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The processing of macronuclear-destined DNA sequences microinjected into the macronuclear anlagen of the hypotrichous ciliate *Stylonchia lemnae*

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ABSTRACT

We describe the construction of a vector carrying the micronuclear versions of two macronuclear DNA molecules, one of which was modified by the insertion of a polylinker sequence. This vector was injected into the polytene chromosomes of the developing macronucleus of *Stylonychia* and its processing during further macronuclear development and its fate in the mature macronucleus were analyzed. In up to 30% of injected cells the modified macronuclear DNA sequence could be detected. While the internal eliminated sequences (IES) present in the macronuclear precursor DNA sequence are still retained in the mature macronucleus, the modified macronuclear DNA sequence is correctly cut out from the vector, telomeres are added *de novo* and it is stably retained in the macronucleus during vegetative growth of the cells. This vector system represents an experimental system that allows the identification of DNA sequences involved in the processing of macronuclear DNA sequences during macronuclear development.

INTRODUCTION

After sexual reproduction in ciliates a new macronucleus is formed by a micronuclear derivative. In hypotrichous ciliates macronuclear development includes the formation of polytene chromosomes, elimination and degradation of many of the micronuclear-specific DNA sequences and the fragmentation of the macronuclear genome into small gene-sized DNA molecules. There exist ~10 000–15 000 different DNA molecules, each being specifically amplified to a copy number between 10^3 and 10^6 per macronucleus (for reviews see 1–3).

Macronuclear precursor sequences occur in clusters of several genes in the micronuclear genome and within a cluster the different macronuclear-destined sequences are separated by short spacer regions. In no case analyzed were telomeric repeats associated with these macronuclear precursor sequences. Very often they are interrupted by short non-coding sequences, the internal eliminated sequences (IES), or by long transposon-like elements. Both types of sequences, IES and transposon-like elements, are eliminated from the genome in the form of DNA circles in the course of polytene chromosome formation (for review see 3,4). The degradation of the polytene chromosomes becomes evident upon the appearance of membranous material surrounding the bands of the chromosomes, which are eventually completely enclosed in vesicles (for review see 2,3). It is assumed that at this stage specific fragmentation of the genome, degradation of DNA and *de novo* addition of telomeres occurs (5–7). Thus in order to create a functional macronuclear DNA molecule, at least the following processes have to occur: excision of IES and transposon-like elements, elimination of micronuclear-specific DNA sequences, amplification of macronuclear DNA molecules, fragmentation of the genome and *de novo* addition of telomeres.

Sequences involved in the excision of IES and transposon-like elements have been characterized (8–11), but little is known about *cis*-acting sequences responsible for specific fragmentation of the genome or *de novo* addition of telomeres. Several DNA sequences that could be possible candidates for directing these processes (see for example 7,12–14) have been described, but no experimental systems to test these hypothesis were available in hypotrichous ciliates. The development of vector systems which can be injected into the macronuclear anlagen of the holotrichous ciliate *Tetrahymena* has contributed significantly to our understanding of DNA rearrangement processes in this ciliate (15–18). No comparable system was available to date in hypotrichous ciliates. In this paper we describe the construction of a vector and its injection into the macronuclear anlagen of the hypotrichous ciliate *Stylonychia* and its fate in the mature macronucleus.

MATERIALS AND METHODS

Cultivation of cells

Stylonychia lemnae were grown in neutral Pringsheim solution and fed daily with the algae *Chlorogonium elongatum* (19). To achieve conjugation, cells of two different mating types were mixed; usually 60–90% mating was observed the next morning. The different stages of macronuclear development were examined by phase contrast microscopy.

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Injection of DNA into the macronuclear anlagen

