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Macronucleus structure and macronucleus development in hypotrichous ciliates

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ABSTRACT **In the course of macronuclear development of the hypotrichous ciliates all genetic information not required for normal growth of the cell is removed from the new macronucleus. This differentiation process involves DNA-splicing, excision of transposons, DNA-fragmentation, selective gene amplification and telomere addition. Since many of the processes observed during macronuclear development, such as DNA-transposition, DNA-rearrangement or selective DNA-amplification, may occur in differentiating cells of higher organisms, this biological system provides an unusual opportunity to study the ways in which DNA-sequences can be manipulated in a differentiating cell.**

KEY WORDS: *DNA-processing, DNA-transposition, DNA-fragmentation, telomeres, minichromosomes*

Introduction

The unicellular ciliated protozoa are among the most complex eucaryotic cells and have been studied for many years as model systems of cellular differentiation processes (for review see Grell, 1973; Jeon, 1986; Gall, 1986).

One characteristic of the ciliate cell is the occurrence of two morphological and functional different nuclei in one cell, macronuclei and micronuclei. While macronuclei represent the vegetative nucleus, making all the RNA required for normal growth of the cell, the micronuclei represent the generative nuclei of the cell. After sexual reproduction (conjugation) a new macronucleus is formed by a micronuclear derivative while the old macronucleus is resorbed. A schematic diagram of macronuclear development in hypotrichous ciliates is shown in Figure 1. It includes an initial DNA synthesis phase leading to the formation of polytene chromosomes; these are subsequently degraded and up to over 90 % of their DNA is eliminated. A second DNA synthesis phase then leads to the vegetative macronucleus (for review see Kraut *et al.*, 1986; Klobutcher and Prescott, 1986; Steinbrück, 1990; Prescott, 1994). This developmental process suggests that macronuclear development in hypotrichous ciliates is accompanied by dramatic DNA reorganization processes and recent work has demonstrated that this developmental process provides the opportunity to study basic biological phenomena such as specific DNA fragmentation, DNA-elimination, DNA-transposition, DNA-splicing and de novo addition of telomeric sequences (for review see Klobutcher and Jahn, 1991; Prescott, 1994). In this paper we will give a short overview of the molecular organization of the hypotrichous ciliate nuclei and review some recent progress in our understanding of molecular mechanisms involved in the differentiation of a new macronucleus.

Macronuclear structure in hypotrichous ciliates

The DNA content and the kinetic complexity of macronuclei and micronuclei from two hypotrichous ciliates are shown in Table 1. While micronuclear DNA reassociates as multiple DNA components indicating the presence of repeated and non repeated sequences, the reassociation kinetics of macronuclear DNA follows a simple second order reaction kinetic. No repetitive DNA could be found in the macronucleus and the kinetic complexity of its DNA is only about 10-14 times the complexity of *E. coli* DNA (for review see Kraut *et al.*, 1986; Prescott, 1994). These results suggest that only part of the micronuclear DNA sequences are present in the macronuclear genome and that most of the chromosomal DNA, that is not essential for the vegetative life cycle of the cells, is specifically eliminated in the course of macronuclear development.

As early as 1971 Prescott and his coworkers reported that the DNA of macronuclei from hypotrichous ciliates is of low molecular weight when investigated by sedimentation analysis and electron microscopy. These results were later confirmed by agarose gel electrophoresis (Fig. 2) and probing the DNA with specific gene probes (reviewed in: Klobutcher and Prescott, 1986). The size of macronuclear DNA-molecules varies between about 0.3 and 20 kb with an average size of 1-3 kb. Some fragments are

Abbreviations used in this paper: IES, internal eliminated sequences; TBE, telomere-bearing element; TEC, transposon Euplotes crassus; Tce, telomere containing element.

