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A subtelomeric DNA sequence is required for correct processing of the macronuclear DNA sequences during macronuclear development in the hypotrichous ciliate *Stylonychia lemnae*

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ABSTRACT

During macronuclear differentiation in ciliated protozoa a series of programed DNA reorganization processes occur. These include the elimination of micronuclear-specific DNA sequences, the specific fragmentation of the genome into small gene-sized DNA molecules, the *de novo* addition of telomeric sequences to these DNA molecules and the specific amplification of the remaining DNA molecules. Recently we constructed a vector containing the modified micronuclear version of macronuclear destined DNA sequences that was correctly fragmented and telomeres were added *de novo* after injection into the developing macronucleus. It therefore must contain all the *cis*-acting sequences required for these processes. We made a series of vectors deleting different sequences from the original vector. It could be shown that at least in the case studied here no micronuclear-specific sequences are required for specific fragmentation of the genome and telomere addition. However, a short subtelomeric sequence at the 3'-end is essential for these processes, whereas no specific cut seems to occur at the 5'-end. In addition, we can show that the processing activity is restricted to a short period of time during macronuclear differentiation and that a preceding transcription is required for correct processing of macronuclear-destined DNA sequences. Possible mechanisms of these processes will be discussed.

INTRODUCTION

In the course of macronuclear development in ciliated protozoa three major genomic changes occur: DNA elimination, DNA fragmentation and selective DNA amplification. The most extreme forms of these changes are observed in hypotrichous ciliates. A schematic diagram of macronuclear differentiation in *Stylonychia lemnae* is shown in Figure 1. After mitotic division of the zygote nucleus formed by the fusion of two haploid micronuclei a first DNA synthesis phase in the developing macronucleus (macronucleus anlagen) leads to formation of polytene chromosomes. During this period excision of internal eliminated sequences (IES) and transposon-like elements occurs (1–4). Soon after, the banded regions of these polytene chromosomes become enclosed into vesicles (vesicle stage), the chromosomes are degraded and as much as >95% of the DNA is eliminated from the nucleus resulting in a DNA-poor stage. The remaining DNA becomes specifically fragmented into short gene-sized DNA molecules, telomeres are added *de novo* and the DNA molecules become selectively amplified in a second DNA synthesis phase leading to the vegetative macronucleus. This vegetative macronucleus contains about 10 000–20 000 different DNA molecules with sizes between 0.4 and 20 kb, each occurring in a defined copy number which varies in the case of *S.lemnae* between ~10^3 and 10^6 copies per macronucleus (reviewed in 3,5,6).

The molecular analysis of the mechanisms involved in some of these genome rearrangement processes is greatly advanced in the holotrichous ciliate *Tetrahymena* and *cis*-acting sequences required for the specific fragmentation of the genome have been identified in this organism (reviewed in 7). Sequence analysis of *Euplotes* macronuclear DNA molecules revealed a consensus sequence in the subtelomeric region of many DNA molecules which was regarded as a candidate fragmentation sequence in this organism (8,9). Based on sequence data from more than 40 gene sized DNA molecules no consensus sequence could be detected in the subtelomeric regions of *Stylonychia* DNA molecules. Instead, a highly ordered sequence organization, including inverted repeats and palindromic sequences, was found in the subtelomeric regions and a model was proposed as to how this sequence organization could be involved in DNA processing (10). However, further analysis of *cis*-acting sequences required for DNA fragmentation and telomere addition was hindered in hypotrichous ciliates by the lack of efficient transfection and selection systems for these cells. Recently, we constructed a vector which contains the micronuclear version of two macronuclear DNA sequences flanked by micronuclear-specific DNA sequences. One of these precursor sequences was modified by the insertion of a

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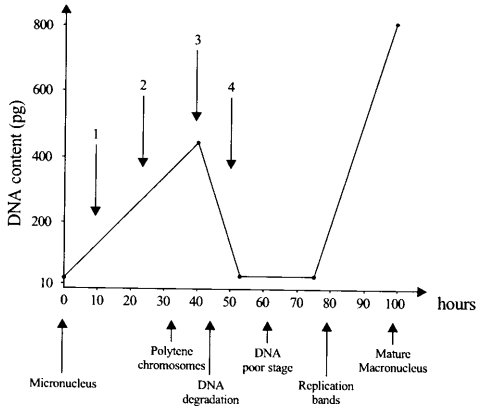

Figure 1. Schematic diagram of macronuclear development in *S.lemnae* (modified after 12,31). Arrows indicate the different time points of DNA injection into the macronuclear anlagen.

polylinker which allows its specific detection by PCR and Southern analyses. When this vector was injected into the macronuclear anlagen no IES excision occurred (11), but the macronuclear precursor sequences were correctly fragmented and telomeres were added *de novo*. Therefore, this vector must contain all sequences required for specific fragmentation of the DNA and *de novo* telomere addition (2,11). We made different deletion constructs of this vector, injected them into various stages of the developing macronucleus and showed that for specific fragmentation of the analyzed sequence no micronuclear-specific DNA sequences are required, but that all sequence requirements for this process are contained within the macronuclear precursor molecule.

MATERIALS AND METHODS

The hypotrichous ciliate *S.lemnae* was grown in neutral Pringsheim solution and fed with the algae *Chlorogonium elongatum* (12). To achieve conjugation, cells of two different mating types were mixed and the different stages of macronuclear development were determined by phase contrast microscopy. In general, the different vectors were injected into the fully developed macronucleus using a slightly modified procedure to that described earlier (11). The DNA concentration used for injection was lowered to 5–20 μg/ml and only ~0.05–1 pl were injected into one nucleus. Under these conditions 10–50 copies of the vectors should be injected each time. This decrease in DNA concentration and injection volume led to a significant higher survival rate of injected cells and in >90% of injected cells the vector DNA could be detected by PCR analysis. However, the hybridization signal observed on Southern blots becomes weaker under these conditions, suggesting that the concentration of the processed vector correlates to the amount of DNA injected. Cells were then allowed to finish macronuclear development and after approximately 15–20 cell divisions DNA was isolated from vegetative macronuclei. In some cases, earlier or later stages of macronuclear anlagen were injected, also sometimes DNA was already isolated from exconjugant cells only 4–10 h after injection. In general, at least 30 cells were injected with each construct; the efficiency of injection was determined to be at least 90%.