Injection of DNA into the polytene chromosome stage of the macronuclear anlage was performed using Eppendorf femtotips (diameter ~0.5 µm) connected to a Zeiss micromanipulator and an Inject and Matic (Geneva) pressure supply. When using needles with larger diameter, cells tend to lyse. However, the needles can be used for only a few injections (up to 10) before they become plugged. The threshold pressure used was 0.08 bar, the injection pressure was between 0.15 and 0.2 bar and the injection time was 0.5 s. Under these conditions 0.1–0.5 pl were injected into the nucleus. When a lower threshold or injection pressure was applied, polytene chromosomes become sucked out of the cells. Higher pressure or longer injection times led to a significant decrease in survival of the cells. Injection into the macronuclear anlagen results in a short increase in volume of the nucleus and thus can be optically controlled. The DNA concentration used for injection varied between 10 and 50 µg/ml in Pringsheim solution. Higher concentrations of DNA led to blocking of the needles within a few seconds. Since the vector used for injection has a size of ~6.7 kb (see Results), ~100–500 copies were injected into one nucleus under these injection conditions. For injection, 2–5 cells in the polytene chromosome stage of macronuclear development were placed on a slide covered with 0.05% polylysine. This concentration leads to a partial immobilization of the cells on the slides; concentrations >0.05% seemed to be toxic to the cells and resulted in a significantly lower survival rate. Following injection, individual cells were placed in 0.5 ml tissue culture dishes. About 40% of injected cells survived and developed into vegetative cells. In 5–30% of these cells, mainly dependent on the person injecting, injected DNA could be detected in the vegetative macronucleus by PCR analysis (see Results).

Other methods

PCR followed the procedure described by Saiki *et al.* (20). Reactions were either performed with 0.1–0.5 µg total macronuclear DNA or DNA isolated from 5–20 cells. For this, cells were placed in 500 µl TE, then 10 ng proteinase K and 10 ng RNase were added and incubated at 55°C for 1 h. The solution was transfered to Microcon (Amicon) tubes and centrifuged for 8 min in an Eppendorf centrifuge. The supernatant, normally ~10 µl, was transfered to an Eppendorf tube and incubated for 10 min at 95°C. Aliquots (2 µl) of this solution was used for a PCR reaction. Primers used for PCR analysis are indicated in Figure 2; all primers contained restriction enzyme recognition sequences.

Isolation of DNA and hybridization with specific gene probes was done as described earlier (21). DNA probes were labeled by oligolabeling using either [α-^{32}P]dCTP or digoxigenin–dUTP (22). Digestion with restriction enzymes followed the instructions of the suppliers. For cloning of PCR products, the DNA was digested with the primer-specific restriction enzymes and subsequently ligated into PUC19 (22). Sequencing was done using the primer extension technique (23).

RESULTS

Vector construction

Construction of the vector used for injection is outlined in Figure 1. Clone pCE7 contains a 7 kb micronuclear DNA insert from