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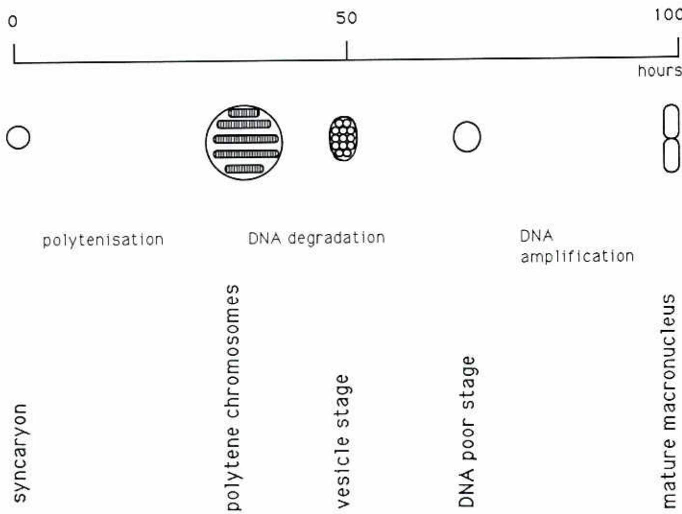

Fig. 1. Schematic diagram of macronuclear development in hypotrichous ciliates *(modified after Ammermann et al., 1974; Kraut et al., 1976).*

seen as prominent bands, indicating that certain DNA molecules occur in higher copy numbers than others. In fact, it was shown for defined sequences, that the copy number of macronuclear DNA-molecules varies between about 10^3 and 10^6 per macronucleus, being always constant for each sequence (see e.g. Helftenbein 1985, Conzelmann and Helftenbein, 1987; Baird and Klobutcher, 1991). The banding pattern of macronuclear DNA seems to be characteristic for different species and has been used as a systematic criterion to identify hypotrichous ciliates (for review see Ammermann, 1990).

Taking all these results together it can be concluded that each macronucleus contains between 10,000 to 20,000 different DNA-molecules, each present in copy numbers between several hundred up to 10^6 per nucleus, i.e. one macronucleus contains several millions individual short DNA-molecules.

Sequence analysis of many macronuclear DNA-molecules (for review see Prescott, 1994) revealed that they always contain one open reading frame, 3' and 5' non coding regions of various length and telomeric repeats consisting of blocs of 5'-C_4A_4 and a 3' protruding G_4T_4 tail which can adopt a special DNA-structure both *in vitro* and *in vivo* (e.g. Lipps, 1980; Oka *et al.*, 1980; Klobutcher *et al.*, 1981; Kaine and Spear, 1982; Lipps *et al.*, 1982; Pluta *et al.*, 1982; Helftenbein, 1985; Conzelmann and Helftenbein, 1987; Harper and Jahn, 1989; Sundquist and Klug, 1989; Williams *et al.*, 1989; Wefes and Lipps, 1990; Fang and Cech, 1991; Kang *et al.*, 1992). Introns are only very rarely found in macronuclear genes (Brünen-Nieweler, 1991; Fang and Cech, 1991; Gray *et al.*, 1991) and, similar to other ciliated protozoan, an unusual genetic code is found in many of these genes (Helftenbein, 1985; Horowitz and Gorovsky, 1985; Preer *et al.*, 1985; Meyer *et al.*, 1992). In the non-coding regions of macronuclear "gene-sized" DNA-molecules sequences which possibly can be related to gene expression and replication are found (Helftenbein *et al.*, 1989). However, so far no replication origin has actually been mapped in macronuclear DNA-molecules. One sequence derived from one subtelomeric region of an over-amplified macronuclear DNA-sequence has been shown to promote plasmid amplification in mouse cells and may represent, *in vivo*, a strong origin of replication (Wegner *et al.*, 1989). Detailed analysis of the subtelomeric regions from *Stylonychia* revealed a highly ordered and conserved sequence organization and it has been speculated that some of these subtelomeric sequences may be involved in the DNA-fragmentation process during macronuclear differentiation (Maercker and Lipps, 1993). A schematic diagram of one macronuclear DNA-molecule from *Stylonychia* is shown in Figure 3.

