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Minichromosomal DNA replication in the macronucleus of the hypotrichous ciliate *Stylonychia lemnae* is independent of chromosome-internal sequences

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Abstract The origins of DNA replication in prokaryotes and eukaryotes are typically defined by *cis*-acting sequences. However, in ciliates, evidence suggests that the replication of short macronuclear minichromosomes may not require such determinants. In hypotrichous ciliates, macronuclei contain millions of gene-sized minichromosomes, which generally have a single protein-coding region, two short noncoding flanks and, on each end, a short telomere consisting of a double-stranded repeat region and a single-stranded 3′ overhang. Electron microscopic studies that showed that replication of minichromosomes initiates at or near telomeres and the discovery of a primase activity synthesizing RNA primers over the whole 3′ telomeric overhang in vitro suggested that minichromosome replication starts directly at telomeres. Conversely, many minichromosomes contain an AT-rich, semi-conserved, palindromic sequence motif in their subtelomeric regions and it has been proposed that this motif is involved in regulating minichromosomal replication. To analyze what sequences or structures of the minichromosomes are essential for DNA replication, we stably transfected genetically modified α1-tubulin-encoding minichromosomes into the hypotrichous ciliate *Stylonychia lemnae*. Cotransfection of mutated and control minichromosomes revealed that noncoding regions can be deleted or replaced with unrelated sequences without affecting minichromosome replication efficiency in vegetatively growing cells. Similarly, replacement of the coding region resulted in a minichromosome that was stably maintained in transfected cells at the same high copy number for many months. In contrast, α1-tubulin-encoding minichromosomes without telomeres were rapidly lost after transfection. Hence, DNA replication of the α1-tubulin-encoding minichromosome does not depend on chromosome-internal sequences but may depend on telomeres.

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Introduction

The nuclear apparatus of ciliates consists of two morphologically and functionally different types of nuclei: generative, transcriptionally silent micronuclei and somatic, transcriptionally active macronuclei (reviewed in Raikov 1982; Prescott 1994). Macronuclei develop from micronuclei after conjugation. During this process, micronuclear DNA is fragmented and to a great extent eliminated. The remaining DNA sequences obtain telomeres de novo and are strongly amplified, leading to high copy numbers of short chromosomes. These processes are most pronounced in hypotrichous ciliates. In *Stylonychia lemnae* approximately 98% of micronuclear DNA sequences are eliminated, and very short chromosomes typically harboring a single gene are amplified to an average copy number of 15,000 (Ammermann 1990). These very short chromosomes have been termed gene-sized molecules (Prescott et al. 1971) or minichromosomes (Hoffman et al. 1995), and are referred to here as macronuclear minichromosomes. There are about 12,000 to 36,000 different macronuclear minichromosomes per cell ranging in size from a few hundred base pairs to 20 kb and those minichromosomes that have been characterized possess the same organization: a protein-coding region flanked by short nontranslated regions, characteristically not longer than 400 bp and termed 5′ DNA leader and 3′ DNA trailer (Prescott 1994). At both ends, a short telomere is present, which consists of the 20 bp double-stranded sequence 3′-$G_4T_4G_4T_4G_4$-5′ and the 16 nucleotide 3′ overhang 3′-$G_4T_4G_4T_4$-5′.

Macronuclear minichromosomes are replicated during vegetative cell growth, and it is not known what se-

quence or structural determinant controls this process. In eukaryotes, determination and initiation of DNA replication are poorly understood. Origins of replication have been studied comprehensively in the yeast *Saccharomyces cerevisiae* and autonomously replicating sequences were identified. The origins are approximately 200 bp long, possess an AT-rich consensus sequence and two or three additional nonconserved auxiliary elements (reviewed in Francon et al. 1999). In addition, it was shown that binding of a multiprotein complex, the origin recognition complex, is essential for the function of an origin (Bell and Stillman 1992). In the holotrichous ciliate *Tetrahymena thermophila*, DNA replication has been analyzed in detail in the 21 kb, palindromic macronuclear rDNA-encoding minichromosome and it was shown that DNA replication initiates within the 5′ nontranscribed spacer (Cech and Brehm 1981; Zhang et al. 1997) and is dependent on *cis*-acting sequence elements (Blomberg et al. 1997; Reischmann et al. 1999). In macronuclear minichromosomes of hypotrichous ciliates, it appears that DNA replication is initiated at or close to telomeres (Murti and Prescott 1983; Allen et al. 1985). Primary structure analysis of *S. lemnae* macronuclear minichromosomes revealed the presence of a semi-conserved AT-rich, palindromic sequence motif in the subtelomeric region of most minichromosomes and it has been proposed that this motif serves as a determinant for minichromosome replication (Helftenbein et al. 1989; Maercker and Lipps 1993). In contrast, the characterization of a telomerase adding single-stranded 3′ overhangs to macronuclear minichromosomes (Zahler and Prescott 1988) and a primase activity in *Oxytricha nova* synthesizing 16 nucleotide RNA primers on 3′ telomeric overhangs in vitro (Zahler and Prescott 1989) led to a model in which telomeres themselves may serve as replication origins in macronuclear chromosomes (Zahler and Prescott 1988, 1989). Thus far, however, a functional analysis of DNA sequences involved in cell cycle-controlled DNA replication of macronuclear chromosomes has not been undertaken in hypotrichous ciliates.