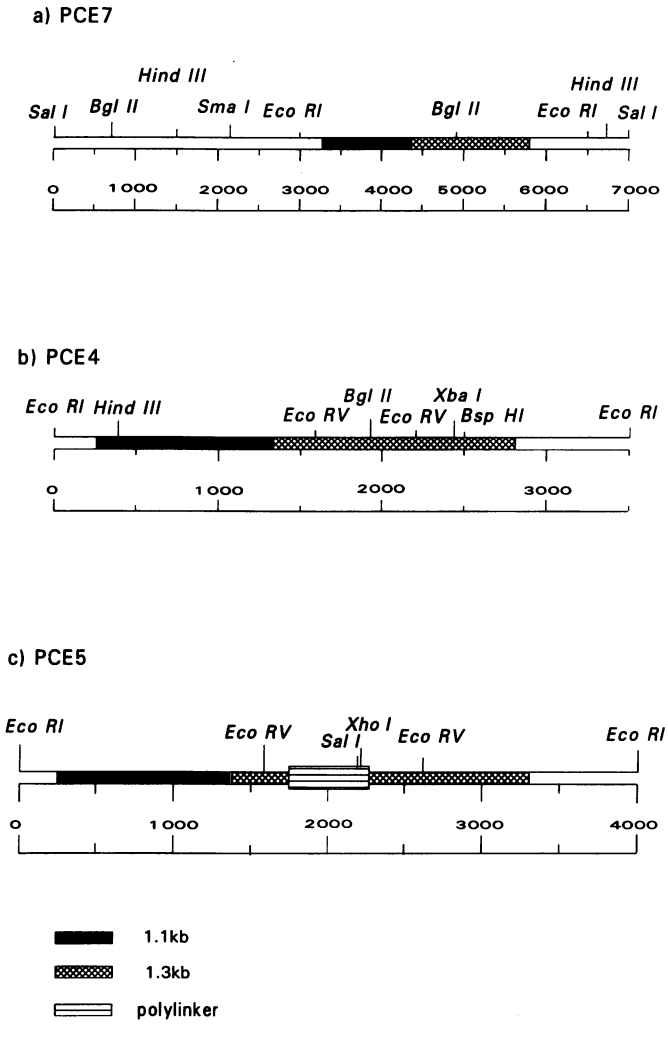

Figure 1. Construction of pCE5. A homology to two macronuclear DNA molecules was found on the 3.5 kb *Eco*RI fragment of pCE7 (**a**). This 3.5 kb *Eco*RI fragment was cloned into pUC19 yielding plasmid pCE4 (**b**). A 503 bp polylinker sequence (indicated as a hatched box) was inserted into the *Bgl*II site of pCE4 yielding plasmid pCE5 (**c**). The localization of the 1.1 and 1.3 kb macronuclear homologous sequence as well as of the polylinker is indicated.

S.lemnae cloned into pUC12 (Fig. 1a). On this insert, homology to two macronuclear DNA molecules with sizes of 1.1 and 1.3 kb were found. Restriction and sequence analysis revealed that these macronuclear-destined sequences are located on a 3.5 kb *Eco*RI fragment. All sequences required for the formation of the 1.3 kb macronuclear DNA molecule have been detected on this 3.5 kb *Eco*RI fragment, whereas 100 bp of the 1.1 kb macronuclear DNA molecule are lacking; they have to be separated from the rest of the gene by a long IES or transposon (24). This *Eco*RI fragment was cloned into pUC19, yielding pCE4 (Fig. 1b). The 503 bp polylinker region isolated from plasmid pSTC (25) was inserted into the *Bgl*II site of pCE4, yielding plasmid pCE5 (Fig. 1c). Thus this final vector contains sequences homologous to two macronuclear DNA molecules with sizes of 1.1 and 1.3 kb. These sequences are interrupted by five IES and separated from each other by an 11 bp spacer sequence. The 503 bp polylinker region is inserted in the open reading frame of the 1.3 kb homologous sequence. In addition, 250 bp of micronuclear-specific DNA

