Developmentally regulated initiation of DNA synthesis by telomerase: evidence for factor-assisted *de novo* telomere formation

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Telomerase serves a dual role at telomeres, maintaining tracts of telomere repeats and forming telomeres *de novo* on broken chromosomes in a process called chromosome healing. In ciliates, both mechanisms are readily observed. Vegetatively growing cells maintain pre-existing telomeres, while cells undergoing macronuclear development fragment their chromosomes and form telomeres *de novo*. Here we provide the first evidence for developmentally regulated initiation of DNA synthesis by telomerase. *In vitro* assays were conducted with telomerase from vegetative and developing *Euplotes* macronuclei using chimeric primers that contained non-telomeric 3' ends and an upstream stretch of telomeric DNA. In developing macronuclei, chimeric primers had two fates: nucleotides were either polymerized directly onto the 3' terminus or residues were removed from the 3' end by endonucleolytic cleavage before polymerization began. In contrast, telomerase from vegetative macronuclei used only the cleavage pathway. Telomere repeat addition onto non-telomeric 3' ends was lost when developing macronuclei were lysed and the contents purified on glycerol gradients. However, when fractions from the glycerol gradient were added back to partially purified telomerase, telomere synthesis was restored. The data indicate that a dissociable chromosome healing factor (CHF) collaborates with telomerase to initiate developmentally programmed *de novo* telomere formation.

Keywords: chromosome healing/development/DNA synthesis initiation/telomerase

Introduction

The integrity of the telomere complex at chromosome ends is essential for genome stability. Chromosomes lacking intact telomeres are detected by DNA damage checkpoints and if not repaired lead to cell cycle arrest (Sandell and Zakian, 1993; Garvik *et al.*, 1995). Aberrant chromosomes that escape this surveillance system are subject to end-to-end fusion or degradation (Gall, 1995).

Telomere synthesis by telomerase is the primary mechanism for sustaining chromosome ends in eukaryotes (Blackburn, 1992). An unusual ribonucleoprotein DNA polymerase, telomerase binds the 3' terminus of the telomere and catalyzes the addition of short repeats of G-rich DNA. This DNA addition counteracts the inability of the cellular replication machinery to duplicate fully chromosome ends (Watson, 1972; Olovnikov, 1973), thereby helping to preserve telomeres throughout the lifetime of an organism.

Telomerase directs DNA polymerization using an essential RNA subunit as a template (Greider, 1995). Although the role of telomerase proteins is less clear, they have been postulated to promote DNA recognition (Collins *et al.*, 1995; Harrington *et al.*, 1995), enzyme processivity (Morin, 1989; Collins and Greider, 1993; Lee and Blackburn, 1993) and catalysis. Two distinct sites in telomerase contact DNA: the templating domain where the 3' terminus of single-stranded DNA hybridizes to the complementary RNA sequence, and the proteinaceous anchor site which binds clusters of dG residues upstream of the DNA 3' terminus (Morin, 1989; Harrington and Greider, 1991; Collins and Greider, 1993; Lee and Blackburn, 1993; Melek *et al.*, 1996) (Figure 1A). Once the DNA 3' terminus binds the active site, synthesis initiates by RNA-directed polymerization of a short stretch of DNA to complete a single telomeric repeat. A translocation event repositions the DNA 3' terminus back at the beginning of the RNA template site, while contact with the upstream DNA is maintained through anchor site interactions. DNA–anchor site contacts facilitate processive addition of telomere repeats by the enzyme (Morin, 1989; Collins and Greider, 1993; Lee and Blackburn, 1993).

Besides maintaining pre-existing telomeres, telomerase can catalyze the synthesis of telomeric repeats directly onto non-telomeric DNA (reviewed in Melek and Shippen, 1996). The formation of a new telomere stabilizes a broken chromosome. Hence, this process is referred to as chromosome healing. Documented in organisms as diverse as yeast (Murray *et al.*, 1988; Kramer and Haber, 1993), worms (Muller *et al.*, 1991), protozoa (Prescott, 1994), plants (McClintock, 1941) and humans (Wilkie *et al.*, 1990), new telomeres can be generated spontaneously following accidental chromosome breakage or artificially induced breakage (Farr *et al.*, 1991; Hanish *et al.*, 1994). Spontaneous telomere formation is very rare compared with the developmentally programmed chromosome fragmentation and telomere healing events associated with conjugation in ciliated protozoa (Prescott, 1994). Massive reorganization of the genome occurs as a copy of the germline micronucleus is converted into a new somatic macronucleus. In *Euplotes crassus*, this developmental program involves chromosome polytenization, extensive site-specific DNA fragmentation,

Fig. 1. DNA processing by the *Euplotes* telomerase. (A) Recognition and elongation of telomeric primers. Telomerase contains three discrete domains of DNA interaction: the anchor site, the RNA template and the cleavage site. Telomeric primers consisting of T₄G₄ repeats (open arrow) interact with both the RNA template and the protein anchor site. The primer 3' terminus is extended by copying the RNA template. The predicted template for *E. crassus* lies between residues 37 and 44 in the RNA (Shippen-Lentz and Blackburn, 1990; Melek *et al.*, 1994). (B) Direct elongation of non-telomeric 3' ends. An upstream telomeric sequence (open box) in a chimeric primer is used to recruit efficiently telomerase to non-telomeric 3' ends (shaded line). The telomeric sequence binds in the anchor site and the primer 3' terminus is delivered to a 'default' site on the RNA template (enlarged bold C) which results in the addition of (dGGGGTTT)_n to the primer (Melek *et al.*, 1996). (C) Cleavage-initiated primer elongation. Chimeric primers whose telomeric sequences contact the RNA template are endonucleolytically cleaved prior to telomere addition. Primer nucleotides that align at or beyond residue 36 in the RNA are removed, whether telomeric or not (Melek *et al.*, 1996).

telomere addition and further rounds of DNA replication. The new macronuclear genome is created in ~100 h (Roth and Prescott, 1985) and contains ~10 million linear DNA molecules, each capped with T₄G₄ telomeric repeats (Klobutcher *et al.*, 1981).