The DNA of the ciliate nuclei is associated with typical eucaryotic histones. However, only small quantities of histone H1 are detected in macronuclear chromatin (for review see Kraut *et al.*, 1986; Cadilla *et al.*, 1986). There is one report describing the absence of histone H3 in the micronucleus and the presence of a high molecular weight protein X in *Stylonychia lemnae*. In the course of macronuclear development this protein X is replaced by a conventional histone H3 (Schlegel *et al.*, 1990). Limited digestion of macronuclear and micronuclear chromatin with micrococcal nuclease revealed a nucleosome repeat of 220 bp in the macronuclei of *Stylonychia* and *Oxytricha* and a repeat length of 200 bp in the micronuclei of *Stylonychia* and 180 bp in the micronuclei of *Oxytricha* (for review see: Kraut *et al.*, 1986). Proteins specifically binding to telomeric DNA and to the 3' single-stranded extension of telomeres have been identified in the macronucleus of several hypotrichous ciliates. A defined DNA-protein complex in this region is formed with nucleosomes phased inwards from this complex (Lipps *et al.*, 1982; Gottschling and Cech, 1984; Gottschling and Zakian, 1986; Steinhilber and Lipps, 1986; Price and Cech, 1987, 1989; Raghuraman and Cech, 1989; Price, 1990; Gray *et al.*, 1991; Fang *et al.*, 1993).

Lysis of macronuclei under low ionic strength releases short chromatin fibers and the length distribution of these fibers corresponds well to the size distribution of macronuclear DNA-molecules (Lipps *et al.*, 1978; Butler *et al.*, 1984; Cadilla *et al.*, 1986). Thus, under these conditions it would be possible to isolate and characterize specific macronuclear "minichromosomes" in their chromatin configuration. However, when macronuclei are gently lysed on a deionized water hypophase only long continuous 30 nm chromatin fibers are observed (Meyer and Lipps, 1981). Possibly, telomere-protein-interactions may lead to a higher order chromatin organization in the macronucleus of hypotrichous ciliates (Lipps *et al.*, 1982). Antibodies against Z-DNA strongly react with the macronucleus but not with the micronucleus of *Stylonychia*. When macronuclear DNA is nicked by gentle DNaseI treatment no more binding of the anti-Z-DNA-antibody is observed, suggesting that macronuclear DNA occurs in

TABLE 1

DNA CONTENT AND DNA COMPLEXITY OF MACRONUCLEAR AND MICRONUCLEAR DNA OF TWO HYPOTRICHOUS CILIATES

	DNA content (pg)		Kinetic complexity	
	Macro-nucleus	Micro-nucleus	Macro-nucleus	Micro-nucleus
Stylonychia lemnae	788	13	3.1×10^{10}	1.45×10^{12}
Oxytricha sp.	116	1.32	3.6×10^{10}	1.5×10^{12}

a supercoiled configuration *in vivo* (Lipps *et al.*, 1982). The exact *in vivo* organization of DNA molecules in the macronucleus still needs to be examined.

DNA-sequence organization in the micronucleus

Micronuclear DNA is contained in many, usually over hundred, small chromosomes and its DNA has chromosomal size. The main results coming from the molecular analysis of the micronuclear genome are: macronuclear precursor genes occur in clusters separated from each other by long non-coding regions, macronuclear precursor genes contain internal sequences, (internal eliminated sequences, IES) not found in the macronucleus, they are not associated with telomeric sequences and a large percentage of the micronuclear genome consists of transposon-like elements.

Several highly repetitious sequence families have been characterized in the micronucleus of different hypotrichous ciliates. For example, a 160 bp tandemly arranged sequence family is part of the highly repetitious sequences in *Stylonychia pustulata* (Dawson *et al.*, 1983), repeats of about the same size have been detected in *Oxytricha fallax* (Dawson *et al.*, 1984). Large, over 11 kb, DNA fragments belonging to a repetitive sequence family of the chromosomal DNA of *Oxytricha* were characterized (Boswell *et al.*, 1983).