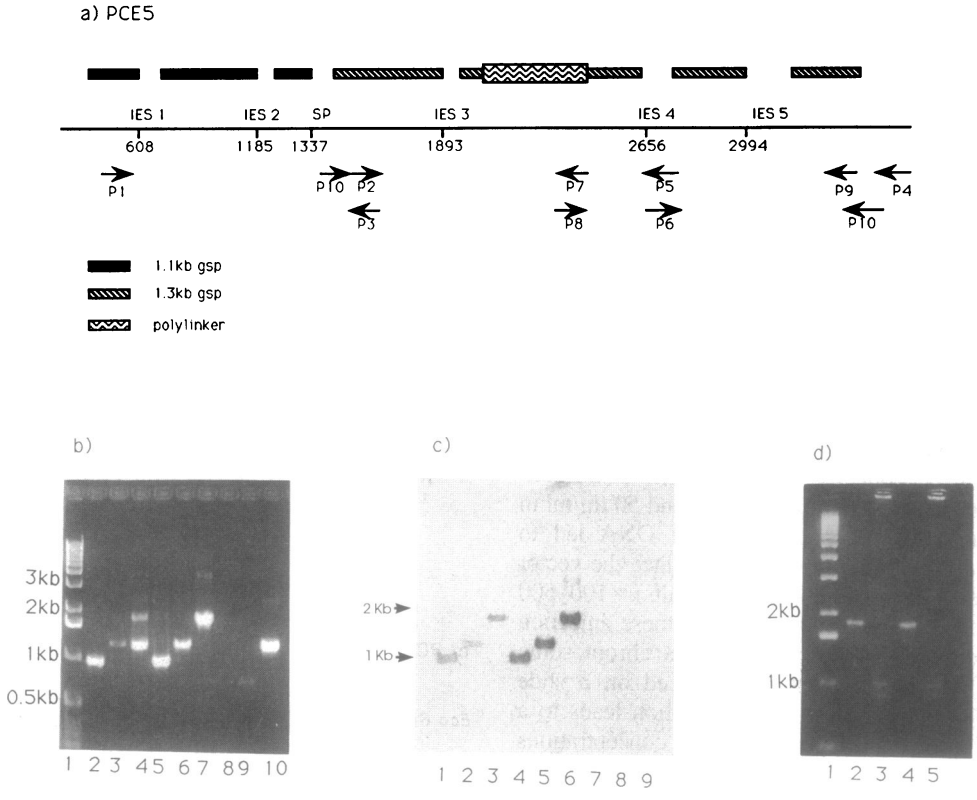

Figure 2. PCR analysis of pCE5-injected cells. (**a**) Schematic diagram showing the positions of macronuclear homologous sequences within clone pCE5 and the position of primers used for PCR. Primers P2, P3 and P9 are derived from the 1.3 kb macronuclear DNA sequence, primer P1 from the 1.1 kb macronuclear DNA molecule, primer P4 from the micronuclear-specific DNA sequences flanking the 1.3 kb homologous sequence, primers P7 and P8 are derived from the polylinker sequence, primers P5 and P6 from IES4 and primer P10 is the telomeric primer. (**b**) Fragments synthesized in PCR from DNA of pCE5-injected cells and from plasmid pCE5. The experimental conditions for PCR were: 18–28 base primers were used, DNA was denatured at 94°C for 60 s, the annealing conditions were 52°C for 120 s, the extension reaction was performed for 120 s at 72°C, DNA was synthesized in 40 cycles. Lane 1, molecular weight marker; lanes 2–4, PCR products from pCE5-injected cells (the primer combinations used are lane 2, P2/P7, lane, 3 P2/P5 and lane 4, P2/P9); lanes 5–7, PCR products synthesized from vector pCE5 (the primer combinations used are: lane 5, P2/P7, lane 6, P2/P5 and lane 7, P2/P9); lanes 8–10, PCR products synthesized from uninjected cells (the primer combinations used are lane 8, P2/P7, lane 9, P2/P5 and lane 10, P2/P9). (**c**) The gel shown in (a) was hybridized with the 503 bp polylinker sequence present in pCE5. Lanes 1–3 are identical to lanes 2–4, lanes 4–6 are identical to lanes 5–7 and lanes 7–9 are identical to lanes 8–10 of (b). (**d**) Restriction digestion of DNA fragments synthesized by PCR. A PCR reaction using the primer combination P2/P9 was carried out using either DNA from pCE5-injected cells or vector PCE5 as a template. The 1830 bp PCR products were digested with *SalI*. Lane 1, molecular weight marker; lane 2, PCR fragment synthesized from pCE5-injected cells; lane 3, fragment shown in lane 2 digested with *SalI*; lane 4, PCR fragment synthesized from vector pCE5; lane 5, fragment shown in lane 4 digested with *SalI*.

sequences flank the 1.1 kb homologous DNA sequence and 700 bp flank the modified 1.3 kb homologous DNA sequence. The organization of the macronuclear precursor sequences and the localization of the polylinker sequence on vector pCE5 are shown in Figure 1 and in detail in Figure 2a. The insertion of a polylinker DNA sequence allows the specific detection of the injected plasmid in the macronucleus by PCR or hybridization analysis.

Injection of vector DNA into the polytene chromosomes of the macronuclear anlagen

Although some of the processes involved in the correct processing of macronuclear precursor DNA sequences may occur before formation of the polytene chromosomes, in this work we decided to inject our vector only into this stage, which immediately precedes the chromosome fragmentation process and *de novo* addition of telomeric DNA. As a control the plasmid pUC19 was injected into cells at the same developmental stage.

Normally ~50–60 cells were injected at one time; results for this study were obtained from 540 injected cells. From these injected cells 220 survived and finished macronuclear development. After 6–8 cell divisions a PCR analysis from DNA of 5–20 cells using a combination of primers derived from the polylinker region (P7 and P8) and from the 1.3 kb macronuclear DNA molecule (P2 and P9) (Fig. 2a) was carried out. Using these primer combinations, P2/P7 or P8/P9, no PCR products were synthesized from uninjected cells or cells injected with pUC19. In 45 pCE5-injected cells PCR fragments with sizes of 980 and 870 bp were synthesized (Table 1 and Fig. 2b). As a positive control a PCR was carried out using pCE5 as a template. To verify that the PCR fragments synthesized from pCE5-injected cells were derived from the injected vector, they were hybridized with the polylinker sequence from pCE5 (Fig. 2c). From these positive clones large cultures were grown and further characterized by PCR, hybridization and sequence analysis. Using pUC19-specific primers the injected pUC19 could never be detected by PCR analysis in the vegetative macronucleus of 60 analyzed clones.