Chromosome healing by telomerase must necessarily be distinct from telomere maintenance, since the former reaction initiates in the absence of extensive Watson–Crick base pairing between the chromosome end and the telomerase RNA template. Given telomerase's need for G-rich primer DNA (Greider, 1995), it is unclear how the enzyme is recruited to a site of new telomere formation *in vivo*. Nevertheless, some aspects of *de novo* telomere synthesis can be

mimicked *in vitro*. Ciliate telomerases will add telomeric repeats directly onto DNA primers that lack any 3'-terminal complementarity to the RNA template (Harrington and Greider, 1991; Wang and Blackburn, 1997). However, the efficiency of this reaction is greatly increased when chimeric primers containing a stretch of telomeric DNA at their 5' terminus are used (Harrington and Greider, 1991; Melek *et al.*, 1996). The upstream sequence, specifically clusters of dG residues (Melek *et al.*, 1996), contacts the anchor site, helping to establish a stable primer:enzyme binary complex (Figure 1B). Since the upstream telomeric sequence enhances a reaction intrinsic to telomerase instead of promoting a novel reaction, chimeric primers are a useful tool for studying non-telomeric 3'-end processing. *In vivo* and *in vitro*, *Euplotes* telomerase initiates synthesis on non-telomeric DNA by the ordered addition of four dG residues followed by four dT residues (Klobutcher *et al.*, 1981; Melek *et al.*, 1996). Thus, the non-telomeric 3' terminus appears to be reproducibly delivered to a 'default' position on the telomerase RNA template during new telomere formation (Melek *et al.*, 1996; Wang and Blackburn, 1997) (Figure 1B).

Euplotes telomerase is capable of processing non-telomeric 3' ends in a second pathway (Figure 1C). With some chimeric primers, the enzyme fails to add telomeric repeats directly onto the non-telomeric DNA. In these cases, elongation is blocked presumably because the non-telomeric 3' terminus becomes positioned outside the polymerization site (Melek *et al.*, 1996). Such a primer is converted into an elongation substrate for telomerase upon removal of the non-telomeric 3' DNA to expose an internal telomeric sequence. DNA cleavage proceeds via an endonucleolytic reaction (Melek *et al.*, 1996) directed by the enzyme's internal RNA template (Collins and Greider, 1993; Autexier and Greider, 1995; Melek *et al.*, 1996). The cleavage activity has been postulated to serve a proofreading role and/or participate in site-specific chromosome fragmentation during ciliate macronuclear development (Collins and Greider, 1993; Melek *et al.*, 1996).

In this study, we examined the two different mechanisms telomerase employs in processing non-telomeric DNA, direct extension and cleavage-initiated extension, as a function of macronuclear development in *Euplotes*. Our results indicate that efficient catalysis of telomeric repeats directly onto non-telomeric 3' ends is a reaction confined to macronuclear development. Telomerase from vegetative macronuclei will not initiate synthesis directly onto non-telomeric DNA. Instead, primers with four or five nucleotides of 3'-terminal complementarity to the RNA template are needed for elongation. Upon purification on glycerol gradients, extracts from developing macronuclei lose the capacity to extend directly non-telomeric 3' ends. However, *de novo* telomere formation can be restored by the addition of side fractions from the gradient to partially purified telomerase. Hence, the *Euplotes* telomerase acts in concert with at least one dissociable factor to facilitate programmed chromosome healing.

Results

Two pathways for processing non-telomeric 3' ends in developing macronuclei

Euplotes telomerase from developing macronuclei will elongate primers consisting of random DNA, but with

Fig. 2. Telomerase from developing macronuclei can process non-telomeric 3' termini by two pathways. Reactions are shown with $GT_4(G_4T_4)_2$ (lane 1); GT-13, $(G_4T_4)ACTACGCGATCAT$ (lane 2); 7-GT-6, $CACTATC(G_4T_4)GATCAT$ (lane 3); 7-GT-6 (Me), $CACTATC(G_4T_4)GATCAT$ (lane 4); pBR, $CACTATCGACTACGCGATCAT$ (lane 5). Left arrow denotes migration of the $GT_4(G_4T_4)_2$ extended by a single dG residue (see Materials and methods). Right arrow denotes the position of the GT-13 extended by one dG residue (not shown). Closed circles indicate cleavage-initiated elongation products and open circles, direct elongation products. The single band generated in the reaction with pBR corresponds to the addition of a dG residue.

extremely low efficiency (Figure 2, lane 5). In contrast, chimeric primers with random 3' ends and an upstream cassette of telomeric sequence (Figure 2, lane 2) are extended comparably with completely telomeric primers (Figure 2, lane 1; Melek *et al.*, 1996). We have previously shown that chimeric primer 3' ends can be processed by an endonucleolytic cleavage pathway or by direct nucleotide addition (Melek *et al.*, 1996). The fate of the chimeric primer depends upon the position of the telomeric cassette within the non-telomeric sequence. For example, a chimeric primer (GT-13) bearing a stretch of telomeric sequence at its 5' terminus and 13 non-telomeric nucleotides at its 3' terminus was extended directly by the addition of nucleotides onto the primer 3' terminus (Figure 2, lane 2). In contrast, 7-GT-6, which carries an internal telomeric cassette, seven 5' non-telomeric residues and

six 3' non-telomeric residues, was extended by both direct nucleotide addition and cleavage-initiated elongation (Figure 2, lane 3). Two overlapping banding patterns were observed: one resulting from direct addition onto the non-telomeric 3' end (Figure 2, open circles) and another corresponding to nucleotide addition onto cleaved primers in which the six 3' non-telomeric residues had been removed (closed circles).