Recently, we established an efficient transfection system for vegetatively growing *S. lemnae* cells (Skovorodkin et al. 1999). This system is based on microinjection of a linear, oligonucleotide-marked α1-tubulin-encoding minichromosome into the macronucleus and raising of clonal cell lines from individual transfected cells. In the present study we developed this system further to a cotransfection system in which two differently marked α1-tubulin-encoding minichromosomes were injected. By mutating one of these minichromosomes, we showed that noncoding and coding sequences of the α1-tubulin-encoding minichromosome can be replaced or deleted without affecting DNA replication efficiency. Hence, it appears that only telomeres may be important for replication of minichromosomes in macronuclei. This hypothesis was supported by our finding that minichromosomes without telomeres were lost within the first cell divisions after injection.

Materials and methods

Strains and culture of *S. lemnae*

Experiments were conducted with *S. lemnae* strains EK-64B and 8a, which were obtained by crossing two naturally occurring strains originally isolated at Dorum in Northern Germany and at Peterhoff near St Petersburg in Russia, respectively. Cells were cultured as described and, under our cultivation conditions, divided approximately 1.3 times per day (Ammermann et al. 1974).

DNA oligonucleotides and plasmid construction

The following DNA oligonucleotides were used: Tuba1-10, Tuba1-11, and tag19 (Skovorodkin et al. 1999); NarI-a, 5′-cgccagctcttcttgcatgacgtgacgtga-3′; NarI-b, 5′-CGTCACGTCACGTCATGCAAGAAGAGCTGG-3′; Tub1-16, 5′-GCCGAGCTCGGGCCCCCCAAAACCCCAAAACCCCAGAACAGTGGATTCGG-3′; Tub1-18, 5′-GCCGGATCCGGGCCCCCCAAAACCCCAAAACCCCAATTGAGATAGAATCGAT-3′; Tub1-20, 5′-ATGAGAGAAGTTATTTCAATTC-3′. The gene construct pTuba1e has been described previously (Skovorodkin et al. 1999). In comparison with the sequence of the endogenous α1-tubulin-encoding minichromosome of *S. lemnae* (Helftenbein 1985), Tuba1e contains the 19 bp insertion 5′-TTCCATGGTATGGCGCCAG-3′ at position 279 relative to the 5′ end of the coding strand of the minichromosome, which causes a premature translation stop on the corresponding mRNA. In addition, Tuba1e contains an engineered second BstXI restriction site at the very end of the 3′ DNA trailer. In construct pTuba1f-st (short tag), the cytosine residue at position 212 of Tuba1e (C212) was deleted, CAT1552 was changed to TTA and A1793 to TT by overlapping polymerase chain reaction (PCR) technology, thereby introducing unique NdeI, HpaI and MfeI restriction sites at positions 209, 1551, and 1790, respectively. NdeI and HpaI restriction sites were located just before and after the α1-tubulin-coding region, respectively, whereas the MfeI site replaced the BstXI site of Tuba1e. In the next step, oligonucleotides NarI-a and NarI-b were hybridized to each other and inserted into the NarI restriction site of Tuba1f-st, extending the tag by the 30 bp sequence 5′-CTCTTCTTGCATGACGTGACGTGACGCCAG-3′. The corresponding gene construct was named pTuba1f-lt (long tag). To generate pTuba1g-st and pTuba1g-lt, the minichromosomal sequences of pTuba1f-st and pTuba1f-lt were amplified by PCR using oligonucleotides Tub1-16 and Tub1-18 and were each cloned into the SmaI site of pTZ18U (Biorad) thereby attaching an ApaI restriction site to the telomeric repeats on either end of the minichromosomes. To construct Tuba1g-st/5′3′, the endogenous BstXI site at position 18 and the NdeI site of Tuba1g-st were used to replace the 5′ DNA leader sequence with a 134 bp inverted fragment of the prokaryotic neomycin phosphotransferase gene (*neo*^{r}), extending from coding position 134 to 1. Similarly, the HpaI and MfeI sites of Tuba1g-st were used to replace the 3′ DNA trailer sequence with a 184 bp inverted *neo*^{r}-fragment comprising coding positions 795 to 612. Finally, construct Tuba1-neo was generated by precisely replacing the α1-tubulin-coding region of Tuba1f-st with the *neo*^{r}-coding region.

Generation of minichromosomal DNA and transfection of *S. lemnae*

Minichromosomal DNA was either generated by PCR with the Expand High Fidelity PCR System (Roche) or by ApaI excision from pTuba1g-st, pTuba1g-lt, or pTuba1-neo gene constructs followed by a blunt-end reaction using the Klenow enzyme. Minichromosomes without telomeres were excised with BstXI from construct pTuba1e. All minichromosomes were purified on a 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen). For cotransfection experiments, different minichromosomes were mixed in equimolar amounts. Prior to injection, the DNA was extracted with buffered phenol:chloroform (1:1, v/v), precipitated

with ethanol, filtered through Minisart SRP 4 filters (Sartorius), lyophilized, and resuspended in distilled water at a concentration of 1–3 µg/µl. As previously described in detail (Skovorodkin et al. 1999), transfection of vegetative *S. lemnae* cells was achieved by microinjection of minichromosomal DNA into the macronucleus. Subsequently, cell lines were raised from individual transfected cells.