Table 1. Summary of DNA fragments synthesized by PCR

Primers	Templates	Primers					
		P7(bp)	P5(bp)	P9(bp)	P4(bp)	P10(bp)	P3(bp)
P2	PCE5	980	1230	1830	1930	–	
	DNA injected cells	**980**	**1230**	**1830**	(1430)	**1870**	
	DNA uninjected cells	–	(730)	1190	(1430)	1230	
P8	PCE5			870	970	–	
	DNA injected cells			**870**	–	**910**	
	DNA uninjected cells			–	–	–	
P1	PCE5	1930	2180	2800	2900		
	DNA injected cells	–	–	–	–		
	DNA uninjected cells	–	–	–	–		
P6	PCE5			620	720	–	5470
	DNA injected cells			**620**	–	**660**	–
	DNA uninjected cells			–	–	–	–
P10	PCE5	–	–	–			
	DNA injected cells	**1050**	**1300**	**1900**			
	DNA uninjected cells	–	–	1260			

The table summarizes the results from the PCR analysis performed with macronuclear DNA from pCE5-injected cells (bold letters), from normal macronuclear DNA and from vector pCE5. Conditions for PCR analysis are described in the legend to Figure 2.

The results of the PCR analyses are summarized in Table 1. In all 12 clones analyzed in detail identical PCR products were synthesized; examples are shown in Figure 2. Using the primer combinations P2/P7 and P8/P9 PCR fragments having the same size as those produced using pCE5 as a template were synthesized from DNA of pCE5-injected cells, using the primer combination P2/P9 (both are derived from the 1.3 kb macronuclear DNA molecule) two PCR fragments were synthesized from pCE5-injected cells, one being identical to that synthesized from DNA of uninjected cells and one with an identical size to that synthesized from pCE5. These results suggest that the three IES present in the 1.3 kb homologous sequence of pCE5 are still retained in injected cells, which is supported by a PCR analysis using primers derived from IES4 (see Fig. 2b, lane 3 and Table 1, primer combinations P2/P5 or P6/P9). Occasionally PCR fragments were synthesized from uninjected cells using the primer combinations P2/P5 and P2/P4. These fragments are probably synthesized from contaminating micronuclear DNA and are, in the case of P2/P5, smaller than those from pCE5-injected cells. No PCR products were formed in injected cells with the primer combination P3/P6, demonstrating that the injected vector is not retained in its circular form in the macronucleus. Furthermore, no, or at least no specific, PCR products were synthesized with the primer combinations P2/P4, P1/P7 and P4/P8, demonstrating that the insert of the vector injected into the polytene chromosomes of the macronucleus anlage becomes fragmentated during macronuclear development. This fragmentation, as well as *de novo* addition of telomeres to the modified macronuclear DNA sequence, is also shown by the use of telomeric primers (see Table 1, primer combinations P7/P10 and P8/P10). In both cases fragments of the expected sizes were synthesized. The presence and correct position of the polylinker in the PCR fragments from macronuclear DNA of pCE5-injected clones could also be demonstrated by restriction analysis. When the 1830 bp PCR fragment synthesized using the primers P2/P9 was digested with *Sal*I, a restriction enzyme present in the polylinker, two bands of the expected size were produced (Fig. 2d).

Southern analysis was used to directly detect DNA molecules derived from pCE5 in the macronucleus of injected cells. Macronuclear DNA was separated on agarose gels and either probed with pCE5 or the polylinker region from pCE5. The hybridization pattern of four of these clones is shown in Figure 3. In uninjected cells pCE5 hybridizes to the 1.1 and 1.3 kb macronuclear DNA molecules. In pCE5-injected cells an additional hybridization signal to a 1.95 kb DNA molecule is observed (Fig. 3a). No hybridization is observed in uninjected cells when hybridized with the polylinker region, however, a hybridization to the 1.95 kb DNA molecule is visible (Fig. 3b), demonstrating that it is derived from the injected pCE5. The intensity of this hybridization varies between different clones and may reflect the amount of pCE5 injected into the macronuclear anlage. Since the polylinker present in pCE5 comprises only 503 bp, the observed DNA molecule is ~140 bp larger than expected when processed completely, thus providing further evidence that the IES present in pCE5 were not spliced out during formation of the new macronucleus.