To confirm this interpretation, telomerase was assayed with a modified version of the 7-GT-6 primer which carried a non-hydrolyzable methylphosphonate linkage at the site of cleavage. Because cleavage is endonucleolytic, cleavage-initiated elongation should be blocked with this primer, while direct nucleotide addition to the primer 3' terminus is unaffected (Melek *et al.*, 1996). As expected, cleavage-initiated elongation was eliminated (Figure 2, lane 4) and the remaining pattern of higher molecular weight products corresponded to direct nucleotide addition (Figure 2, lane 2). We conclude that telomerase from developing macronuclei processes non-telomeric DNA by both the cleavage and the direct elongation pathways.

Telomerase from vegetative *Euplotes* macronuclei does not initiate synthesis directly on non-telomeric 3' ends

Chromosome healing in ciliates is confined to a stage in macronuclear development temporally coupled to massive site-specific DNA fragmentation (Prescott, 1994). During vegetative growth, telomerase is not expected to encounter non-telomeric DNA. Instead, the enzyme presumably functions to 'top off' pre-existing telomeres with DNA as a counterbalance to the loss of terminal sequences that results from chromosomal replication. Therefore, it was of interest to determine whether and how telomerase from vegetative cells interacts with non-telomeric DNA.

Macronuclei from vegetatively growing *Euplotes* were isolated and assayed initially for elongation of standard telomeric primers. Elongation reactions displayed properties consistent with telomerase extension, including RNase and protease sensitivity, and predicted changes in product banding profiles when primers bearing different sequence permutations were used (data not shown). Vegetative macronuclei contained less telomerase activity than developing macronuclei. In assays with equivalent numbers of macronuclei, the vegetative reaction products were 5- to 10-fold less abundant than products generated from developing macronuclei (data not shown). This observation correlates with the lower amount of telomerase RNA found in vegetative versus developing *Euplotes* cells (Price *et al.*, 1994).

One obvious distinction between the vegetative and developmental preparations was in the banding profile of elongation products. Vegetative telomerase generated two strong bands of equal intensity within each of the eight nucleotide repeats, one corresponding to the addition of the third dG and the other to the fourth dT (GGGGTTT) (Figure 3A, lane 3). In contrast, telomerase products from developing macronuclei were characterized by a single prominent band which mapped to the third dG residue (Figure 3A, lane 2). The most intense band in the developmental *Euplotes* telomerase profile was previously reported to map to the fourth dG (Shippen-Lentz and Blackburn, 1989); however, further analysis using both 5'- and 3'-

Fig. 3. Differential DNA processing by developmental and vegetative macronuclei. Telomerase from vegetative and developmental macronuclei was analyzed for primer elongation. **(A)** Telomerase from developing and vegetative macronuclei generates different banding profiles. Lane 1, $GT_4(G_4T_4)_2$ extended by a single dG residue (see Materials and methods); lanes 2 and 3, $GT_4(G_4T_4)_2$ reacted with developmental and vegetative macronuclei, respectively. The deduced sequence for the elongation products is indicated. Approximately five times more vegetative macronuclei were needed to generate a signal equivalent to developing macronuclei. **(B)** Elongation of telomeric and chimeric primers by telomerase from vegetative macronuclei. The arrow denotes the position of the $GT_4(G_4T_4)_2$ extended by one dG residue. The asterisk marks the product of a non-specific labeling activity in vegetative macronuclei.

labeled oligonucleotides as controls showed that the strongest band corresponded to the third dG (Figure 3A, and data not shown). Altering nucleotide concentrations in the reaction over a 2500-fold range for dTTP (2 μ M–5 mM) and a 1000-fold range for dGTP (5 nM–5 μ M) did not convert the telomerase product banding pattern from vegetative macronuclei into the pattern seen with developmental macronuclei or vice versa (data not shown). The distinctive banding profiles generated by the vegetative and developmental telomerases suggest an inherent mechanistic difference in the two enzymes.

Vegetative and developmental telomerase preparations were remarkably different in their ability to process non-telomeric DNA. In contrast to telomerase from developing macronuclei, the vegetative telomerase did not elongate

the chimeric primer GT-13 into the typical eight nucleotide repeated ladder of products. Instead, the reaction generated one prominent product band, corresponding to the addition of a dG residue (Figure 3B, lane 2; data not shown). Formation of this product was sensitive to RNase treatment (data not shown). However, since the *Euplotes* telomerase has previously been shown to add a dG residue to primers non-specifically (Shippen-Lentz and Blackburn, 1990; and see Figure 2, lane 5), and since a ladder of products consistent with telomerase synthesis was not generated, we conclude that telomerase from vegetative macronuclei did not initiate productive elongation on the GT-13 primer.

The inability of the vegetative telomerase to add telomeric repeats directly onto non-telomeric DNA was further substantiated in reactions with 7-GT-6. This primer was

extended, but only by cleavage-initiated elongation (Figure 3B, lane 3). For comparison, the banding profile of the telomeric control primer GT₄(G₄T₄)₂ (Figure 3B, lane 1) should be identical to the profile obtained with GT-13 or 7-GT-6 if these primers are extended by direct nucleotide addition pathway (see Figure 2). The vegetative telomerase did not react with the methylphosphonate-substituted version of 7-GT-6, 7-GT-6 (Me), which cannot be cleaved by telomerase. Hence, telomerase from vegetative macronuclei failed to utilize the direct addition pathway.

We tested whether the vegetative telomerase required a longer stretch of upstream telomeric DNA to extend a non-telomeric 3' terminus. Conceivably, the increased length of telomeric DNA would stabilize the primer–telomerase complex. The *Tetrahymena* telomerase from cells undergoing development needs a relatively long 5' telomeric sequence (TTGGGGTTGGGG) to extend efficiently a non-telomeric 3' end (Harrington and Greider, 1991). Vegetative macronuclei from *Euplotes* were assayed with 21 nucleotide chimeric primers containing a non-telomeric 3' terminus and increasing amounts of telomeric sequence at their 5' ends. The telomeric sequence ranged from 12 nucleotides for GTG-9 to 20 nucleotides for GTGTG-1. The deduced alignments for these primers on the vegetative telomerase RNA template are shown in Figure 4A.

