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The telomeres of replicating macronuclear DNA-molecules of the hypotrichous ciliate *Stylonychia lemnae*

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Summary

While the telomeres of higher eukaryotic cells show heterogenity in their length no such length heterogenity is observed in the macronuclear DNA of hypotrichous ciliates. Since the analysis of the telomere structure of replicating DNA molecules might give an insight in the mechanisms of telomere length regulation *Stylonychia* cell cultures were synchronized, the replicating DNA was labeled with BrdU and isolated by immuno-precipitation. The 3‘– as well as the 5‘–end of these replicating DNA was labeled and both ends were sequenced using the chemical degradation method by Maxam and Gilbert (1977). The telomere length of the BrdU labeled DNA was identical to that of macronuclear DNA of starved cells. Possible explanations for this observation are discussed.

Key words: cell synchronization, replication band, BrdU-labeling, gene-sized DNA molecules, *Stylonychia*

Introduction

Telomeres, the specialized structures at the ends of eukaryotic chromosomes, protect chromosomes from end to end fusion and exonucleolytic digestion, furthermore they are essential for the replication of the ends of a linear DNA molecule. Loss of telomeres would lead to a progressive shortening of the DNA during each round of replication (for review see Kipling, 1995). Telomere DNA consists of simple repetitive sequences from which the 3‘–strand is always G–rich. It has now been shown for a number of organisms that the G-rich strand is longer than its complement and it is generally believed that this is the usual structure of telomeric sequences (Klobutcher et al., 1981; Henderson and Blackburn, 1989; Wellinger et al. 1993, 1996; Henderson, 1995; Makarov et al., 1997). This 3‘-overhang acts as a template for the enzyme telomerase which guarantees the integrity of the telomeres. Telomerase adds telomeric repeats to the 3‘-end of telomeres, thus avoiding shortening of telomeres during replication (for review see: Greider, 1995) although other as yet poorly described mechanisms of telomere maintenance seem to occur (Reddel et al., 1997). Telomere DNA length varies from less than 50bp to over 20kb. Due to the action of telomerase, which adds a variable number of telomeric repeats to the ends, telomere length variation is observed in all organisms with the exception of the macronuclear gene-sized minichromosomes in hypotrichous ciliates (Klobutcher et al., 1981).

Like all other ciliated protozoa hypotrichous ciliates such as *Oxytricha*, *Euplotes* or *Stylonychia* contain two morphologically and functionally different types of nuclei, the macronucleus and the micronucleus. After sexual reproduction, the conjugation, a new macronucleus is formed from a micronuclear derivative in a series of well defined events. These include the formation of polytene chromosomes, degradation of these chromosomes, elimination of a high percentage of DNA and subsequent amplification of the remaining DNA to form the vegetative macronucleus (for review: Ammermann, 1971; Kraut et al., 1986; Prescott, 1984). In the course of this macronuclear differentiation macronuclear DNA becomes specifically fragmented into gene-sized DNA molecules and telomeric sequences are added *de novo* (Roth and Prescott, 1985). While telomere length variation is observed in the micronuclear DNA (Jahn, 1988), no such length variation is observed in macronuclear DNA. In *Stylonychia lemnae* the telomeric sequence is 5‘-$C_4A_4C_4A_4C_4$ with a 16mer 3‘-G_4T_4 overhang (Klobutcher et al., 1981; Lipps and Erhardt, 1981). The lack of telomere length variation suggests a tight control of the telomere length during macronuclear DNA replication which takes

place in a morphologically distinct region, the replication band (Olins et al., 1981; Lin and Prescott, 1985). The presence of telomerase was demonstrated in the replication bands of *Oxytricha* (Fang and Cech, 1995).

Telomere length homogenity can be explained by different mechanisms: for example telomerase could add a defined number of repeats or telomerase adds a variable number of repeats to the 3'-end but the telomeres are trimmed postreplicative by a specific exonuclease. In order to distinguish between these possibilities we synchronized *Stylonychia* cells, labeled and isolated nascent DNA and sequenced the ends.