DNA analysis

Total DNA was prepared as previously described (Skovorodkin et al. 1999) from 10 cells collected 3 or 7 days after transfection, from 100 cells collected 14 days after transfection, or from approximately 100,000 cells 30 or more days after transfection. In these DNA preparations, the relative amounts of transfected and tagged α1-tubulin DNAs versus endogenous α1-tubulin DNA were analyzed by coamplifying an α1-tubulin-specific DNA fragment spanning the tag insertion site by competitive PCR. The competitive PCRs were carried out either with the oligonucleotide pair Tuba1-10 and Tuba1-11 or with the pair Tub1-20 and Tuba1-11. Amplification products were separated on denaturing 6% polyacrylamide, 50% urea gels and visualized by silver staining using the Biorad silver stain kit. For both primer pairs, the robustness of the competitive PCR assay was tested by varying the minichromosomal input ratio over a tenfold range. In all cases, matching results were obtained in the exponential and nonexponential reaction phase (data not shown). Alternatively, minichromosomal DNA was separated on a 1.5% agarose gel, blotted onto nylon membrane, and analyzed by Southern hybridization. For the specific detection of both short- and long-tagged α1-tubulin-encoding minichromosomes, the digoxigenin-end-labeled oligonucleotide tag19 was used. The digoxigenin-labeled probe neo-cod detected Tuba1g-st/5′3′ minichromosomes and was generated by random-primed labeling of the NdeI-HpaI restriction fragment of pTuba1-neo, which comprised the whole *neo*^{r}-coding region. Finally, the same labeling procedure was used with the HpaI-MfeI restriction fragment of Tuba1f-st, which spans the α1-tubulin 3′ DNA trailer to generate a probe for the detection of endogenous α1-tubulin DNA and Tuba1-neo. In Southern blots, digoxigenin-labeled probes were detected by an enzyme immunoassay with luminescence using the DIG Luminescent Detection Kit (Roche). Southern blot signals and silver-stained PCR products were densitometrically analyzed by the E.A.S.Y Win32 imaging system (Herolab).

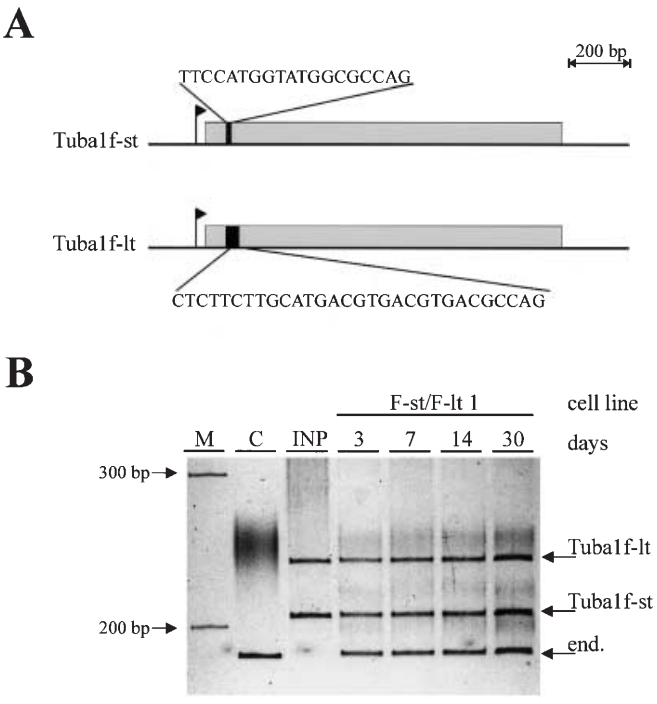

Fig. 1 Cotransfection of two differently marked α1-tubulin-coding minichromosomes. **A** Schematic drawing to scale of the Tuba1f-st and Tuba1f-lt minichromosomes. The α1-tubulin-coding region is represented by the *gray rectangle* and tag insertions by *black boxes*. The 19 bp sequence present in both tags is given above the Tuba1f-st drawing and the sequence of the 30 bp extension of the long tag is indicated below Tuba1f-lt. The *flag* indicates the transcription initiation site. **B** Total DNA of cell line F-st/F-lt 1 prepared 3, 7, 14 or 30 days after transfection was analyzed by competitive polymerase chain reaction (PCR) with oligonucleotides Tuba1-10 and Tuba1-11. Amplification products were separated on denaturing 6% polyacrylamide, 50% urea gels and visualized by silver staining. As controls, total DNA of nontransfected cells (lane *C*) and of the DNA mix used in transfections (*INP*) were analyzed. *Arrows* on the right indicate the Tuba1f-lt, Tuba1f-st, and endogenous α1-tubulin DNA (*end.*) products. *M* marker