All the primers in this series were elongated by the vegetative telomerase, but with different efficiencies and only by cleavage-mediated extension (Figure 4B). For example, the five 3' non-telomeric nucleotides were removed from GTGT-5 before primer extension (Figure 4B, lane 3). This interpretation was confirmed when GTGT-5 was reacted with [³²P]dGTP and ddTTP (Figure 4B, lane 7). The products ran below input, migrating at 18–21 nucleotides. The *Euplotes* telomerase typically does not pause strongly upon addition of the first dG. Therefore, only four DNA products resulting from the addition of two, three, four and five nucleotides are visible (GGGGddT). These results indicate that GTGT-5 aligned on the telomerase RNA template relative to the cleavage site, as indicated in Figure 4A.

Reactions with GTGTG-1 generated the same product profile as with GTGT-5 (Figure 4B, lanes 4 and 8), indicating that five residues were removed from this primer 3' terminus (four dG residues and a non-telomeric nucleotide) before elongation. Interestingly, the GTGTG-1 reaction was much less efficient than the GTGT-5 reaction. This was particularly evident in reactions with dGTP and ddTTP (Figure 4B, lane 8). Since the same DNA substrate for telomerase elongation is produced following cleavage of these primers, sequences that will be eliminated appear to influence strongly primer utilization by telomerase (see below).

Thirteen nucleotides were removed from the 3' terminus of GTG-9 prior to elongation (Figure 4B, lane 2). The products of the dGTP and ddTTP reaction had an apparent size of 10–13 nucleotides (Figure 4B, lane 5), resulting from the removal of 13 nucleotides followed by the addition of 2–5 residues. Thus, GTG-9, like GTGTG-1, aligned on the RNA template so that the most 3' dG residues as well as the adjacent non-telomeric DNA that extended past the cleavage site were eliminated (Figure 4A).

As a control, we tested how telomerase from developing macronuclei processed the same primer series (Figure 4C). GTG-9 was extended only by direct addition (Figure 4C, lanes 2 and 5). In contrast, GTGT-5 and GTGTG-1 were elongated by a combination of direct addition and cleavage-initiated extension, generating two overlapping banding profiles (Figure 4C, lanes 3 and 4). Products running both above and below the 22 nucleotide marker were obtained from reactions with dGTP and ddTTP (Figure 4C, lanes 6 and 7). The banding profile generated by the developmental telomerase with GTGTG-1 was more complex than expected for extension by direct nucleotide addition or cleavage (Figure 4C, lane 4). A new banding pattern with an eight nucleotide periodicity was apparent. Since this profile was offset by one nucleotide from the direct elongation pattern, we interpret this to mean that the non-telomeric 3' residue caused a misalignment of a subset of primers on the RNA template during an early elongation cycle.

Taken together, our results uncover striking differences in DNA processing by telomerase from different stages of the *Euplotes* life cycle. Although both the vegetative and developmental telomerases readily cleave primers to eliminate non-telomeric DNA, the vegetative enzyme lacks the capacity to initiate synthesis on non-telomeric 3' ends.

Vegetative telomerase extends primers bearing 4–5 bp of 3'-terminal complementarity to the telomerase RNA template

The inability of telomerase from vegetative macronuclei to add telomeric repeats onto non-telomeric DNA implies that productive elongation initiates only on primers with 3' ends that can hybridize to the RNA template. To explore this idea, we determined the minimal terminal DNA sequence requirements for vegetative telomerase using primers with 3' ends that could form base pairs with the *Euplotes* RNA template, but whose 5' ends were non-telomeric (Figure 5). The primer N-GGTTT is 21 nucleotides in length and terminates in the sequence ...GGTTT 3', allowing it to form a five base pair duplex with the RNA template. This primer was elongated by vegetative telomerase, but short products accumulated in the reaction (Figure 5, lane 5). Note that this build-up of shorter products was not observed in reactions with a completely telomeric primer (Figure 5, lane 1). N-GGTT, which carries four nucleotides of 3' complementarity to the telomerase RNA template, was extended very poorly with a banding profile that was inconsistent with typical telomerase elongation (Figure 5, lane 6). N-GTT was not extended at all (Figure 5, compare lanes 7 and 8). We assayed primers that terminated in varying numbers of dG residues, reasoning that the ability to form G-C base pairs would increase the stability of primer interaction with the RNA, and might facilitate initiation with fewer base pairs. A primer terminating in four dG residues, N-GGGG, was extended (Figure 5, lane 2), but, as with N-GGTTT, the reaction was less processive than with a completely telomeric primer. In contrast, N-TGGG which terminates in one dT residue and three 3' dGs was not elongated (Figure 5, lane 3). These data indicate that the 3'-terminal sequence of a primer is a major determinant for extension by telomerase from vegetative macronuclei and that a

Fig. 4. Vegetative telomerase does not add telomeric repeats directly onto non-telomeric 3' ends. (A) Model for primer alignment on the *Euplotes* RNA template. The arrow indicates the position of cleavage (Melek *et al.*, 1996). Primer nucleotides extending at or beyond the cleavage site in the RNA are removed prior to telomerase elongation. Differential processing of DNA by telomerase from vegetative macronuclei (B) or developmental macronuclei (C) is shown. Assays were conducted with [³²P]dGTP and dTTP or ddTTP as indicated. Primer sequences are: GTG-9, (G₄T₄G₄)CGCGATCAC; GTGT-5, (G₄T₄G₄T₄)ATCAC; GTGTG-1, (G₄T₄G₄T₄G₄)C. The arrow indicates the migration of GT₄(G₄T₄)₂ primer upon extension by a single dG residue. Brackets indicate the sizes of cleavage-initiated elongation products. Asterisk marks the product of a non-specific labeling activity in vegetative macronuclei. In (C), cleavage-initiated elongation products are denoted by closed circles and direct elongation products by open circles.