Material and Methods

Vegetative *Stylonychia lemnae* cultures were kept at room temperature (21–23°C) and fed daily with the algae *Chlorogonium elongatum* (Ammermann et al., 1974). Synchronization of cells followed in principle a protocol from Ammermann (unpublished) by growing *Stylonychia* under physiological stress. The detailed procedure is described in the results and discussion section (Fig. 1a).

As soon as replication bands were visible in more than 70% of DAPI stained macronuclei (0.5µg/ml for 10 minutes), replicating DNA was labeled by the addition of 1mM of the nucleotide analog BrdU (5'-bromo-2'-deoxyuridine, Sigma) to the culture medium for 15–45 minutes. The successful incorporation of the BrdU was detected by binding of an anti-BrdU monoclonal antibody (Sigma) and visualized by a FITC-labeled anti-mouse IgG (Dianova, Fig. 1c). Macronuclear DNA was isolated from these cultures (Ammermann et al., 1974) and BrdU-labeled DNA isolated by immunoprecipitation: DNA was denatured at 95°C for 5 minutes and immediately placed in an ice-cold water bath. The DNA solution was then adjusted to 10mM Na_2HPO_4, 0.14M NaCl, 0.05% TritonX-100, pH7.4 and 1µg/ml BrdU antibody and 0.3%BSA were added. Precipitation was achieved by the addition of 1mg/ml goat anti-mouse IgG (Sigma). After incubation at room temperature for 60 minutes the precipitate was collected by centrifugation (13000 rpm, Heraeus/Sepatech Biofuge 13, 15 minutes at room temperature). DNA from this pellet was isolated by proteinaseK digestion and subsequent phenol:chloroform extraction (Ammermann et al., 1974). After precipitation with 2.5vol. ethanol the pellet was resuspended in distilled water (1mg/ml).

After labeling the 3'-end of the DNA with [a-^{32}P]ddATP by terminal transferase or the 5'-end with [g-^{32}P]dATP by T4 kinase (Sambrook et al., 1989) both ends were sequenced according to the chemical degradation method as described by Maxam and Gilbert (1977). The sequence reactions were loaded on a 20% urea-polyacrylamide gel (Sambrook et al., 1989). After electrophoresis the gel was fixed with 7% acetic acid for 15 minutes, dried at 70°C and exposed to a Kodak Xomat film. The autoradiography was analyzed with a Bio-Rad GS-700 imaging densitometer using the molecular analyst software (Bio-Rad).

Results and Discussion

A schematic diagram of the optimized synchronization procedure is shown in Fig. 1a. To vegetative unsynchronized *Stylonychia* cultures grown at room temperature (21–23°C) the food organism *Chlorogonium* was given in excess and the cultures were placed in a 12°C incubator. After 24 hours the cells were collected on a 30µm gauze to remove all algae. Cells were then resuspended in Pringsheim medium (Ammermann et al., 1974) and placed in a 20°C incubator for 24 hours. Cells were then refed and again incubated for 24 hours at 12°C. They were then again collected on a 30µm gauze, resuspended in Pringsheim medium and incubated at 20°C. 18–22 hours later replication bands were visible in over 70% of the macronuclei. At this stage BrdU was added to the culture medium as described in materials and methods. As shown in Fig. 1c, the BrdU was incorporated exclusively into newly synthesized, nascent DNA which is localized in the rear zone of the replication band (Olins et al., 1981). BrdU-labeled DNA was then isolated as described in materials and methods. Thus using this technique, we were able to isolate newly replicated and replicating DNA.

BrdU-labeled DNA was isolated from 5–10l cell culture, the 3'- and 5'-end labeled (Sambrook et al., 1989) and sequenced using the chemical degradation technique of Maxam and Gilbert (1977). As a control macronuclear DNA from starved cells was isolated, labeled and sequenced. The results of the sequence analysis of the 3'-end are summarized in Fig. 2, where only the analysis of the G- and T-reaction is shown. In no case longer telomeres or telomere heterogenity could be observed. The 3'-telomere strand always consisted of exactly 36bp of a G_4T_4-repeats, similarly the sequence of the 5'-strand was a 20mer consisting of C_4A_4-repeats identical to the telomere sequence of starved cells (data not shown). This result was independent of the length of the BrdU pulse and could be verified in six independent sequence reactions.