Results

Cotransfection of two differently marked minichromosomes

To analyze the sequence of the *S. lemnae* α1-tubulin-coding macronuclear minichromosome for determinants of DNA replication, we developed a cotransfection system that enabled us to compare the replication efficiency of two transfected minichromosomes with each other. In our previous study, we microinjected the α1-tubulin-encoding minichromosome Tuba1e directly into the *S. lemnae* macronucleus and showed that this minichromosome was stably maintained in clonal cell lines in amounts comparable to that of the endogenous α1-tubulin-coding minichromosome for the experimental time span of approximately 80 cell divisions (Skovorodkin et al. 1999). Tuba1e can be distinguished from the endogenous α1-tubulin-coding minichromosome by a 19 bp unrelated tag sequence that was inserted into the protein-coding region. In this study, Tuba1e was further developed to Tuba1f-st, a cassette-type vector in which unique restriction sites were introduced at the borders between the coding and noncoding regions and between the noncoding regions and telomeres. For cotransfection, we generated a second α1-tubulin-coding minichromosome, Tub1f-lt, in which the tag was extended to a length of 49 bp (Fig. 1a). Our previous results indicated that minichromosomes lacking the 3′ overhang were as efficient in transfection as minichromosomes possessing perfect ends (Skovorodkin et al. 1999). Therefore, we generated both Tuba1f-st and Tuba1f-lt minichromosomes by PCR, mixed them in equimolar amounts and microinjected the DNA mix directly into the macronucleus of vegetative cells. Clonal cell lines were raised from each transfected cell and three cell lines were obtained that contained detectable amounts of transfected DNA (Fig. 1 and data not shown). On days 3, 7, 14, and 30 after transfection, total DNA was prepared from

transfected cells and the relative amounts of the endogenous α1-tubulin DNA, Tuba1f-st and Tuba1f-lt were determined by competitive PCR amplification of a 184 bp sequence of the α1-tubulin gene that spans the site of tag insertion. The PCR products were separated by denaturing polyacrylamide electrophoresis and visualized by silver staining. As shown for cell line F-st/F-lt 1 in Fig. 1, the ratio of amounts of Tuba1f-st and Tuba1f-lt amplification product was the same in the DNA mix used for transfection (Fig. 1, lane 2, INP) and in the genomic DNA of transfected cells (Fig. 1, compare lane 2 with lanes 3–6). This ratio did not change during the 30 day experimental phase, demonstrating that both Tuba1f-st and Tuba1f-lt minichromosomes were transfected and maintained in the cells with the same efficiency. Moreover, both transfected minichromosomes remained in similar amounts relative to endogenous α1-tubulin DNA, suggesting that the latter was not preferentially replicated in transfected cells (Fig. 1, lanes 3–6). Hence, the cotransfection assay proved to be suitable for the analysis of DNA replication sequence determinants in the α1-tubulin-coding minichromosome.

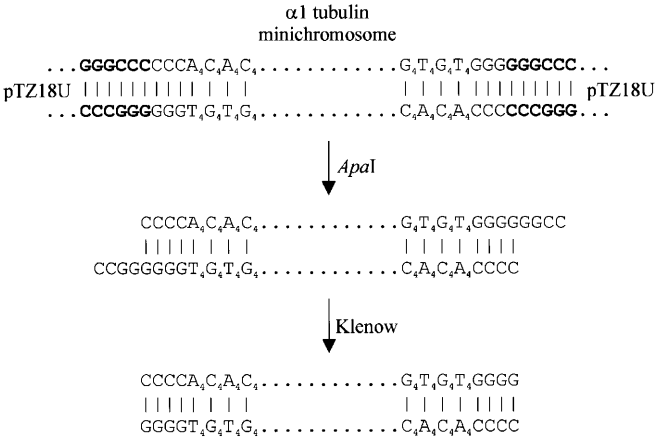

Fig. 2 Preparation of α1-tubulin-encoding minichromosomes for transfection. Sequences of α1-tubulin-coding minichromosomes including the 20 bp double-stranded telomeric repeat regions were fused on both sides to ApaI restriction sites (*bold letters*) and cloned into pTZ18U. To generate minichromosomes with correct double-stranded telomeres for transfection, minichromosomal sequences were excised from gene constructs by ApaI digestion, blunt-ended with Klenow enzyme, and gel purified

The 5′ and 3′ noncoding regions of the α1-tubulin-coding minichromosome lack sequence determinants for cell cycle-controlled DNA replication