Fig. 5. Vegetative telomerase requires primers with a minimum of 4–5 base pairs of 3'-terminal complementarity to the telomerase RNA template. Telomerase reactions with vegetative macronuclei are shown with the following primers: GT₄(G₄T₄)₂; N-GGGG, CACTATCGACTACGCGAGGGG; N-TGGG, CACTATCGACTACGCGATGGG; GT-N-TGGG, (G₄T₄)ACTACGCGATCATGGG; N-GGTTT, ATTGCTCTGAGTTTACGGTTT; N-GGTT, AATTGCTCTGAGTTTACGGTT; N-GGT, GAATTGCTCTGAGTTTACGGTT. The asterisk marks the product of a non-specific labeling activity in vegetative macronuclei. The arrow indicates the position of the primer GT₄(G₄T₄)₂ extended by one dG residue.

minimum of 4–5 bp of complementarity to the RNA template are needed to initiate productive elongation.

We investigated whether sequences upstream of the primer 3' terminus influenced DNA recognition by vegetative telomerase. As mentioned above, a primer that bears the sequence TGGG on its 3' terminus is not extended. However, when we changed the first eight nucleotides to GGGGTTTT on this primer 5' terminus (GT-N-TGGG), the vegetative enzyme elongated the primer in a processive reaction (Figure 5, lane 4). The overall efficiency was somewhat lower than observed with a completely telomeric primer (Figure 5, lane 1), indicating that the vegetative enzyme required additional telomeric sequence for maximum product formation. Nevertheless, the upstream stretch of telomeric DNA enhanced both primer recognition and the processivity of elongation by the vegetative telomerase. This observation argues strongly that telomerase from vegetative macronuclei contacts DNA upstream

from a primer 3' terminus independently from the RNA template and accordingly must possess an anchor site as it has been previously defined (Morin, 1989; Collins and Greider, 1993; Lee and Blackburn, 1993; Melek *et al.*, 1994).

***De novo* telomere formation by telomerase**

To investigate the biochemical basis for developmental differences in DNA processing by telomerase, we tested whether vegetative macronuclei contained a diffusible inhibitor of DNA synthesis onto non-telomeric DNA. Macronuclei were lysed and mixing experiments with the soluble fraction were performed. No inhibition in the ability of the developing macronuclear lysate to extend the GT-13 primer was observed in the presence of vegetative macronuclear lysate (data not shown). Moreover, when telomerase from vegetative macronuclei was purified on a glycerol gradient (see below), it did not gain the capacity to initiate DNA synthesis on non-telomeric 3' ends (data not shown). These data argue that vegetative macronuclei do not contain a diffusible inhibitor of *de novo* telomere formation.

We considered two other models to explain the ability of telomerase to initiate synthesis on non-telomeric DNA: a specific initiation factor present in the developing macronucleus or chemical modification of telomerase during development. To address these possibilities, telomerase from developing macronuclei was subjected to glycerol gradient centrifugation. We reasoned that if telomerase formed a stable complex with an additional factor during macronuclear development, the enzyme might be detectably larger than in vegetative macronuclei. This approach also allowed us to test whether the *de novo* telomere formation capacity co-purified with other telomerase activities, including primer cleavage. Gradient fractions were assayed for telomerase activity using a standard telomeric primer. Activity peaked in fractions 18–24 (Figure 6A, lanes 18–24), as did telomerase RNA (data not shown). The enzyme activity was significantly reduced in the gradient fractions relative to the starting material (Figure 6A, compare lane S with lanes 18–24). This decrease was due in part to incubation of the extract at 4°C for 24 h, as required for centrifugation (data not shown). Telomerase from developing and vegetative macronuclei sedimented as particles of the same size (*S* value = 16–17; apparent molecular mass = 375–400 kDa) (Figure 6A and B), somewhat larger than the sizes reported for the *Tetrahymena thermophila* and *Euplotes aediculatus* telomerases (Collins *et al.*, 1995; Lingner and Cech, 1996).

Gradient fractions from developing macronuclei were assayed for non-telomeric DNA processing. To enhance our ability to detect cleavage products after enzyme purification, we used a modified version of 7-GT-6 (7-GT-6λ) in the assays, which bears different 5' and 3' non-telomeric DNA sequences and is processed more efficiently by the cleavage pathway (E. Greene and D. Shippen, in preparation). The primer cleavage and elongation activity peaked in the same fractions as extension of the telomeric primer (Figure 7, lanes 18–22).

In contrast, the capacity to add telomeric repeats directly onto non-telomeric 3' ends was lost upon purification. Gradient fractions assayed with GT-13 generated only a single prominent product band corresponding to the addi-

Fig. 6. Glycerol gradient centrifugation of developmental and vegetative telomerase. Macronuclear lysates were analyzed on glycerol gradients. Even-numbered fractions from developmental and vegetative extracts were assayed for telomerase activity using $GT_4(G_4T_4)_2$ (**A** and **B**, respectively). 150 kDa = alcohol dehydrogenase, 200 kDa = α -amylase and 443 kDa = apoferritin. Molecular weight standards were run in parallel gradients. Numbers below lanes denote fractions taken from the top of the gradient. S = starting material. The recovery control for each telomerase reaction from gradient fractions, ^{32}P -labeled TTTTGGGG is designated RC.