The presence of the polylinker sequence and the IES in the processed macronuclear version of the modified macronuclear precursor sequence from pCE5 were further demonstrated by sequence analysis. The 1830 bp PCR product using the primers P2/P9 was digested with *Sal*I/*Xho*I, cloned in pUC19 and partially sequenced (Fig. 4a–c). As shown in Figures 4d and e, sequences identical to the polylinker, as well as to IES5, are found.

Finally, we determined the stability of the modified macronuclear DNA molecule during vegetative growth. At 4 weekly intervals the presence of this molecule was determined by PCR analysis using single cells. Its presence could be demonstrated throughout the vegetative life of the cells, i.e. for >8 months. After

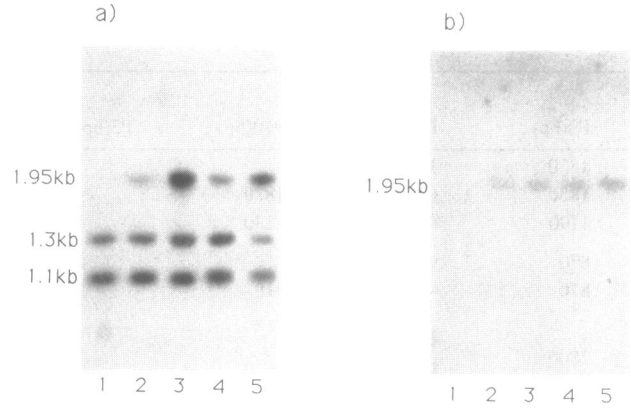

Figure 3. Hybridization of pCE5 and the polylinker sequence from pCE5 to total macronuclear DNA from uninjected and pCE5-injected cells. (**a**) Hybridization with pCE5. Lane 1, macronuclear DNA from uninjected cells; lanes 2–5, macronuclear DNA from different pCE5-injected clones. (**b**) Hybridization with the polylinker sequence from pCE5. Lanes are identical to those of (a).

this time cells age or have to conjugate. As expected, this molecule was lost after conjugation.

DISCUSSION

In an attempt to establish an experimental system for the identification of *cis*-acting DNA sequences involved in the processing of macronuclear DNA sequences during macronuclear development of hypotrichous ciliates, we constructed a DNA vector, injected it into the macronuclear anlage and analyzed its fate in the mature macronucleus. The vector constructed contained the micronuclear versions of two macronuclear genes and short adjacent micronuclear-specific sequences. By the insertion of a 503 bp polylinker region in one exon of a macronuclear precursor sequence, specific detection of this modified DNA molecule in the macronucleus was possible by PCR or Southern analysis. Although important molecular events might occur at different stages of macronuclear development, we decided to inject our construct only into one defined stage of the macronuclear anlagen, the polytene chromosome stage, which immediately precedes the fragmentation process.

The injection procedure we describe allows the injection of a sufficient number of cells at one time for a subsequent statistical analysis. However, due to the fragility of *Stylonychia* exconjugant cells, the range of DNA concentrations which can be injected are, compared with *Tetrahymena* (15), very limited. Further analysis showed that the modified macronuclear DNA sequence could be detected in up to 30% of injected cells and probably with increasing injection experience this figure could be even higher.

The presence of the modified macronuclear DNA molecule from pCE5 in the macronuclei of injected cells was demonstrated by PCR, hybridization and sequence analysis. It was stably retained in the macronucleus during vegetative growth, demonstrating that all sequences required for replication were present. When pUC19 was injected into the macronuclear anlagen it could never be detected in the mature macronucleus. The processing and stability of the injected pCE5 must depend on the presence of the ciliate DNA sequences present on this vector. By PCR and sequence analysis it could be shown that the IES present in the