The aim of this study was to get an insight into the mechanism of telomere length regulation in hypotrichous ciliates. While in all other eukaryotic nuclei telomere length variation is observed, the macronuclear telomeres of hypotrichous ciliates consist always of an exact number of telomeric repeats suggesting a very tight control mechanism. Replication of macronuclear DNA occurs in the replication band. Since the presence of telomerase could be demonstrated in this structure (Fang and Cech, 1995), telomerase independent telomere maintenance mechanisms seem to be unlikely to occur. Several explanations for telomere length homogeneity are possible. For example telomerase adds a defined number of telomeric repeats to

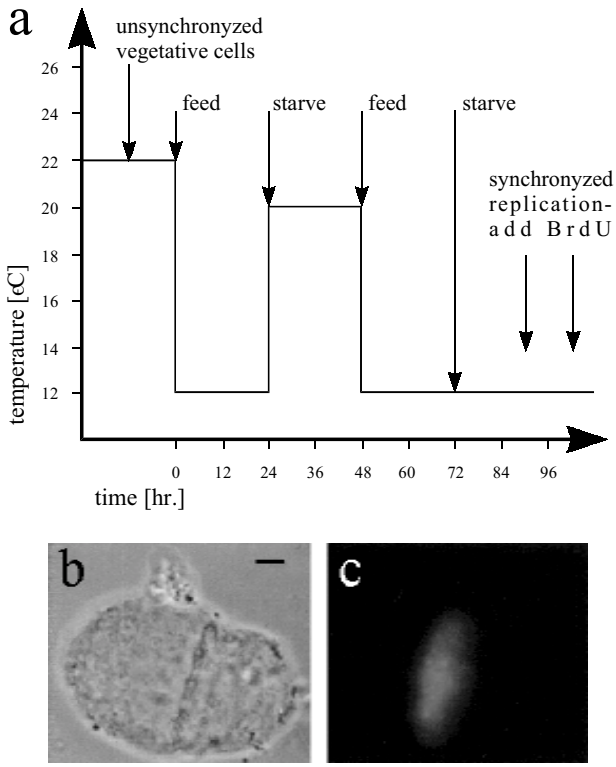

Fig. 1. Synchronization of *Stylonychia* cells and labeling of replicating DNA with BrdU. **a.** Schematic diagram of the optimized synchronization procedure (for details see results and discussion). **b**. Macronucleus showing the replication band (scale bar: 1µm). **c.** The same macronucleus as in fig. b after incorporation of BrdU into replicating DNA shown by antibody staining as described in materials and methods.

the 3'-end which are subsequently removed during the replication process. In this case the 3'-telomeric strand should be longer than that of non-replicating DNA molecules but still show length homogeneity. However, no telomerase with these properties has been described yet. In fact, it has been demonstrated that the *Oxytricha* macronuclear telomerase adds two to seven tandem repeats of the sequence GGGGTTTT (Zahler and Prescott, 1988). Alternatively, telomerase could add a variable number of telomeric repeats to the 3'-end and the telomeres are then trimmed to their homogeneous length by a specific exonuclease as suggested by Zahler and Prescott (1988). The 3'-telomeric strand of replicating DNA then should be longer and heterogeneous in length. Further mechanisms are possible but all have in common that the 3'-telomeric strand of replicating DNA should change in length compared to that of non-replicating DNA. In our study neither