In the next step, the cotransfection assay was employed to analyze sequence determinants of DNA replication in vegetative cells. To avoid potential nonspecific effects of unfaithful minichromosome production by PCR, we developed our gene constructs further and cloned the minichromosomal sequences between two ApaI restriction sites. Excision of minichromosomal DNA from these constructs with ApaI and a subsequent blunt-end reaction with the Klenow enzyme generated perfect 20 bp double-stranded telomeric repeat sequences at both ends of the minichromosomes (Fig. 2). The gene constructs containing the short and long tags were named pTuba1g-st and pTuba1g-lt, respectively. In order to find out whether there are any sequence determinants for DNA replication in the noncoding regions of the α1-tubulin-coding minichromosome, we replaced both the 5′ leader and 3′ trailer with unrelated sequences of the prokaryotic neomycin phosphotransferase gene (*neo*^{r}; Fig. 3a). The corresponding minichromosome, Tuba1g-st/5′3′, marked with the short tag was cotransfected with minichromosome Tuba1g-lt, which contained the wild-type flanking regions and the long tag. Five out of 11 microinjections were successful, giving rise to cell lines G-st5′3′/G-lt 1–5. Total DNA prepared from transfected cells 3, 7, 14, 30, and 40 days after transfection was analyzed by competitive PCR (Fig. 3b). Since this pair of transfected minichromosomes had only the α1-tubulin-coding region and the telomeric repeat sequences in common, a shorter fragment than in our first analysis was amplified. The product of the endogenous α1-tubulin gene had a length of 91 bp and, since it spanned the tag insertion site, the products of Tuba1g-st/5′3′ and Tuba1g-lt were expected to have lengths of 110 bp and 140 bp, respectively. As expected, DNA of nontransfected control cells gave rise to a single PCR product corresponding to the endogenous α1-tubulin DNA (Fig. 3b, lane 2, C) whereas all three products were detectably amplified from DNA of transfected cells (Fig. 3b, lanes 4–12). As shown for cell line G-st5′3′/G-lt 1, the product ratio of Tuba1g-st/5′3′ and Tuba1g-lt DNA did not change during the 40 day experimental phase (Fig. 3b, lanes 4–8) and closely resembled the ratio obtained with the DNA mix that was injected into the cells (Fig. 3b, lane 3, INP). The same result was obtained with DNA of the other four cell lines, which was prepared 40 days after transfection (Fig. 3b, compare lane 2 with lanes 9–12). Hence, DNA of both transfected minichromosomes was propagated in transfected cells at the same rate. Since the amounts of products derived from transfected minichromosomes did not change in comparison with the product of endogenous α1-tubulin over time (Fig. 3b, lanes 4–8, and data not shown), we conclude that endogenous and both transfected α1-tubulin-coding minichromosomes were replicated with the same efficiency. In sum, these results suggest that the noncoding sequences of the α1-tubulin-coding minichromosome do not contain determinants for DNA replication.

However, it may be possible that the coding region of Tuba1g-st/5′3′ became fused to the α1-tubulin 5′ DNA leader or 3′ DNA trailer by homologous recombination, thereby linking the short tag to a potential replication origin. To exclude this possibility and to verify the results obtained by competitive PCR, we carried out a Southern blot analysis of DNA obtained from cell lines G-st5′3′/G-lt 40 days after transfection. Macronuclear minichromosomes were separated on a 1.5% agarose gel, blotted

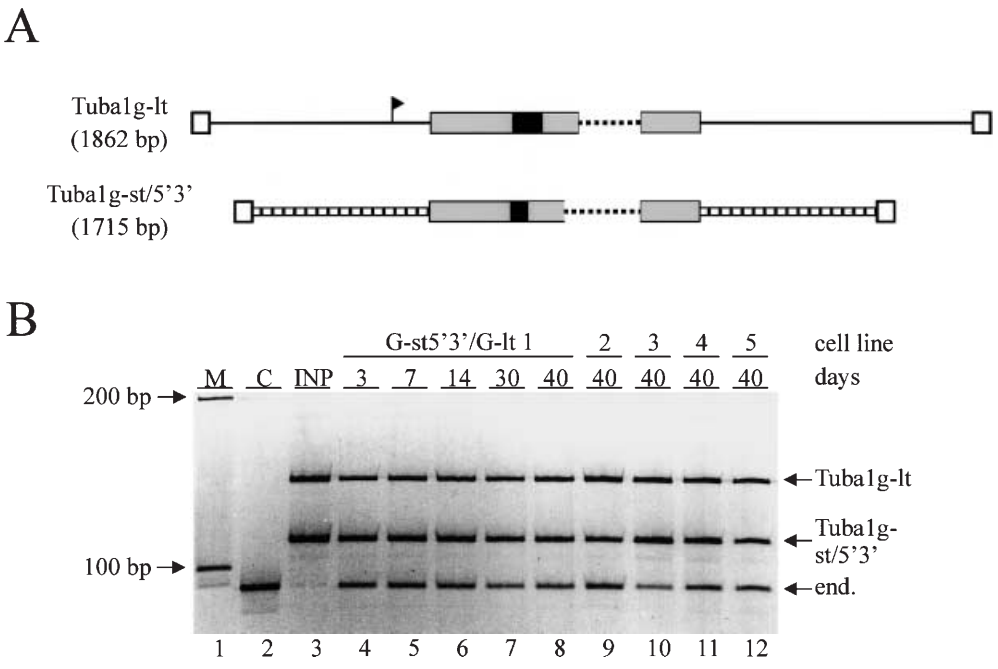

Fig. 3 Cotransfection of Tuba1g-lt and Tuba1g-st/5′3′ minichromosomes. **A** Schematic outline of Tuba1g-lt and Tuba1g-st/5′3′ minichromosomes. The α1-tubulin-coding region, tag sequences and double-stranded telomeric repeats are indicated by *gray*, *black* and *open rectangles*, respectively. Noncoding regions of the α1-tubulin-determining minichromosome are represented by *black lines*, whereas substitutions of these regions with neomycin phosphotransferase sequences are indicated by *stippled lines*. The *flag* indicates the transcription start site. **B** An equimolar mix of Tuba1g-lt and Tuba1g-st/5′3′ minichromosomes was transfected into vegetative *Stylonychia lemnae* cells and total DNA of transfected cell lines G-st5′3′/G-lt 1 to 5 was analyzed by competitive PCR using oligonucleotides Tuba1-11 and Tuba1-20. For cell line G-st5′3′/G-lt 1, DNA prepared 3, 7, 14, 30, or 40 days after transfection (*lanes 4–8*) and for cell lines G5′3′-H2–5, DNA prepared 40 days after transfection (*lanes 9–12*) was analyzed. DNA of nontransfected cells (*C*) and of the mix used for transfection (*INP*) was investigated for comparison. On the right, *arrows* indicate amplification products of Tuba1g-lt, Tuba1g-st/5′3′, and endogenous α1-tubulin DNA (*end.*), which were separated by denaturing gel electrophoresis and stained with silver. *M* marker