The DNA from injected cells was characterized by PCR, sequence and Southern analyses. For PCR analysis the DNA from 10–30 cells was isolated (11) and dissolved in 40 μl TE. Aliquots of 10 μl were used for one PCR reaction carried out as described by Saiki *et al.* (13). Primers used for construction of the vectors and PCR analysis are summarized in Table 1 and indicated in Figure 2. They all carry a restriction site for cloning in pUC 19 or pGem7ZF (for details see Fig. 2). The PCR program used was described earlier (11). Following PCR reactions samples were separated on a 1% agarose gel. When required, the gel was blotted onto nylon membranes (Qiagen) and hybridized with random primed probes (14), labeled either with digoxigenin-dUTP (Boehringer Mannheim) or [^{32}P]dCTP. For Southern analysis total macronuclear DNA isolated from approximately 10 000 cells was separated on a 1% agarose gel, blotted onto nylon membranes and hybridized with a digoxigenin-dUTP or [^{32}P]dCTP labeled probe under conditions described earlier (11). For sequence analysis the PCR products obtained using the primer combination P9/P548 were cloned in pCR2.1-TOPO (Invitrogen) and sequenced according to Sanger *et al.* (15). Inhibition of RNA synthesis in *Stylonychia* cells was achieved with 50 μg/ml Actinomycin D.

RESULTS

The original vector pCE5 (Fig. 2a) carries two macronuclear precursor sequences homologous to a 1.1 and 1.3 kb macronuclear DNA molecule (16, GenBank accession nos X72955 and X72956). Two IES are found in the 1.1 kb and three IES in the 1.3 kb precursor sequence. These sequences are separated by an 11 bp spacer. When this vector was injected into the developing macronucleus it was correctly fragmented and telomeres were added, but no IES excision was observed (2,11). These results demonstrated that all sequences required for correct DNA fragmentation and telomere addition are present on this vector. Based on vector pCE5 several deletion constructs were made and functionally tested by injection into the macronuclear anlagen. All vectors (Fig. 2b) were constructed by PCR amplification of the desired fragment using pCE5 (Fig. 2a) as a template. The primers used for these constructions are indicated in Figure 2b and summarized in Table 1.

All vectors were injected into the macronuclear anlagen in the polytene chromosome stage. Cells were then allowed to finish macronuclear development and the presence of the processed modified macronuclear precursor sequence in the vegetative macronucleus was tested by PCR analysis performed with the DNA isolated from 10–30 vegetative cells. To control PCR conditions all different primer combinations were first tested with the various vectors. Vector DNA (10^{-8} μg) was used as a template, which corresponds to the approximate amount of vector injected into the cells. The presence of the modified 1.3 kb DNA molecule in injected cells was then further demonstrated by Southern analysis with DNA isolated from macronuclei of these cells. For PCR analysis seven primers, P5, P7, P9, P10, P20, P548 and P549 were used. P7, P9 and P20 are derived from the 1.3 kb precursor sequence, P5 is located in IES4, P548 and P549 are derived from the polylinker sequence inserted into the 1.3 kb precursor

