNA fragments were performed according to Sambrook *et al.* (1989) and the suppliers' instructions. Polymerase chain reactions followed the procedure by Saiki *et al.* (1988). The primers used are indicated in the figures.

Acknowledgments

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