onto nylon membrane and first detected with the 5′ end-labeled oligonucleotide tag19 (Fig. 4a). This probe specifically detected minichromosomes carrying the long or the short tag sequence and did not cross-react with DNA of control cells (Fig. 4a, lane C). As indicated in Fig. 3a, Tuba1g-st/5′3′ has a size of 1715 bp whereas Tuba1g-lt is 1862 bp long. Correspondingly, tag19 hybridization detected two bands at the expected positions in DNA of transfected cells (Fig. 4a, lanes 1 to 5). The ratio of these two bands correlates with the ratio of the DNA mix that was used in transfection (Fig. 4a, lane INP) confirming the competitive PCR results (Fig. 3) that both minichromosomes were replicated with the same efficiency in transfected cells. Moreover, when the same blot was reprobed with *neo*^{r} sequences only the lower Tuba1g-st/5′3′ band became visible, demonstrating that Tuba1g-st/5′3′ DNA remained intact in transfected cells and was not rearranged by recombination events (Fig. 4b). Finally, to exclude the unlikely possibility that the *neo*^{r}-coding sequences present in Tuba1g-st/5′3′ contain a replication origin for the *S. lemnae* α1-tubulin-coding minichromosome, we deleted the noncoding regions of Tuba1g-st and fused the telomeric repeats directly to the α1-tubulin-coding region. Analysis by competitive PCR of DNA prepared from five independently transfected cell lines showed that this truncated minichromosome was replicated as efficiently as Tuba1g-lt (data not shown). Hence, we concluded that the 5′ leader and 3′ trailer of the α1-tubulin-coding minichromosome in *S. lemnae* do not contain sequence determinants for cell cycle-controlled DNA replication.

Replacement of the α1-tubulin-coding region does not interfere with DNA replication

Although unlikely, it is possible that the α1-tubulin-coding region harbors sequence determinants important for minichromosome replication. To exclude this possibility, we replaced the α1-tubulin-coding region with that of neomycin phosphotransferase, a prokaryotic protein. The corresponding minichromosome was termed Tuba1-neo and was microinjected into *S. lemnae*. Clonal cell lines were kept for 2 years in the absence of neomycin selection and the DNA of these cell lines was regularly analyzed for the presence of the Tuba1-neo minichromosome (Fig. 5 and data not shown). Southern analysis data of two cell lines that contain very different amounts of Tuba1-neo are shown in Fig. 5. In this blot, the endogenous α1-tubulin-coding minichromosome and Tuba1-neo were simultaneously detected with a probe spanning the 3′ trailer of this chromosome excluding the telomeres. While cell line NEO1 contained severalfold more