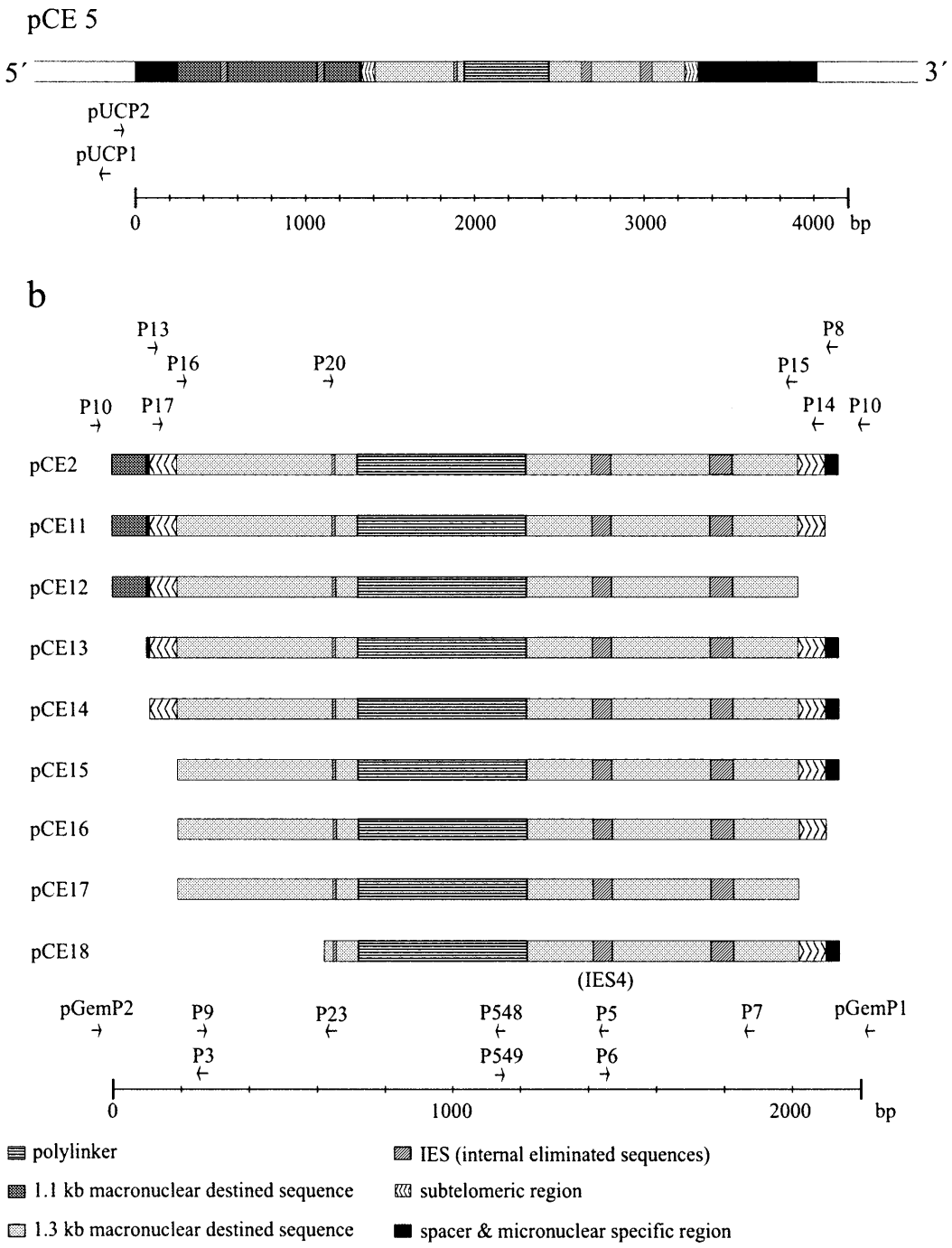

Figure 2. Schematic diagram of vector constructs used for injection into the macronuclear anlagen. (**a**) Vector pCE5, this vector contains two macronuclear precursor sequences homologous to a 1.1 and a 1.3 kb macronuclear DNA molecule (24). The 1.1 kb homologous precursor sequence contains two IES, the 1.3 kb homologous sequence contains three IES. The two precursor sequences are separated by an 11 bp spacer and flanked by micronuclear-specific DNA sequences on both sides. The 1.3 kb homologous precursor sequence was modified by the insertion of a 500 bp polylinker sequence (11). (**b**) Vectors pCE2–pCE18, different deletion constructs derived from vector pCE5. Primers used for the construction of these vectors and for PCR analysis after injection into the developing macronucleus are indicated above and below these constructs and are listed in Table 1.

sequence and P10 is a telomeric primer (Table 1, Fig. 2b). Results of these PCR analyses are shown in Figure 3a and b. Using the combination P9/P548 (in the case of pCE18 primer P9 was replaced by primer P20) only PCR fragments from cells containing the vector were obtained; no PCR product was obtained in non-injected cells (Fig. 3a and b, lanes 1, 4, 7, 10).

Table 1. Primers used for construction of the various deletion constructs (Fig. 2b) and for PCR analyses of uninjected and injected cells

Primer	Sequence 5'→ 3'	derived from
P5	CTCATTCTTATTATAATCCCAATATAAGCAC	IES4 in the 1.3 kb macronuclear precursor
P7	CAACTCCCTGCAACATACAC	1.3 kb macronuclear precursor
P8	GCGGGATCCATCTTCATTAAA	micronuclear sequences flanking the 3'end of the 1.3 kb macronuclear precursor
P9	GGCTCGAGTTGCTACTTCTAGATATTC	1.3 kb macronuclear precursor
P10	CCCCAAAACCCCAAAACCCC	telomeres
P13	GGATCCGATCATAAACTGATTACTCAGG	spacer region between the 1.1 kb and 1.3 kb precursor sequences
P14	CCGAGGTACCGCGGTCAGAAATAATAGTTG	3'-end of the 1.3 kb precursor sequence
P15	CGGAGGTACCCGATATTTAAAATCATTAATC	3'-region of the1.3 kb precursor sequence
P16	CGCAGGATCAAATCAAACAAATAAATACG	5'-region of the 1.3 kb precursor sequence
P17	CCGCGGATCCGATTCTTTAGAATAATATTT	5'end of the 1.3 kb precursor sequence
P20	CCGCAGGATCCTTGAGAGTCTGCCATTTAA	coding region of the 1.3 kb macronuclear DNA-molecule
P23	GTTAAATGGCAGACTCTCTCAAGAAGAAATGC	coding region of the 1.3 kb macronuclear DNA-molecule
P548	CTGCAGGTCGACTCTAGAGCTC	polylinker sequence
P549	GAGCTCTAGAGTCGACCTGCAG	polylinker sequence
pUCP1	GTCGACCTGCAGGCATGCAAGCTT	pUC
pUCP2	CGGGTACCGAGCTCGAATTC	pUC
pGemP1	CGCATGCTCCTCTAGACTCGAGGAATTCGG	pGem
pGemP2	GCTATGCATCCAACGCGTTGGGAGCTCTCC	pGem