tion of one dG residue (Figure 8A, lanes 16–26). The GT_{13} product peaked in the same fractions as cleavage-initiated elongation and extension of telomeric primers (Figures 6A and 7), indicating that telomerase was responsible for the incorporation. However, upon long exposure of the film, only a very faint ladder of elongation products with an eight nucleotide-repeated banding profile was detected. The failure of telomerase to extend efficiently GT_{13} was not due to a labile factor in the extract. Diluting the extract and incubating it at 4°C in glycerol for 24 h resulted in only a slight decrease in GT_{13} product length, but the distinctive telomerase product ladder was still obtained (data not shown). The result obtained with the GT_{13} primer was not caused by a complete loss of enzyme processivity; both the telomeric primer and the cleavage primer were extended by the addition of several GGGTTTT repeats following purification (Figures 6A and 7). The behavior of the partially purified developmental telomerase was essentially identical to the vegetative telomerase with respect to 3' end processing of non-telomeric DNA. The only significant difference between the two preparations was in the banding pattern of elongation products. These remained distinct (Figure 6A and B).

De novo telomere formation capacity was also severely reduced when macronuclear extracts from developing cells were purified on spermine agarose, heparin agarose or DEAE (data not shown). Taken together, these data argue that a dissociable factor in developing macronuclei enables telomerase to add telomeric repeats directly onto non-telomeric DNA and this factor is lost during purification. To explore this possibility further, we tested whether *de novo* telomere synthesis capacity (ability to extend GT_{13}) could be restored to the partially purified telomerase by adding fractions from a glycerol gradient. Telomerase from developing macronuclei was purified over phenyl-Sepharose, a glycerol gradient and then spermine-agarose. As expected, the purified enzyme failed to generate an elongation ladder with GT_{13} (Figure 8B, lane 1). However, addition of any one of the glycerol gradient fractions,

Fig. 7. Cleavage activity co-purifies with telomerase. Even-numbered glycerol gradient fractions of developmental telomerase were assayed with 7-GT-6 λ , TCGTTCA(G_4T_4)GAATTC. S = starting material. The recovery control is designated RC. Arrows denote the migration of 16-residue and 25-residue oligonucleotides used as molecular size markers.

1–9, which do not themselves display the telomerase RNA (data not shown) or telomerase activity (Figures 6A, 7 and 8B, lanes 7–11) RNA, allowed the purified telomerase to extend GT_{13} by the addition of several G_4T_4 repeats (Figure 8, lanes 2–6). From these findings, we conclude that telomerase collaborates with at least one *trans*-acting factor in the developing macronucleus to initiate synthesis on non-telomeric 3' ends.

Discussion

Developmentally regulated DNA synthesis by telomerase

The generation of a new macronucleus in *Euplotes* demands synchronous and efficient telomere formation on thousands of chromosome fragments. Although mutagenesis studies in *Tetrahymena* confirm that telomerase

Fig. 8. Elimination and reconstitution of *de novo* telomere formation. (A) Loss of *de novo* telomere formation upon telomerase purification. Fractions from the glycerol gradient shown in Figure 6A were assayed for the extension of GT-13 primer. The recovery control is designated RC and the arrow indicates the migration position of a 22 nucleotide size marker. (B) Reconstitution of *de novo* telomere formation. Developing macronuclear extracts were extensively purified as described in Materials and methods, and the peak activity was mixed with glycerol gradient fractions as indicated and assayed for GT-13 extension (lanes 2–6). Lanes 7–11 show reactions with individual glycerol gradient fractions and, lane 1, the reaction with purified telomerase alone. Arrows highlight elongation products.

lays down the first telomeric repeat on a new chromosome end (Yu and Blackburn, 1991), the range of biochemical activities presently ascribed to this enzyme do not fully account for *de novo* telomere formation. In the milieu of a developing macronucleus, telomerase must initiate synthesis on DNA that cannot base pair with the RNA template and it must do so with extremely high efficiency. The mechanisms for telomerase recruitment to a fractured chromosome and DNA positioning in the enzyme active site for elongation remain enigmatic.

In this study, we investigated how the *Euplotes* telomerase processes non-telomeric DNA 3' ends. We found that telomerase initiates synthesis on non-telomeric DNA only during a specific stage in the ciliate life cycle which is characterized by massive chromosome fragmentation and new telomere formation. Telomerase in a nucleus where all the DNA is capped by telomeres (the vegetative macronucleus) does not catalyze nucleotide addition onto non-telomeric 3' ends. Instead, the enzyme requires DNA 3' ends that terminate in at least 4–5 telomeric nucleotides. 3'-Terminal sequence complementarity to the RNA template appears to be a general requirement for most telomerase enzymes (reviewed in Melek and Shippen, 1996). The human telomerase demands that primer 3' termini have the capacity to form 2–4 base pairs with the RNA for *in vitro* elongation (Morin, 1991). Furthermore, a limited stretch of complementarity to a known or predicted telomerase RNA template is found at the break site of virtually all spontaneous chromosome healing events described to date, including those from humans, *Plasmodium* and *Ascaris* (Wilkie *et al.*, 1990; Muller *et al.*, 1991; Flint *et al.*, 1994; Mattei and Scherf, 1994).

Telomerase from vegetatively growing *Euplotes* and other telomerases seeking to maintain tracts of perfect telomeric repeats do not need the capacity to initiate synthesis on non-telomeric DNA. In fact, such an activity might be deleterious. Studies in *Tetrahymena* and yeast indicate that single point mutations in telomeric DNA are not tolerated and lead to serious disruptions in telomere length regulation, senescence and death (Yu *et al.*, 1990; Singer and Gottschling, 1994; McEachern and Blackburn, 1995). Ciliate telomerases achieve a high degree of accuracy by appropriate alignment of 3'-terminal nucleotides on the chromosome end onto the telomerase RNA template and high fidelity of polymerization during each round of elongation (Autexier and Greider, 1994, 1995; Gilley *et al.*, 1995; Gilley and Blackburn, 1996). The telomerase cleavage activity may further enhance enzyme fidelity by ensuring faithful primer translocation after each repeat is synthesized (Collins and Greider, 1993; Melek *et al.*, 1996). Failure to translocate at the proper time would generate imperfections in the telomere repeat array as residues beyond the templating domain are copied into DNA. Since telomerase appears to be non-processive *in vivo* (Yu and Blackburn, 1991), the enzyme will have an opportunity to correct a translocation error by removing mismatched residues on the primer 3' terminus when the enzyme contacts that chromosome end the next time. In support of this interpretation, telomerase from vegetative *Euplotes* cells eliminates non-telomeric residues on a primer 3' terminus prior to initiating polymerization. If telomerase initiated DNA synthesis directly on nucleotides that did not match the RNA template, such residues would become 'sealed' within the telomere.