Part of the micronuclear repetitious DNA-sequences are families of elements resembling transposons. Different classes of transposon-like elements have been described in the micronuclei of various hypotrichous ciliates (for review see Klobutcher and Jahn, 1991). A schematic drawing showing the different classes is shown in Figure 4. In *Euplotes crassus* two related families of transposon-like elements, TEC1 and TEC2 (Fig. 4 a) were characterized in the micronuclear genome. About 30.000 copies of these elements are found in the micronucleus and are absent in the macronucleus. They have a size of about 5.3 kb with 700-725 bp terminal inverted repeats and a 5' TA 3' target site duplication. Very often, these sequences interrupt macronuclear precursor sequences (Jahn *et al.*, 1988a,b, 1989; Baird *et al.*, 1989; Krikau and Jahn, 1991; Tausta *et al.*, 1991). *Oxytricha fallax* contains about 1.000 copies of the transposon-like element TBE-1 (telomere bearing element) in its micronuclear genome (Fig. 4b). It has an approximate size of 4 kb with 77 bp terminal inverted repeats surrounded by a 3 bp direct repeat, which appears to be the equivalent of a target site duplication. Telomeric C_4A_4-repeats are found at the ends of the inverted repeats (Herrick *et al.*, 1985; Hunter *et al.*, 1989). Both elements, TEC and TBE, appear to encode transposase proteins (Doak *et al.*, 1993). Another telomere containing element (TCE) was described in the micronucleus and the polytene chromosomes of *Stylonychia lemnae* (Fig. 4c). About 1.500 copies of this element are found in the micronucleus. It has a size of about 7 kb and is flanked by 2 kb direct repeats. Immediately adjacent to one of these repeats, an 18mer of the C_4A_4 telomeric repeat sequence is localized. In addition, sequences homologous to a 2.6 kb macronuclear DNA-molecule are found in this element. This sequence family is highly conserved showing almost 90 % homology to each other (Stoll *et al.*, 1993). It seems very likely that many of the other repetitive sequence families in hypotrichous ciliates will be shown to have transposon-like character.

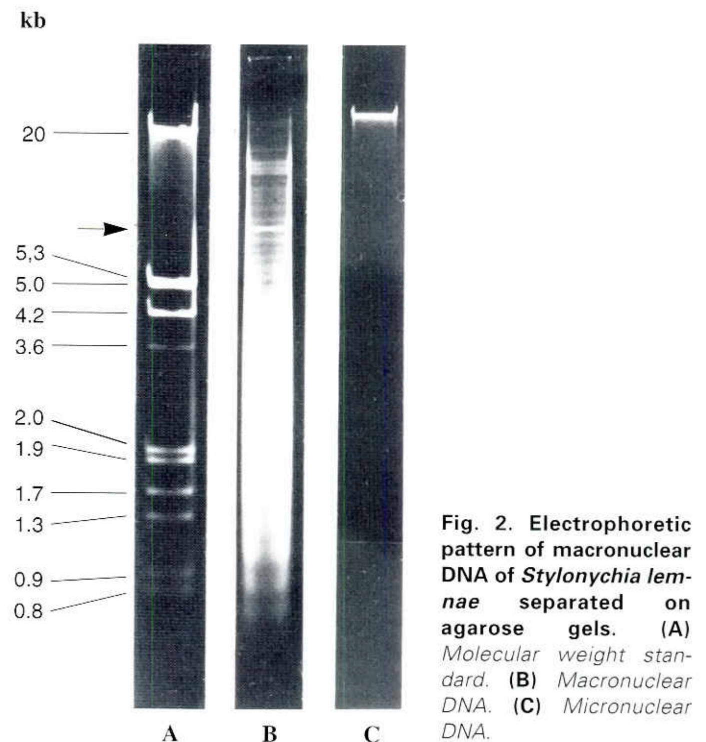

Fig. 2. Electrophoretic pattern of macronuclear DNA of *Stylonychia lemnae* separated on agarose gels. (A) *Molecular weight standard.* **(B)** *Macronuclear DNA.* **(C)** *Micronuclear DNA.*

The evolution and possible biological function of all these transposon-like elements is not clear; however, at least part of the TCE is transcribed at a specific stage during macronuclear development (Stoll *et al.*, 1993).