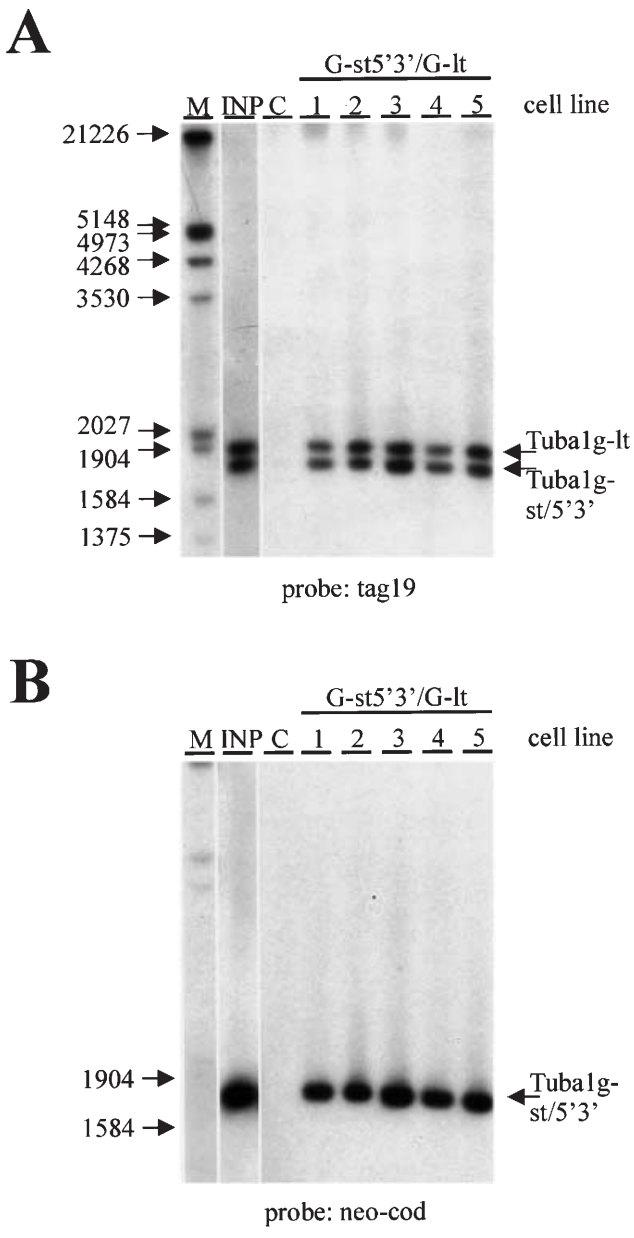

Fig. 4 Southern analysis of total DNA from cell lines G-st5′3′/G-lt. **A** Total DNA of nontransfected cells (*C*) and of cell lines G-st5′3′/G-lt 1–5 prepared 40 days after transfection was separated on a 1.5% agarose gel and blotted onto nylon membrane. For comparison, the DNA mix used in transfections was analyzed. Tuba1g-lt and Tuba1g-st/5′3′ minichromosomes were specifically detected with the 5′ end-labeled oligonucleotide tag19, which is complementary to both tag sequences as indicated on the *right*. *M* marker λ DNA, digested with HindIII and EcoRI. The fragment sizes in base pairs are shown on the left. **B** The same blot was stripped and minichromosomal DNA containing *neo*^{r}-coding sequences was detected with the probe neo-cod

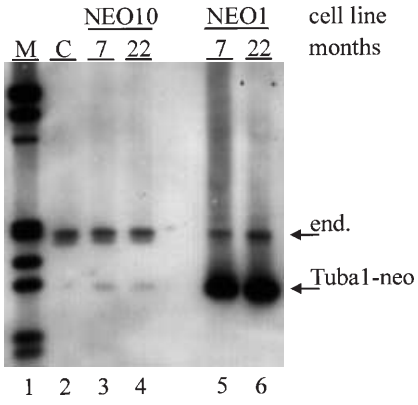

Fig. 5 Southern analysis of total DNA from cell lines NEO1 and NEO10. Cells of *S. lemnae* were transfected with Tuba1-neo minichromosomes and total DNA was prepared from transfected cell lines NEO10 and NEO1 7 and 22 months after transfection. Minichromosomal DNA of these preparations and of total DNA prepared from nontransfected control cells (*C*) was separated on a 1% agarose gel and blotted onto a nylon membrane. Endogenous α1-tubulin-coding minichromosomes and Tuba1-neo minichromosomes were detected with a probe spanning the 3′ noncoding sequence of the α1-tubulin-encoding minichromosome. The weak band just below the α1-tubulin-coding minichromosome corresponds most likely to the endogenous α2-tubulin-coding minichromosome

Tuba1-neo DNA than endogenous α1-tubulin DNA (Fig. 5, lanes 5, 6), cell line NEO10 contained much less of the transfected minichromosome (Fig. 5, lanes 3, 4). Nevertheless, the amount of Tuba1-neo in both cell lines did not change significantly over a 15 month period (Fig. 5, compare lanes 3 and 5 with lanes 4 and 6, respectively). Therefore, we conclude that the α1-tubulin-coding region does not contain sequence or structural information relevant to DNA replication.

Rapid loss of α1-tubulin-coding minichromosomes lacking telomeres after transfection

Thus far, we have shown that all sequences between the telomeric repeats of the *S. lemnae* α1-tubulin-coding minichromosome can be replaced or deleted without diminishing the cell cycle-controlled replication rate as compared with a cotransfected, tagged wild-type minichromosome or with the endogenous minichromosome. This suggested that the telomeres might be important for replication. In accordance with this hypothesis, transfected Tuba1e minichromosomes lacking telomeres were rapidly lost after transfection in three independently transfected cell lines in contrast with cotransfected Tuba1g-lt minichromosomes with telomeres (Fig. 6, compare lanes 4–9 with lane 2). As shown for the cell line E/G-lt 1, the amount of Tuba1e minichromosomes was drastically reduced to a faint band 3 days after transfection (Fig. 6, lane 4), demonstrating that telomeres are of crucial importance for stable transfection of minichromosomes. Although this result argues that telomeres are important for minichromosome replication, the fast decline of Tuba1e DNA in transfected cells may equally well have been caused by DNA degradation. Surprisingly, a faint but distinct band of Tuba1e DNA remained detectable for the whole experimental period in all three cell lines (Fig. 6, lanes 5–9). Possibly, transfected cells were able stably to maintain Tuba1e minichromosomes at a low level by adding telomeres to them de novo.