Consistent with the notion that the telomerase cleavage activity serves a proofreading role, this function appears to be an inherent property of the enzyme. It co-fractionates with telomerase on glycerol gradients (Figure 7) and over five consecutive purification steps (E.Greene and D.Shippen, in preparation). Furthermore, the *Euplotes* telomerase from all stages of macronuclear development and vegetative growth performs the cleavage reaction (E.Greene and D.Shippen, unpublished data; this study).

Finally, cleavage activities have also been reported for the *Tetrahymena* and yeast telomerases (Collins and Greider, 1993; Autexier and Greider, 1995; Cohn and Blackburn, 1995).

***Euplotes* telomerase as a multi-subunit complex with dissociable factors**

The behavior of the *Euplotes* telomerase is altered during programmed chromosome healing to permit DNA synthesis on non-telomeric DNA. We considered several models to account for regulated synthesis initiation. We found no evidence for a diffusible inhibitor of *de novo* synthesis in vegetative macronuclei, although it is feasible that telomerase from vegetative macronuclei is modified in a manner that prevents efficient synthesis on non-telomeric DNA. One surprising difference in the behavior of telomerase from the two different developmental stages is the distinctive profile of the elongation products. Since this property co-purifies with enzyme activity, it appears to be inherent to the different telomerase preparations. Thus, developmentally regulated modifications of telomerase remain a distinct possibility.

The data presented here provide strong evidence for a novel 'initiation' factor in the developing macronucleus of *Euplotes*. This activity, which we have named chromosome healing factor (CHF), enables telomerase to begin synthesis on non-telomeric DNA. CHF is apparently loosely associated with telomerase, as it is disengaged from the ribonucleoprotein during the first steps of purification of macronuclear extracts. However, the ability to form telomere repeats on non-telomeric DNA can be restored to purified telomerase by adding side fractions from a glycerol gradient. Although the efficiency of reconstitution is relatively low, it is reproducible and does not require the addition of multiple gradient fractions. Nevertheless, the data leave open the possibility that other factors are involved in mediating the developmental switch in telomerase behavior.

In *Euplotes*, CHF dramatically modifies telomerase-DNA interactions to eliminate effectively the requirement for Watson-Crick base-paired alignment of DNA on the RNA template for synthesis initiation. Interestingly, a similar developmental switch in DNA specificity has not been documented for *Tetrahymena* telomerase. Wang and Blackburn (1997) detected no differential DNA processing in preparations from vegetative and developing cells. However, these authors reported that the *Tetrahymena* enzyme has an intrinsic ability to initiate DNA synthesis on non-telomeric DNA which they speculate may be sufficient for *de novo* telomere formation. Since the *Tetrahymena* study examined DNA synthesis using partially purified enzyme preparations, it is conceivable that an activity stimulating new telomere formation in the developing macronucleus was lost during purification. Alternatively, a novel mechanism may have evolved in *Euplotes* to optimize programmed telomere formation, as >100-fold more telomeres are created during macronuclear development in this organism (Prescott, 1994).

Regulated initiation of DNA synthesis by the *Euplotes* telomerase has obvious parallels to transcription initiation by RNA polymerase II (Pol II). Purified Pol II will not initiate transcription selectively; it requires at least 50 polypeptides to identify a promoter and initiate synthesis

at a specific site on the DNA template (Conaway and Conaway, 1993). Although these factors can assemble with Pol II in an ordered fashion to form an initiation complex *in vitro* (Conaway and Conaway, 1993; Buratowski, 1994), recent studies indicate that Pol II exists as a holoenzyme *in vivo*, with the majority of general transcription factors pre-assembled (Kim *et al.*, 1994; Koleske and Young, 1994; Ossipow *et al.*, 1995). Detection of the higher order complex is credited in part to special enzyme preparation conditions (Koleske and Young, 1995; Ossipow *et al.*, 1995), since Pol II molecules from most crude whole-cell extracts are not associated with other polypeptides (Kolodziej *et al.*, 1990).

Our data provide strong evidence that *Euplotes* telomerase exists as a higher order complex *in vivo* during programmed chromosome healing. Chromosome breakage and new telomere formation are temporally coupled in ciliates (Yao *et al.*, 1990; Fan and Yao, 1996). Hence, we and others have argued that telomerase is likely to be part of a multisubunit complex (Yao *et al.*, 1990; Fan and Yao, 1996; Melek *et al.*, 1996) that recognizes specific sequences in the DNA (Baird and Klobutcher, 1989; Yao *et al.*, 1990), catalyzes cleavage and forms telomeres on the nascent ends. Telomerase could conceivably be directly involved in chromosome fragmentation using its inherent endonuclease activity (Melek *et al.*, 1996), but there is currently no direct evidence to support or refute this hypothesis. Once cleaved, the DNA becomes a telomerase substrate, presumably, in part, through association with CHF. We speculate that CHF helps telomerase capture a non-telomeric 3' end, deliver it into the active site, and position it stably at the 'default' alignment site on the RNA template for elongation (Melek *et al.*, 1996). Accordingly, CHF would be responsible for the precision of new telomere formation in *Euplotes*; all telomeres initiate with the same nucleotides (Klobutcher *et al.*, 1981). Clearly many aspects of the programmed chromosome healing process are unknown. However, our identification of a new activity involved in directing non-telomeric DNA into the polymerization site of telomerase should provide an entry point for dissecting this mechanism.