Macronuclear precursor sequences occur in the micronuclear genome in clusters of two to several genes. The individual clusters are separated by long (over 10 kb) non-coding sequences. Within a cluster, short spacer regions (0-600 bp) are found between the different macronuclear destined sequences (Boswell *et al.*, 1983; Klobutcher *et al.*, 1986; Jahn *et al.*, 1988a,b; Jahn, 1991; Eder *et al.*, 1993). An interesting sequence organization was described in a spacer region in *Stylonychia lemnae*; since only few sequence data are available no general rules about spacer sequence organization can be made at this point (Eder *et al.*, 1993). In no case analyzed are telomeric repeats associated with macronuclear precursor sequences, so that these sequences must be added to macronuclear sequences in the course of macronuclear development. Very often, macronuclear destined sequences are interrupted by short non-coding sequences (internal eliminated sequences, IES). Usually the different segments that comprise the macronuclear DNA-molecules are arranged in a linear order (Klobutcher *et al.*, 1986; Jahn *et al.*, 1988a,b; Jahn 1991; Bierbaum *et al.*, 1991; Eder *et al.*, 1993). However, in the case of the actin gene and the genes coding for telomere-binding proteins, the DNA-segments that will form the macronuclear DNA-molecules are scrambled in the micronuclear genome implying an extensive reorganization process during macronuclear development in order to create functional genes (Greslin *et al.*, Mitcham *et al.*, 1992; Prescott; 1992, 1994).

The internal eliminated sequences (IES) interrupting macronuclear precursor sequences are small (10-550 bp)

sequences and composed of unique DNA. On average one IES is found per 1 kb of macronuclear precursor sequence. The general structure of a typical IES is shown in Figure 4 d. Almost all of the IES are bordered by 2-7 bp direct repeats and very often imperfect inverted repeats are found near the ends (Klobutcher *et al.,* 1984; Herrick *et al.,* 1987; Ribas-Aparicio *et al.,* 1987; Jahn *et al.,* 1988a,b; Baird *et al.,* 1989; Hunter *et al.,* 1989; Bierbaum *et al.,* 1991; Eder *et al.,* 1993). This sequence organization led to the suggestion that IES may be degenerate versions of transposable elements (for review see: Klobutcher and Jahn 1991).

Macronuclear differentiation

While the morphological events occurring during macronuclear development are well described (for review see: Ammermann *et al.,* 1974; Kraut *et al.,* 1986), the molecular processes (summarized in Fig. 5) are still poorly understood.

Macronuclear differentiation starts with the mitotic division of the syncaryon. The factors determining which of the daughter nuclei will develop into the new macronucleus are unknown in hypotrichous ciliates. The first DNA reduction event can occur as early as the first DNA synthesis phase (Fig. 1). In *Stylonychia lemnae* only about 30% of the chromosomes enter the polytenization stage; the rest become pycnotic and eventually disintegrate (Ammermann *et al.,* 1974; Meyer and Lipps, 1981). The number of polytene chromosomes, having a 100-200 polyteny, has been estimated to be about 50-100 and the total number of bands in the polytene chromosomes is around 10.000-15.000 (Ammermann *et al.,* 1974). In *Oxytricha* all chromosomes become polytenized, the macronuclear anlage contains about 120 polytene chromosomes with a total number of bands of about 10.000 (Spear and Lauth, 1976). So far, no comparison of sequence copy numbers has been made between the micronucleus and the polytene chromosomes of the macronuclear anlagen. However, injection of macronuclear precursor sequences into the macronuclear anlage suggest that selective amplification of macronuclear destined sequences occurs during the first DNA-synthesis phase (Wen *et al.,* 1995). Sequences involved in the control of copy number have not yet been described.