All the PCR products synthesized from DNA of injected cells were cloned and sequenced. In all cases an almost identical sequence was obtained which includes the polylinker as well as IES3 present in the 1.3 kb homologous precursor sequence; insertions of 1 bp or mutations are most probably due to PCR or sequencing mistakes (Fig. 4). Also the primer combination P9/P5 leads only to a PCR product from vector containing cells (Fig. 3a and b, lanes 2, 5, 8, 11); however, in PCR analysis from 10 to 30 cells sometimes an additional, 500 bp smaller fragment is observed which is synthesized from micronuclear DNA. With the primer combination P9/P7 two PCR fragments are amplified in vector containing cells, a 1190 bp fragment is synthesized from the endogenous 1.3 kb macronuclear DNA molecule and a 1830 bp fragment is synthesized from the injected vector which contains the polylinker sequence and three IES (Fig. 3a and b, lanes 3, 6, 9, 12). Using this primer combination sometimes only the smaller fragment was amplified, probably because the injected vector was present in a very low copy number in relation to the endogenous 1.3 kb macronuclear DNA molecule. To test whether the vectors occur as circular products, a PCR reaction with DNA from transfected cells using the primer combination P3/P6 was performed. In no case was a PCR product obtained from injected cells, in agreement with previous observations (11). To demonstrate that the injected constructs were fragmented correctly and telomeres were added, a PCR reaction was made using a telomeric primer (P10) and a primer derived from the polylinker region (P548, P549). With all vector constructs, which gave the expected PCR products using the other primer combinations, a signal was observed above a strong background. One example is shown in Figure 3a, lane 14. The specificity of this band was demonstrated by hybridization with the polylinker (Fig. 3b, lane 14). The processed vectors were further detected by Southern hybridization; examples are shown in Figure 3c. While in uninjected cells (Fig. 3c, lane 1) only a hybridization to the 1.1 and the 1.3 kb macronuclear genes is observed, similar to previous observations (11), in transfected cells the processed vector is detected as an additional 1.95 kb molecule, which gives, due to the low amount of DNA injected, a significantly weaker signal compared with the endogenous molecules (Fig. 3c, lanes 2–4).

Results from all PCR experiments, using macronuclear DNA from cells injected with the different constructs as a template, are summarized in Table 2. The first vector tested was pCE2. On the 5'-end the 1.3 kb macronuclear precursor sequence is flanked by an 11 bp spacer and 100 bp of the 1.1 kb precursor sequence. On its 3'-end it contains 43 bp micronucleus-specific DNA sequences. As demonstrated by PCR and Southern analyses this vector was correctly fragmented and the modified 1.3 kb DNA molecule replicated in the macronucleus as a linear molecule, suggesting that, as in the case of pCE5, telomeric sequences were added *de novo* to this molecule. This result shows that only very few, if any, micronucleus-specific DNA sequences are required for correct processing of the injected construct. In fact, the observation that also vector pCE11, which contains no micronuclear-specific sequences, was correctly processed demonstrates that no flanking micronuclear DNA sequences are required for these processes (Fig. 3a and b, lanes 7–9). Deletion of sequences from the 1.1 kb macronuclear precursor sequence (vector pCE13) and deletion of the spacer (vector pCE14) had no effect on vector processing, demonstrating that no interaction between subtelomeric