Materials and methods

Isolation of E.crassus macronuclei

Euplotes crassus were cultured with the algae, *Dunaliella salina*, and mated as described previously (Roth *et al.*, 1985). Developing macronuclei containing active telomerase were isolated from cells ~64 h after mating and were purified on Percoll-sucrose gradients (Shippen-Lentz and Blackburn, 1989). To isolate vegetative macronuclei, *Euplotes* cells were grown until all the algae were consumed, starved overnight, then fed with dense algae ($A_{800} = 0.35$) and collected 1–2 days after refeeding. Cells were lysed by the addition of Triton X-100 to a final concentration of 0.5% and douncing after incubation in a hypotonic buffer [10 mM Tris-HCl pH 7.5, 2.5 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] for 3 min at room temperature. Vegetative macronuclei were purified on 35–100% Percoll-sucrose gradients essentially as described for developing macronuclei isolation. Purified developing or vegetative macronuclei were resuspended to ~10⁵/ml in TMG buffer (10 mM Tris-HCl pH 7.5, 3 mM MgCl₂, 10% glycerol) plus a cocktail of protease and RNase inhibitors at a final concentration of 12.5 µg/ml antipain, 1.7 µg/ml aprotinin, 10 µg/ml chymostatin, 10 µg/ml E-64, 1 µg/ml leupeptin, 7 µg/ml pepstatin, 10 mM vanadyl ribonucleoside complex and stored at -80°C until use.

Telomerase assays

Five microliters of *Euplotes* macronuclei (2×10^5 nuclei/ml) were assayed in 20 μ l reactions containing 0.4 μ M primer; 5–10 mM MgCl_2 ; 20 mM EGTA; 50 mM Tris–HCl pH 8.0; 1 mM spermidine; 1 mM DTT, 0.1 mM dTTP and 0.25 or 0.5 μ M [^{32}P]dGTP (800 Ci/mmol), unless otherwise stated. Reactions were incubated at 30°C for 1 h. DNA was precipitated and products were resolved on 10% sequencing gels and subjected to autoradiography.

Oligonucleotide preparation

DNA oligonucleotides were obtained from Gibco-BRL or Glaxo-Wellcome Inc. Methylphosphonate oligonucleotides were synthesized and purified as described previously (Hogrefe *et al.*, 1993; Melek *et al.*, 1996). All oligonucleotides were purified on 20% denaturing polyacrylamide gels before use. To generate plus one product markers, gel-purified oligonucleotides were reacted with [^{32}P]dGTP and terminal deoxynucleotidyl transferase (Boehringer Mannheim) for 15 min at 37°C as per the manufacturer's instructions. Following labeling, the plus one product was excised from a 20% denaturing gel and eluted overnight in TE (10 mM Tris–HCl pH 7.5; 1 mM EDTA).

Macronuclei lysis

Macronuclei from either developing or vegetative macronuclei were lysed by French press or sonication, respectively. Developing macronuclei were subjected to 16 000 p.s.i. and lysis was monitored by microscopy. When complete lysis was achieved, the vegetative or developmental lysates were spun for 15 min in a microfuge to remove membrane fractions and the supernatant stored at –80°C until use.

Telomerase purification

For glycerol gradient purification, macronuclear lysates were loaded onto a 15–35% glycerol gradient and spun for 24 h at 29 k.r.p.m. at 4°C. Fractions of 200 μ l were collected and 20 μ l were assayed in 40 μ l reactions as described above. To gauge the size of the telomerase particle, protein molecular weight standards were run in parallel gradients and detected by Bradford assays. Telomerase used in the reconstitution experiment was subjected to more extensive purification. Nuclear lysate (~10 mg protein) from developing macronuclei was loaded onto a 1 ml phenyl-Sepharose (PSP) column pre-equilibrated with TMG (30 mM Tris–HCl pH 7.5, 1 mM MgCl_2 , 10% glycerol, 1 mM DTT and 0.1 mM PMSF) + 0.5 M potassium glutamate (KGlu). The column was washed with 10 vols TMG + 0.5 M KGlu and activity eluted with TMG + 2% Triton X-100. Active fractions were frozen on dry ice/MeOH and stored at –80°C. Aliquots (200 μ l) of PSP-purified telomerase were loaded onto six 9 ml glycerol gradients (15–35% glycerol, 30 mM Tris–HCl pH 7.5, 1 mM MgCl_2 , 2 mM DTT and 0.2 mM PMSF). The gradients were centrifuged at 29 k.r.p.m. for 24 h at 4°C. Fractions of 200 μ l were collected from the top of the gradients, like fractions combined and acetylated bovine serum albumin (BSA) added to 0.1 mg/ml. Fractions active for telomerase were loaded onto a 0.5 ml spermine–agarose column pre-equilibrated with TMG + 0.5 M KGlu. The column was washed with 10 vols TMG + 0.5 M KGlu and activity eluted with 4 vols TMG + 1.5 M KGlu. Active fractions were dialysed into 30 mM Tris–HCl pH 7.5, 1 mM MgCl_2 , 50% glycerol, 1 mM DTT, 0.1 mM PMSF and acetylated BSA added to 0.1 mg/ml. Fractions were stored at –20°C until use.

Telomerase reconstitution experiment

Fifteen microliters of purified telomerase were mixed with 30 μ l aliquots of glycerol gradient fractions and ATP was added to 2 mM. The mixture was held at 37°C for 5 min then 30°C for an additional 10 min; 2 $\times$ telomerase reaction cocktail was added, the samples incubated for 1 h at 30°C and then processed as described above.

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