In spread electron microscopical preparations of the developing macronucleus it became apparent, that during formation of the polytene chromosomes a dramatic reorganization of chromatin occurs. An increasing percentage of 30 nm chromatin fibers becomes organized into loop-like structures which

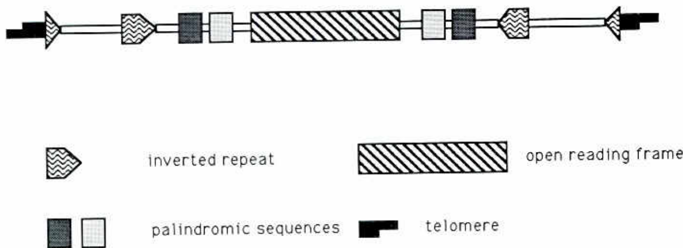

Fig. 3. Schematic diagram of a gene-sized macronuclear DNA-molecule from *Stylonychia lemnae* *(modified after Maercker and Lipps, 1993).*

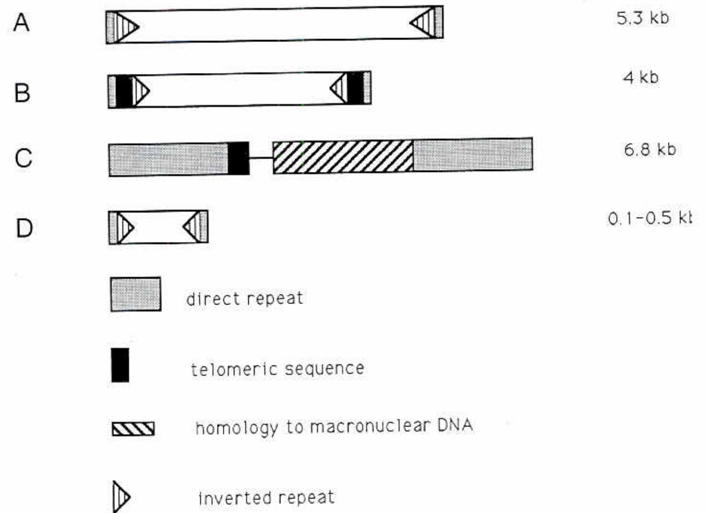

Fig. 4. Schematic diagrams of transposon-like elements and internal eliminated sequences hypotrichous ciliates. (A) *TEC,* **(B)** *TBE,* **(C)** *TCE,* **(D)** *IES (modified after Klobutcher and Jahn, 1991; Stoll et al., 1993).*

are interconnected by a 10 nm chromatin axis. Later, these 30 nm chromatin loops are released from the axis in the form of chromatin rings and eventually become degraded while the 10 nm chromatin axis remains intact (Meyer and Lipps, 1980; 1981). This morphological observation possibly can be related to the en masse excision of transposon-like elements and internal eliminated sequences in the course of polytene chromosome formation. In *Euplotes crassus* the elements TEC1 and TEC2 are eliminated from the genome during a discrete time period and their excision precedes the removal of IES. Both elements show similar excision mechanisms: They appear as free circles following excision. The junction of these free circular forms consists of both copies of the target site direct repeats with a few, normally 10, additional nucleotides between them. This implies that excision involves staggered cuts in the chromosome that are subsequently filled in and therefore the two excision products share some of the same nucleotides (Baird *et al.,* 1989; Jahn *et al.,* 1989; Tausta and Klobutcher, 1989, 1990; Krikau and Jahn, 1991; Tausta *et al.,* 1991; Klobutcher and Jahn, 1991; Jaraczewski and Jahn, 1993; Klobutcher *et al.,* 1993). The TBE-1 elements are also excised as circles but the junction contains only one direct repeat and no flanking DNA (Hunter *et al.,* 1989; Williams *et al.,* 1993).