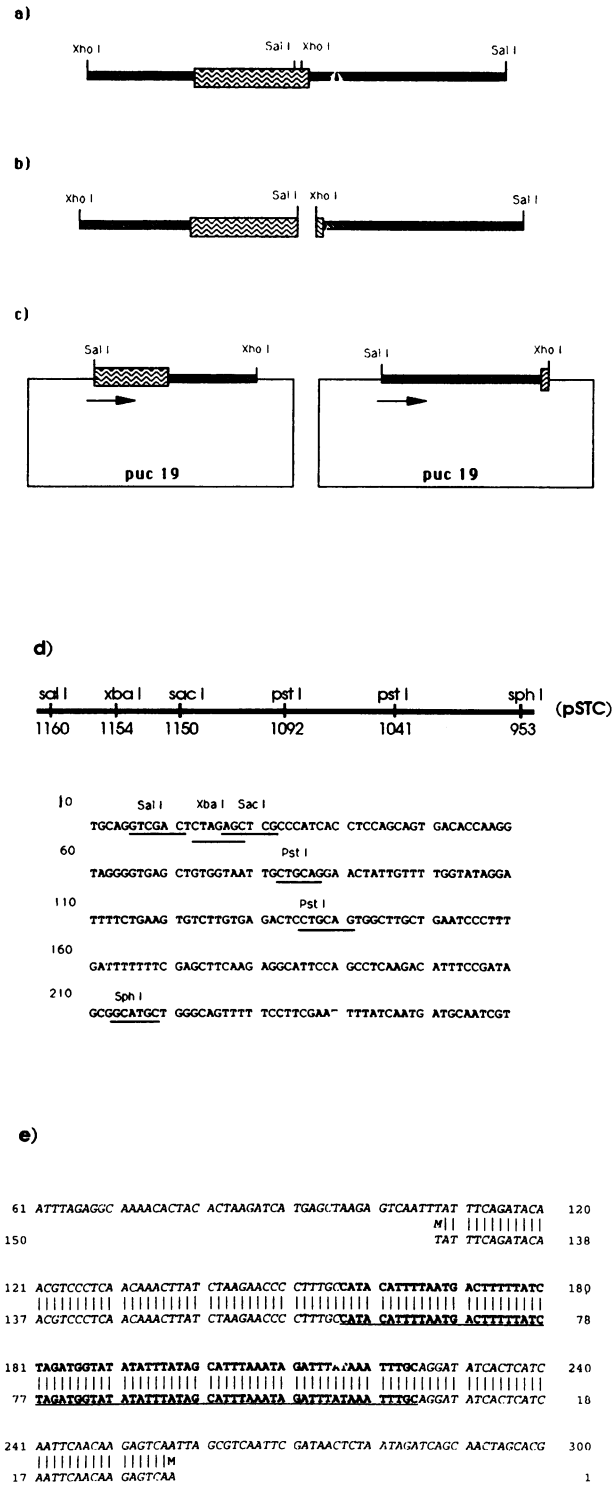

Figure 4. Sequence analysis of PCR products from pCE5-injected cells. A PCR reaction using the primer combination P2/P9 was performed (**a**); the 1830 bp fragment was digested with *SalI*/*XhoI* (**b**) and cloned into pUC19 (**c**) and sequenced. The sequence direction is indicated by an arrow, the position of the polylinker region by a box. (**d**) Comparison of the restriction map of the polylinker sequence from pSTC with the sequence from the PCR product. (**e**) Comparison of the sequence from vector pCE5 (underlined letters indicate IES5) with the sequence from the PCR product.

macronuclear precursor sequence were not spliced out from our injected molecule. Previous experiments (26–28) showed that the IES are excised from the genome before formation of the polytene chromosomes. Since we injected our vector into the fully developed polytene chromosomes our observation is in good agreement with these previous results. It may well be that for the excision of IES a defined chromatin configuration or *trans*-acting factors, no longer available in the polytene chromosome stage, are required. Using our vector system it would now be possible to determine the exact timing of the different molecular events throughout macronuclear development. In Southern blots hybridization signals of different intensities were observed in different clones, which probably can be explained by different amounts of vector injected. Normally macronuclear DNA molecules occur at a very defined copy number in the vegetative macronucleus. This seems not to be true for DNA injected into the polytene chromosome stage of the macronuclear anlagen and therefore control of copy number might occur at an earlier stage during macronuclear development. By hybridization and PCR analysis we could show that the modified macronuclear DNA molecule is cut out from the vector and telomeres are added *de novo*. All *cis*-acting sequences required for correct fragmentation of the genome and *de novo* addition of teloremic sequences must therefore be present on our vector. Thus the experimental system we describe may not only prove useful for the identification of those sequences, but may also lead to the construction of other vectors that could facilitate the analysis of basic molecular events during cell differentiation.

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