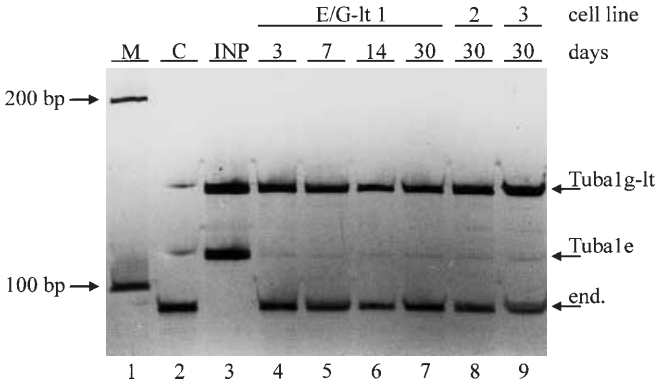

Fig. 6 Minichromosomes without telomeric repeat sequences are rapidly lost after transfection. Cells of *S. lemnae* were transfected with a mix of Tuba1g-lt and Tuba1e minichromosomes (*INP*) and clonal cell lines E/G-lt 1, E/G-lt 2, and E/G-lt 3 were raised from single transfected cells. Total DNA was prepared from nontransfected cells (*C*) and from E/G-lt cells 3, 7, 14 or 30 days after transfection, and analyzed by competitive PCR using oligonucleotides Tuba1-11 and Tuba1-20. Coamplified PCR products were separated on a denaturing 6% polyacrylamide, 50% urea gel and visualized by silver staining. On the right, PCR products of Tuba1g-lt, Tuba1e, and the endogenous α1-tubulin gene (*end.*) are indicated by *arrows*. *M* marker

Discussion

We have developed a stable cotransfection assay for vegetatively growing *S. lemnae* cells based on oligonucleotide-marked α1-tubulin-coding minichromosomes as vectors. This assay system allowed us to monitor exactly the fate of a mutated minichromosome in comparison with a cotransfected, unaltered control minichromosome as well as with the endogenous minichromosome in transfected cell lines. Complete deletion of both the 5′ and 3′ noncoding sequences of the α1-tubulin-coding minichromosome or their substitution with unrelated sequences from the protein-coding region of a prokaryotic gene resulted in minichromosomes that were as efficiently maintained in transfected cells as a marked wild-type control minichromosome. Similarly, the coding region of the α1-tubulin-determining minichromosome could be replaced without affecting the replication rate in vegetative *S. lemnae* cells. Moreover, Southern analysis of DNA from transfected cells revealed that introduced minichromosomes remained intact and were not detectably rearranged by DNA recombination events that may have fused tags to wild-type sequences containing replication origins. Therefore, we concluded that the *S. lemnae* α1-tubulin-coding minichromosome, with the exception of the telomeric repeats, does not possess any sequence determinant for cell cycle-controlled DNA replication. Since all sequences between the telomeres could be replaced or deleted without affecting the replication rate, it is most unlikely that replication of other macronuclear minichromosomes of *S. lemnae* requires such elements.

These findings discount assumptions made in previous studies that noncoding sequences of *S. lemnae* macronuclear minichromosomes harbor determinants for DNA replication (Helftenbein et al. 1989; Maercker and Lipps 1993). On the other hand, our data strongly support the replication model proposed by Zahler and Prescott (1988, 1989). In their model, primase synthesizes RNA primers on telomeric 3′ overhangs and DNA polymerase starts replication at the beginning of the double-stranded region. Subsequently, the RNA primer sequence is degraded and single-stranded 3′ telomeric overhangs are added by telomerase. In accordance with our results, DNA replication in this model does not depend on internal sequence determinants.

By cotransfection experiments, we demonstrated the importance of telomeres for stable transfection. However, it may still be possible that telomeres merely protect minichromosomes from degradation and DNA replication starts randomly and independently of sequence within minichromosomes. Alternatively, multiple redundant replication origins may exist in protein-coding and noncoding regions. Although we cannot exclude these possibilities, they are unlikely because electron microscopic analyses have shown that in macronuclear minichromosomes DNA replication starts exclusively at or near telomeres (Murti and Prescott 1983; Allen et al. 1985).

Our results are also in close agreement with results obtained in the holotrichous ciliate *Paramecium tetraurelia*. This organism was able to replicate transfected, linearized prokaryotic plasmid DNA (Gilley et al. 1988) or restriction fragments of phage lambda DNA (Kim et al. 1992), both lacking *Paramecium*-specific sequences, at least to such an extent that the introduced DNA was detectable 25 cell fissions after transfection. However, in these studies it remained unclear how efficient replication of introduced DNA was relative to that of endogenous chromosomes. In fact, some results indicated that endogenous chromosomes were more efficiently replicated than prokaryotic DNA fragments (Gilley et al. 1988). Conversely, our cotransfection experiments demonstrated that mutated minichromosomes were as efficiently replicated as the cotransfected control and the endogenous minichromosome.

In spite of our results, some macronuclear minichromosomes of hypotrichous ciliates may contain sequence information influencing the rate of DNA replication. In several independent studies on *S. lemnae* (Steinbrück 1983; Wegner et al. 1989; Kreyenberg et al. 1998) and *O. nova* (Harper et al. 1991), a strong increase in some macronuclear minichromosomes was observed under prolonged vegetative growth. Since this macronuclear overamplification is restricted to only a few minichromosomes and overamplified minichromosomes do not contain aberrant telomeres, determination of overamplification may be promoted by internal sequences or structures.

In conclusion, our study demonstrated that in vegetatively growing *S. lemnae* cells macronuclear α1-tubulin-coding minichromosomes do not contain nontelomeric sequence determinants for DNA replication. Most likely,

the DNA replication machinery utilizes in a unique way the telomeric structure for initiation of minichromosome replication.

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