The degradation of the polytene chromosomes becomes evident in electron microscopic thin sections by the appearance of membranous material surrounding the bands of the chromosomes. In later stages, bands are completely enclosed in vesicles (for review see Klobutcher and Prescott, 1986; Kraut *et al.,* 1986). It is assumed that in this stage fragmentation of the genome, degradation of DNA and eventually telomere addition occurs. In contrast to the holotrichous ciliate *Tetrahymena* very few data about DNA fragmentation and telomere addition exist in hypotrichous ciliates (for review see: Klobutcher and Jahn, 1991). In *Oxytricha* multiple fragmentation can be used, leading to different size-versions of macronuclear DNA-molecules

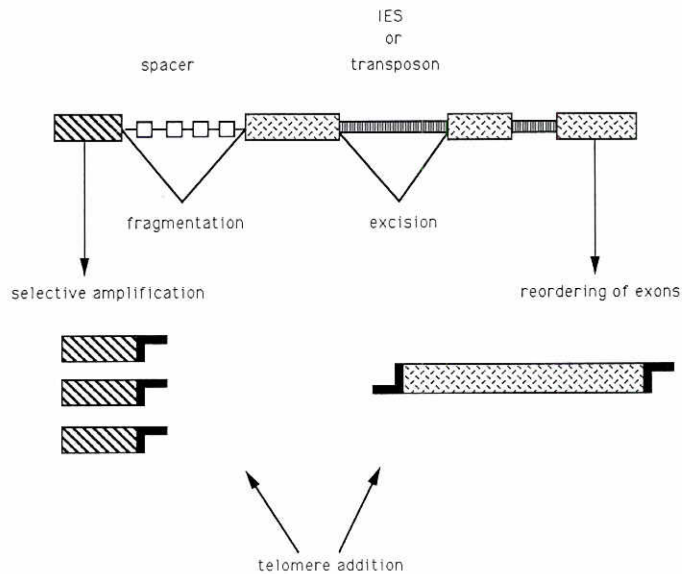

Fig. 5. Molecular events occurring during macronuclear differentiation in hypotrichous ciliates.

(Herrick *et al.,* 1987; Baird and Klobutcher, 1989). In contrast, *Euplotes crassus* uses only unique fragmentation sites. In this species a conserved sequence was found in the subtelomeric regions of macronuclear DNA-molecules which may be a candidate for a cis-acting sequence directing the fragmentation process (Baird and Klobutcher, 1989). A systematic analysis of the subtelomeric regions of *Stylonychia* macronuclear DNA-molecules revealed a highly ordered and common sequence organization and a model was proposed how some of these sequences may be involved in the DNA-fragmentation process (Maercker and Lipps, 1993). It is obvious, that for further identification of cis- and trans-acting factors responsible for specific fragmentation of the genome either cell-free systems or efficient transfection systems, as in the holotrichous ciliate *Tetrahymena*, have to be developed and both approaches are currently being established in hypotrichous ciliates (Wen *et al.,* 1995).

Following fragmentation of the genome telomeres have to be added de novo to macronuclear DNA-sequences. Initially, telomeres are added in an extended form and later trimmed to their normal size (Roth and Prescott, 1985; Vermeesch *et al.,* 1993; Vermeesch and Price, 1994). The mechanisms of this telomere addition are not yet clear. An enzyme, the telomerase, is capable of synthesizing telomeric repeats in a template independent way. This enzyme adds telomeric repeats to pre-existing telomeres *in vitro* (Greider and Blackburn, 1985), but experiments done in exconjugants of *Tetrahymena* suggest that telomerase is also able to add telomeres de novo to linear DNA *in vivo* (Yu and Blackburn, 1991). Whether other cofactors or cis-acting sequences associated with chromosomal ends are required for the de novo addition of telomeres still has to be determined. Once telomeres are added to macronuclear DNA-molecules they are probably protected from exonucleolytic digestion. Finally, a second round of replication then will amplify these molecules leading to the vegetative macronucleus containing only the minimum information needed for the regulation of normal ciliate cell growth.

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