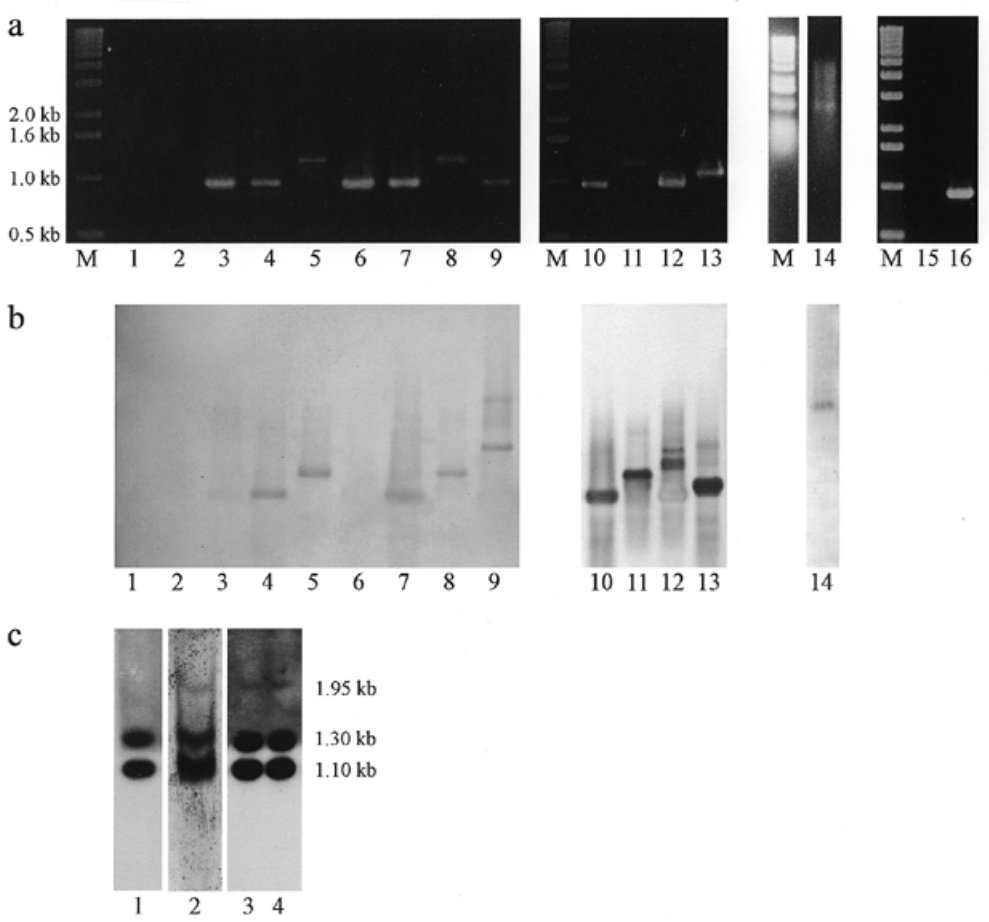

Figure 3. Injection of the different vector constructs into the developing macronucleus. The presence of the injected constructs and their processing was analyzed in the macronuclear DNA of injected cells by PCR analysis and Southern hybridization. (**a** and **b**) Examples of a PCR analysis of control cells and injected cells, a summary of the results obtained by PCR analysis and Southern hybridization is given in Table 2. All vectors were injected into the polytene chromosome stage of macronuclear anlagen, injection conditions are described in Materials and Methods. After injection the cells were allowed to finish macronuclear development. DNA was isolated from 10–30 cells and used as a template for PCR analysis. Primers used were P9, P548, P5, P7 and P17. (a) The PCR products were separated on a 1% agarose gel. M, molecular weight marker (1 kb ladder, Gibco BRL); lanes 1–3, PCR products from uninjected control cells using the primer combinations P9/P548 (lane 1), P9/P5 (lane 2) and P9/P7 (lane 3); lanes 4–6, PCR products from cells injected with vector pCE5 using the primer combinations P9/P548 (lane 4), P9/P5 (lane 5) and P9/P7 (lane 6); lanes 7–9, PCR products from cells injected with vector pCE11 using the primer combination P9/P548 (lane 7), P9/P5 (lane 8) and P9/P7 (lane 9); lanes 10–13, PCR products from cells injected with vector pCE15 using the primer combinations P9/P548 (lane 10), P9/P5 (lane 11), P9/P7 (lane 12) and P17/P548 (lane 13); lane 14, PCR products from cells injected with vector pCE13 using the telomeric primer P10 in combination with primer P548; lanes 15 and 16, PCR products from a mixture of vector pCE15 and total cellular DNA. 100 ng of total DNA were mixed with 10^{-2} ng vector DNA. Primer combinations were P17/P548 (lane 15) and P9/P548 (lane 16). (b) The gel was hybridized with a Dig-labeled probe of the polylinker sequence inserted into the 1.3 kb homologous precursor sequence. Using Dig-labeled probes the intensity of the signal does not necessarily reflect the absolute amount of DNA present in the gel. Moreover in some cases non-specific PCR products cross-hybridizing with the probe were obtained. One example is shown in lane 12. (**c**) Southern analysis of macronuclear DNA from uninjected cells and cells injected with different vector constructs. After injection cells were allowed to finish macronuclear development. Macronuclear DNA was isolated from about 10 000 cells, separated on a 1% agarose gel, blotted onto nylon membranes and hybridized with a ^{32}P-labeled pCE5 probe. Lane 1, macronuclear DNA from uninjected cells; lane 2, macronuclear DNA from cells injected with vector pCE5; lane 3, macronuclear DNA from cells injected with pCE13; lane 4, macronuclear DNA from cells injected with pCE15.

regions of different precursor sequences or with the spacer is required for DNA processing.

After injection of vector pCE12, which lacks 70 bp of the 3'-subtelomeric sequences of the 1.3 kb macronuclear precursor molecule, no processed product could be detected in injected cells. This may be explained by the fact that either the deleted subtelomeric sequences are required for correct processing or for subsequent amplification and replication of the processed product. To distinguish between these possibilities vectors pCE5 and pCE12 were injected in 20–25 exconjugants in the polytene chromosome stage. These cells were then allowed to continue macronuclear development for another 6 h. DNA was prepared from these cells and a PCR analysis was performed using primers either derived from the modified 1.3 kb precursor sequence (P9/P548 and P9/P7) or by using one primer from the 1.3 kb precursor sequence and the others from pUC19 sequences in the case of pCE5 (pUCP1/P20 and pUCP2/P23) or from pGem in the case of pCE12 (pGemP1/P20 and pGemP2/P23) flanking the insert. As shown in Figure 5 after injection of pCE5 PCR products were only obtained using

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                          550
                          |
a) endogenous sequence    GAGATGCTGC CATTTA~CAT T********* ***ACAATGA TTTGAGTT~A
                          |||||||||| |||||| ||| |            |||||||| |||||||| |
b) modified sequence      GAGATGCTGC CATTTAACAT TTGGAGTTTG AGGACAATGA TTTGAGTTTA

                          586                                                628
                          |                                                  |
a) endogenous sequence    GACTCAGTT~ CTAGCTGTT~ ATGGACGTAA TTTAGGGATA CAAGA*****
                          |||||||||  ||||| |||  |||||||||| |||||||||| |||||
b) modified sequence      GACTCAGTTT CTAGCAGTTT ATGGACGTAA TTTAGGGATA CAAGATCCCC


a) endogenous sequence    ********** ********** **

b) modified sequence      GTCAGTGTTT TCTAATGGGT AC


IES3 = TGGAGTTTG AGG
start of polylinker = TCCCC GTCAGTG....
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Figure 4. Sequence analysis of PCR products synthesized from transfected cells using the primer combination P9/P548. A similar sequence was obtained from all cells transfected with the different constructs. (**a**) Endogenous sequence of the 1.3 kb macronuclear DNA molecule (24), (**b**) sequence obtained from a PCR product (primer combination P9/P548) from injected cells. IES3, not present in the endogenous macronuclear sequence, is underlined, the polylinker sequence is shown in bold.

Table 2. Summary of PCR reactions using the different vectors as template

Template	Primer combinations											Processed
	P9/P548	P9/P5	P9/P7 end.	P9/P7 exog.	P17/P548	pUCP1/ P20	pUCP2/ P23	pGemP1/ P20	pGemP2/ P23	P10/P548	P10/P549	
pCE5	+ 18/20	+	+	(+)	n.t.	-	-	n.t.	n.t.	+	+	+
pCE2	+ 17/21	+	+	(+)	n.t.	n.t.	n.t.	n.t.	n.t.	+	+	+
pCE11	+ 25/30	+	+	(+)	n.t.	n.t.	n.t.	n.t.	n.t.	+	+	+
pCE12	- 0/45	-	+	-	n.t.	n.t.	n.t.	+	+	n.t.	n.t.	-
pCE13	+ 23/25	+	+	(+)	n.t.	n.t.	n.t.	n.t.	n.t.	+	+	+
pCE14	+ 22/25	+	+	(+)	n.t.	n.t.	n.t.	n.t.	n.t.	+	+	+
pCE15	+ 28/33	+	+	(+)	+	n.t.	n.t.	n.t.	n.t.	+	+	+
pCE16	+ 17/24	+	+	(+)	+	n.t.	n.t.	n.t.	n.t.	+	+	+
pCE17	- 0/47	-	+	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-
pCE18	P20/P548 - 0/43	P20/P5 -	P20/P7 +	P20/P7 -	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-