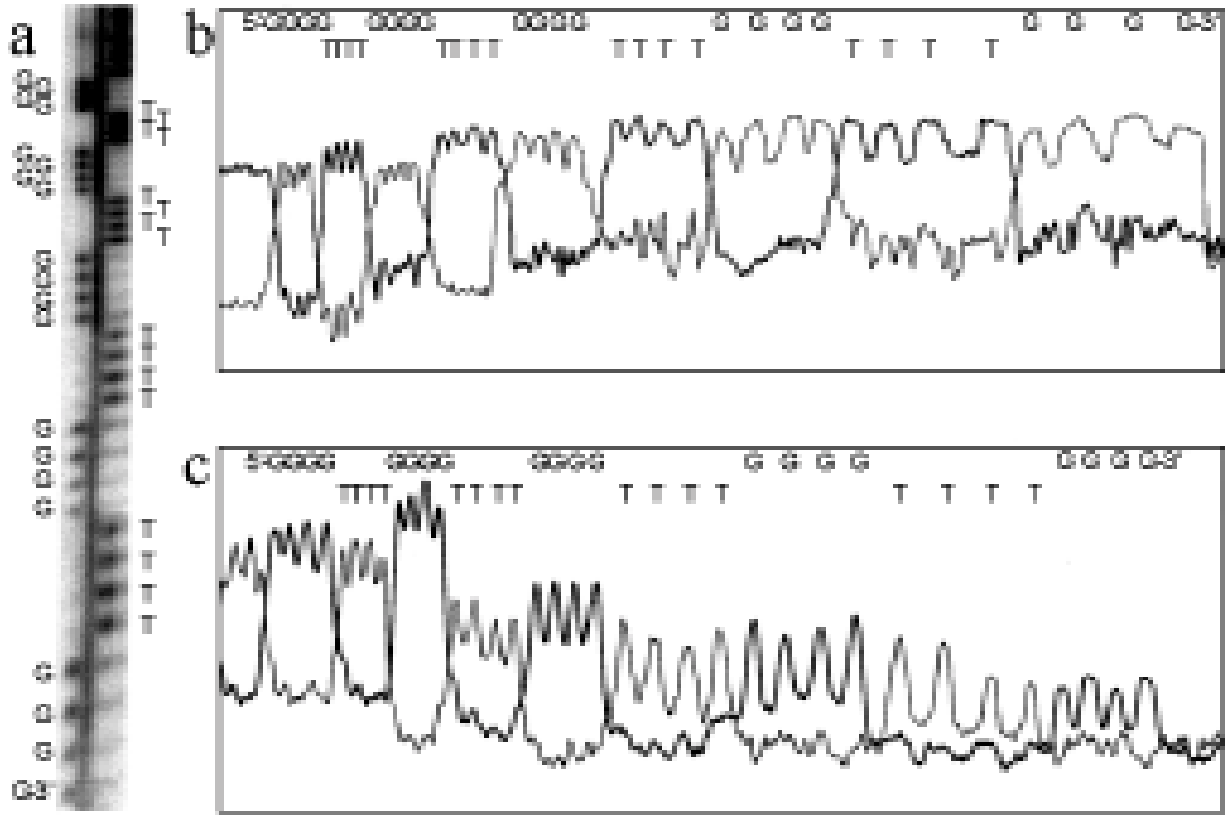

Fig. 2. Sequence analysis of the 3'-telomeric strand. Sequence analysis was performed as described in materials and methods. **a.** 20% urea polyacrylamide gel showing the 3'-sequence of the immuno-precipitated BrdU-labeled macronuclear DNA. Only the G- and the T-reaction are shown. **b** and **c.** Densitometric analysis of the G- and T-reaction separated on a 20% urea polyacrylamide gel (**b** – macronuclear DNA isolated from starved cells, **c** – immuno-precipitated BrdU-labeled macronuclear DNA).

homogeneous nor heterogeneous increase in length could be observed in the 3'-telomeric strand; the same was true for the 5'-strand. Since this result was also obtained when very short BrdU-pulses were applied (15min) it seems unlikely that we only sequenced the telomeres of fully replicated and processed DNA molecules. We therefore have to assume that the process of telomere length regulation in the macronuclei of hypotrichous ciliates is immediately associated with the replication process, both in time and space. Therefore to understand the mechanisms of telomere length regulation it will be necessary to isolate and characterize the replication machinery occurring in the macronuclei of hypotrichous ciliates.

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