The primer combination P9/P548 was used to determine the ratio of positive cells (first number) versus the number of all cells tested (second number). Using the primer combination P9/P7 PCR products from the endogenous, as well as in many cases the injected exogenous DNA (+), could be obtained. Various primer combinations were not relevant for specific constructs and therefore not tested (n.t.).

primers from the modified 1.3 kb macronuclear precursor sequence (P9/P548 and P9/P7, Fig. 5, lanes 1 and 2). No PCR product was synthesized using a combination in which one primer is derived from the 1.3 kb macronuclear precursor sequence and the other from pUC19 (pUCP1/P20 and pUCP2/P23, Fig. 5, lanes 3 and 4), strongly suggesting that pCE5 is fragmented at this time point. In control experiments using vector pCE5 as template strong PCR products were achieved, demonstrating that the lack of PCR products obtained from injected cells was not due to PCR conditions. However, after injection of pCE12 PCR products from all primer combinations were obtained (Fig. 5, lanes 5–8), demonstrating that

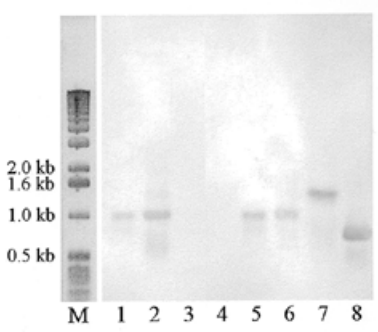

Figure 5. Functional analysis of the 3'-subtelomeric sequence of the 1.3 kb homologous macronuclear precursor sequence. Vectors pCE5 and pCE12 were injected into the macronuclear anlagen in the polytene chromosome stage of 20–30 cells, 4 h later DNA was isolated and used for PCR analysis. M, molecular weight marker (1 kb ladder); lanes 1–4, PCR analysis of cells injected with vector pCE5 using the primer combinations P9/P548 (lane 1), P9/P7 (lane 2), pUCP1/P20 (lane 3) and pUCP2/P23 (lane 4); lanes 5–8, PCR analysis of cells injected with vector pCE12 using the primer combinations P9/P548 (lane 5), P9/P7 (lane 6), pGemP1/P20 (lane 7) and pGemP2/P23 (lane 8).

the deleted 70 bp 3'-subtelomeric sequences are indeed required for specific DNA fragmentation and telomere addition but not just for amplification of this DNA molecule. Moreover, since PCR products were obtained with primers derived from pGem sequences flanking the insert on the 3'- and the 5'-end, this deleted 3'-subtelomeric DNA sequence is not only involved in a cut at the 3'-end but also at the 5'-end. Therefore, this sequence is either the only specific sequence required for processing of macronuclear DNA or the failure to fragment at the 5'-end is due to the fact that it has to interact specifically with a sequence located at a similar position in the 5'-subtelomeric region, as suggested by the model of Maercker and Lipps (10). To test this hypothesis, 69 bp of the 5'-subtelomeric sequences were deleted in vector pCE15. Injection of this vector into the macronuclear anlagen still led to a processed product in the vegetative macronucleus (Fig. 3a and b, lanes 10–12). This result strongly argues against the previously suggested model in which the two subtelomeric regions interact specifically resulting in a loop-like structure subsequently resolved by specific nucleases (10). Similarly, vector pCE16 also became processed, demonstrating that neither the micronuclear-specific DNA sequences nor the 5'-subtelomeric region are required for specific DNA fragmentation and telomere addition. Only after deletion of >500 bp of the 5'-sequences from the 1.3 kb precursor molecule (vector pCE18) no more processed product could be detected by PCR analysis in the macronucleus of vegetative cells. Therefore, our results clearly demonstrate that no interactions between the two subtelomeric regions are required for processing although so far we can not definitely rule out the possibility that the 3'-subtelomeric sequence interacts with a sequence located further downstream of the 5'-subtelomeric region. No processed product could be detected after injection of vector pCE17, which lacks the micronuclear-specific DNA sequences as well as both subtelomeric regions. This observation is in agreement with the results obtained after injection of vector pCE12, which suggested that the 3'-subtelomeric region is essential for processing. In *Oxytricha nova* the use of alternative telomere addition sites has been described during macronuclear differentiation (17). Although this phenomenon has never been observed in *S.lemnae* (unpublished), we analyzed the processed product from pCE15 by PCR, sequence analysis and Southern hybridization to examine this possibility. When a primer combination was used in which one primer is derived from the polylinker sequence (primer P548) and the other one from the deleted 69 bp subtelomeric sequences (primer P17) we still could detect a PCR product (Fig. 3a and b, lane 13). Southern analysis demonstrated that the processed product had the same size as that from vector pCE5 (Fig. 3c, lane 4). At present we cannot explain this result. To test the probability that this is due to a PCR artefact by template switching, we performed control PCR reactions in which vector pCE15 DNA was mixed with total cellular DNA from uninjected cells. Under our experimental conditions about 10–50 vector molecules are injected into one cell. The macronucleus contains about 10 000 endogenous copies of this sequence and at least 10 000 different DNA molecules are present in one macronucleus. Therefore the ratio of injected DNA to total cellular DNA is at least below 10^{-6}. We mixed 100 ng of cellular DNA with 10^{-1}–10^{-4} ng pCE15 vector DNA and performed a PCR reaction using the primer combinations P17/P548 and P9/P548. While a PCR reaction using the primer combination P9/P548 leads to the expected PCR product, no such product was obtained using the primer combination P17/P548 (Fig. 3a, lanes 15 and 16). Only by increasing the total concentration of DNA by at least 10-fold and the ratio of vector DNA to cellular DNA to at least 1:10, low amounts of unspecific P17/P548 PCR products were sometimes amplified. This result makes a PCR-artefact very unlikely and probably other, so far uncharacterized mechanisms, could be postulated. Eventually a sequence analysis of the 5'-end of the processed product will give further insight into the mechanisms involved in this process.

A specific processing activity should be restricted to a short period during macronuclear development, furthermore it may depend on a preceding transcriptional activity. We therefore determined the time period during macronuclear differentiation until which the processing of an injected vector can be observed and analyzed whether transcription is necessary for processing of macronuclear-destined DNA sequences. We found that vectors injected in the very early anlagen stage up to the polytene chromosome stage (Fig. 1, stages 1–3) are correctly fragmented and telomeres are added. However, as soon as exconjugants enter the vesicle stage of macronuclear development (Fig. 1, stage 4) injected vectors are no longer processed. Finally we determined whether vector processing is dependent upon transcriptional activity. RNA synthesis was therefore inhibited at various time points during and after conjugation. When Actinomycin D is added at the beginning of mating, conjugation pairs can separate but do not form macronuclear anlagen. Inhibition at the very end of conjugation or at early stages of macronuclear development leads to exconjugants which are still able to form polytene chromosomes suggesting that by the end of conjugation all RNA required for the formation of polytene chromosomes is already synthesized. However, these exconjugants stop macronuclear development in the polytene chromosome stage, which may indicate that for further macronuclear differentiation a preceding transcription is necessary. To analyze whether this transcriptional activity is

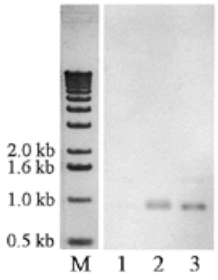

Figure 6. DNA processing after inhibition of RNA synthesis with Actinomycin D. Analysis of DNA fragmentation was performed as described in Materials and Methods. The primer combination P8/P20 was used for PCR analysis. Lanes 1 and 2, control cells without Actinomycin D: lane 1, exconjugants 50 h after cell separation; lane 2, exconjugants 35 h after cell separation; lane 3, exconjugants where RNA synthesis was inhibited with Actinomycin D 65 h after cell separation.

required for specific DNA fragmentation, exconjugants of the same developmental stage were collected and either allowed to complete macronuclear differentiation or Actinomycin D was added ~15 h after separation of conjugants. After various time intervals cells were lysed and 20–30 macronuclear anlagen were collected under the inverted microscope thus avoiding any contamination with micronuclei. PCR analysis was performed using primers derived from micronuclear-specific sequences (P8) and from the 1.3 kb molecule (P20). As shown in Figure 6, fragmentation has completed in normal development ~40–42 h after cell separation (Fig. 6, lanes 1 and 2); however, in cells where RNA synthesis was inhibited a PCR fragment was obtained even 65 h after separation of conjugant cells (Fig. 6, lane 3), demonstrating that no DNA fragmentation had occurred.

DISCUSSION

Although programed DNA reorganization and DNA elimination processes are frequently observed in eukaryotic cells (for examples see 18–25), they never occur to the same extent as those described in hypotrichous ciliates. In the course of macronuclear differentiation as much as >95% of micronuclear DNA sequences are eliminated, the remaining DNA becomes specifically fragmented into short gene-sized DNA molecules and telomeres are added *de novo* (reviewed in 5). While in the distantly related holotrichous ciliate *Tetrahymena thermophila* a sequence element (chromosome breakage sequence element, Cbs-element) located in the developmentally eliminated DNA flanking the macronuclear-destined sequence has been shown to be necessary and sufficient to direct chromosome fragmentation and telomere addition (reviewed in 7), very little is known about *cis*-acting sequences involved in DNA processing in hypotrichous ciliates. Based on sequence data from individual macronuclear DNA molecules and adjacent micronuclear DNA sequences, models for DNA fragmentation and telomere addition have been proposed. In *Euplotes crassus* a 10 bp AT-rich conserved sequence located either in the subtelomeric region of macronuclear DNA molecules or in micronuclear DNA flanking these molecules has been identified. A model for chromosome fragmentation and telomere addition was suggested that involves a staggered cut in the chromosome (9). No such consensus sequences were found in *S.lemnae*, although due to the high AT-content of subtelomeric regions several sequences similar but not identical to the *E.crassus* sequence can be detected at various locations in subtelomeric regions. However, in *S.lemnae* a highly ordered subtelomeric sequence organization is found in all macronuclear DNA molecules. Previously, a model was proposed (10) in which inverted repeats present in the two subtelomeric regions of one macronuclear-destined DNA molecule can interact forming a loop-like structure, which can be resolved by an endonuclease such as restriction endonuclease type III (26), or an endonuclease recognizing a tetrad structure such as described by Lilley and Kemper (27). In the case of the 1.3 kb macronuclear molecule a 4 bp inverted repeat (5'-CCAG) is present adjacent to both telomeric sequences (position 3 at the 3'-end, position 6 at the 5'-end). A 12 bp palindromic sequence is found at position 100 in the 3'-region and an 8 bp palindromic sequence at position 60 in the 5'-region. In addition, a 10 bp CA repeat at position 60 is present only in the 3'-subtelomeric region (16).

In this paper we used a biological approach to identify *cis*-acting sequences involved in DNA processing in hypotrichous ciliates. These experiments were based on a previously constructed vector system that was shown to contain all sequence requirements for correct DNA fragmentation and telomere addition (11). Various sequence elements of the original vector pCE5 were deleted; the new constructs were injected into the macronuclear anlagen and the processing of the macronuclear-destined DNA sequence present on these vectors was examined by PCR, sequence and Southern analyses. Constructs in which all micronuclear-specific DNA sequences adjacent to the macronuclear precursor sequences were deleted, or in which only the 1.3 kb homologous macronuclear sequence were present became correctly fragmented and telomeres were added. Thus, unlike in *Tetrahymena* where the Cbs required for fragmentation resides in the micronuclear sequences flanking the macronuclear-destined sequence (7), in *Stylonychia* sequences involved in fragmentation and telomere addition (Cbs) reside within the analyzed precursor molecule itself. In addition, obviously no interactions between sequences of different precursor molecules occurring within this cluster in the micronuclear genome are necessary for these processes. Of course, we cannot generalize this statement since it may well be that some Cbs are located outside the precursor sequences. Using a similar approach as described here, but inserting very many different macronuclear-destined sequences and their flanking micronuclear-specific DNA into such a vector system, could solve this question. When the 3'-subtelomeric region of the 1.3 kb homologous precursor sequence was deleted we never could observe any processed product in the vegetative macronucleus. Since we could demonstrate that such a vector becomes indeed not fragmented in the course of macronuclear development we have strong evidence that the Cbs must reside within these 69 deleted base pairs. The only sequence peculiarities found in the 3'-subtelomeric region is a short repeat, which is also present in the 5'-subtelomeric region and, in addition, a 10 bp CA-repeat at position 60, which has the capacity of forming a Z-DNA structure (28). Since no consensus sequences are found in the subtelomeric regions of *Stylonychia* macronuclear DNA molecules, one could speculate that the Cbs in hypotrichous ciliates is not determined by a sequence but rather by a

sequence motif or a DNA structure. Surprisingly, after deletion of the 5'-subtelomeric region as well as after deletion of all micronuclear-specific DNA sequences (vectors pCE15 and pCE16) we could still observe DNA processing by PCR and Southern analyses. Only after deletion of several hundred base pairs from the 5'-end a processed product could no longer be detected in the vegetative macronucleus. According to these results no interaction between the two subtelomeric regions is required for correct processing although at present we cannot rule out the possibility that the 3'-subtelomeric Cbs interacts with another sequence located downstream of the 5'-subtelomeric region. This observation is in contrast to our previously suggested model in which two specific sequences located in both subtelomeric regions would be required for correct processing (10), but fits more with the data recently described by Klobutcher *et al.* (9). There is an even more surprising result we cannot explain at present: Vector pCE15, in which the 5'-subtelomeric region of the 1.3 kb homologous macronuclear precursor sequence was deleted, became not only correctly fragmented and telomeres added, but the processed product contained again the previously deleted subtelomeric region. Several explanations are possible. It could be a PCR artefact in which one primer binds to the vector and the other primer derived from the 5'-subtelomeric sequence binds to the endogenous sequence. However, our control PCR experiments make this possibility very unlikely as unspecific PCR products were only obtained using very high concentrations of both total and vector DNA. In addition, on Southern blots the processed product seemed to have the same size as the product synthesized e.g. from pCE5 injected cells. Unfortunately, due to the weak signal we obtain on Southern blots, we could not perform a restriction digest to determine the very exact size of the 5'-terminus of the processed molecule. Another possibility could be that recombination events between the injected vector and the endogenous precursor sequence take place. But again this should be a rare event and one would not expect a PCR product of the intensity we observe. Further experiments are required to definitely exclude an experimental artefact, only then could one speculate that hypotrichous ciliates possess some so far uncharacterized mechanism to correct the processed product.

Finally, we determined whether RNA synthesis is required for processing macronuclear destined DNA sequences. When RNA synthesis was inhibited exconjugants never developed beyond the polytene chromosome stage. Indeed, it could be shown that transcription is required for completion of macronuclear differentiation and no DNA fragmentation is observed after inhibition of RNA synthesis. This failure of DNA processing after inhibition of transcription with Actinomycin D could be due to the fact that e.g. *trans*-acting factors required for these processes are transcribed during this time period or that specific transcripts themselves are needed for correct processing. Further sophisticated experiments will be necessary to distinguish between these possibilites. Finally, it also has to be determined which of the nuclei in the exconjugant cell, i.e. old macronucleus, macronuclear anlagen or micronuclei, becomes transcribed during macronuclear development and the specific transcripts have to be characterized.

From the sequence data available for *Stylonychia* and the results presented in this study, the following picture for DNA processing in *Stylonychia* and probably for hypotrichous ciliates in general emerges. As described in other ciliates DNA fragmentation and *de novo* addition of telomeric sequences are temporally closely related (reviewed in 3) and presumably require the same *cis*-acting DNA sequences. At least in the case studied here this sequence is located in one subtelomeric region of the macronuclear precursor sequence and this sequence is essential for DNA fragmentation and telomere addition. No interactions with micronuclear-specific DNA sequences or other precursor sequences present in this gene cluster are necessary for these processes. No consensus sequence could be detected in the subtelomeric regions of the gene-sized macronuclear DNA molecules of *Stylonychia* studied so far. It may well be that in hypotrichous ciliates a Cbs is not necessarily defined by its primary sequence but by a sequence motif or a structure it can adopt. Our results suggest that one specific cut is made at the 3'-end, the cut at the other side can be corrected by some so far unknown mechanism. Possibly a transcript from the old macronucleus could act as a template for DNA repair, similar to an RNA-based model of evolution recently suggested by Herbert and Rich (29). Epigenetic modifications of germ line sequences by nucleic acids derived from the old macronucleus were also hypothesized by Meyer and Duharcourt (30). Processing activity is restricted to a defined period during macronuclear development and RNA synthesis is required for specific DNA fragmentation and completion of macronuclear differentiation. Still, some important questions, such as the nature of *trans*-acting factors involved in DNA processing, the possibly existing mechanism for correcting fragmentation and the characterization of the transcripts required for processing, have to be answered.

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