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An Overhanging 3′ Terminus Is a Conserved Feature of Telomeres

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The reactivity of single-stranded thymidines with osmium tetraoxide was used to demonstrate the existence of a terminal overhang of the G-rich strand of telomeres from two distantly related eucaryotes, the ciliated protozoan *Tetrahymena* spp. and the acellular slime mold *Didymium* spp. Conservation of a G-strand overhang at the molecular terminus of telomeres is consistent with our suggestion that an unusual DNA structure formed by the G-strand overhang is important for telomere function (E. Henderson, C. C. Hardin, S. K. Wolk, I. Tinoco Jr., and E. H. Blackburn, Cell 51:899–908, 1987).

Telomeres are essential for chromosome stability (6). These specialized structures at chromosomal termini have highly conserved features. They consist of simple repeated DNA sequences complexed with proteins which specifically recognize these sequences (1, 4, 8, 9, 11, 12, 23). All telomeric DNA sequences have distinctly guanosine- and cytosine-rich strands, with the G-rich strand oriented 5′ to 3′ toward the molecular terminus of the chromosome (3).

Previous studies have shown that in four species of hypotrichous ciliates, the G-rich strand extends beyond the C-rich strand at the terminus of the telomere DNA duplex, constituting a 3′ overhang (19, 22). Hypotrichous ciliates have a fixed number of repeats of the telomeric repeat sequence, facilitating examination of the lengths of both strands by direct sequencing. Telomeres from all other species appear to have variable numbers of repeats, making comparison of G- and C-strand lengths impossible by direct sequencing. We have used base-specific modification of thymidine by osmium tetraoxide (10) to circumvent this limitation. This technique has allowed us to analyze the structures of the telomeres of the linear ribosomal RNA genes (rDNA) of *Tetrahymena* spp. and the slime mold *Didymium* spp. By using the osmium reagent, we show that, like those previously reported for hypotrichous ciliates (19, 22), the telomeres of the rDNA from *Tetrahymena* and *Didymium* spp. have G-strand overhangs extending approximately 12 nucleotides beyond the end of the telomere duplex. These overhangs are also present in the unusually long telomeres of *Tetrahymena* spp., induced by prolonged maintenance of cultures under exponential growth conditions (20, 24). Furthermore, the natural ends of *Tetrahymena* rDNA are recognized and elongated by the telomere-specific enzyme telomerase.

Osmium tetraoxide can mediate scission of single-stranded DNA at thymidine residues. Under appropriate conditions, osmium tetraoxide preferentially reacts with thymidine residues that are not hydrogen bonded to adenine. We optimized these conditions by using synthetic DNA oligonucleotides. An oligonucleotide composed of the *Tetrahymena* telomeric, G-rich strand sequence $d(TTGGGG)_4$ was radiolabeled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase and gel purified. This radiolabeled DNA was cleaved with piperidine (1 M; 90°C for 30 min) after osmium modification of thymidine residues (3.3% OsO_4 in water). These control experiments were carried out with a higher concentration of OsO_4 than those used in the experiments described below to demonstrate that, even at these high concentrations, OsO_4 was single strand specific. Under all conditions tested, single-stranded, radiolabeled $d(TTGGGG)_4$, incubated either alone (Fig. 1, lanes 1 through 6) or in the presence of an unlabeled, noncomplementary oligonucleotide d(TTTTATTCCTAAAAAAAGTT) (Fig. 1, lanes 13 through 18), was efficiently cleaved, as measured by the disappearance of the full-length oligonucleotide. The 5′ terminal osmium-modified thymidines at the bottom of the gel resolve into a single species corresponding to the 5′ terminal thymidine after extensive cleavage. The slight resistance to cleavage seen with the noncomplementary oligonucleotide at 0°C (Fig. 1, lane 13) was probably due to a small degree of chance base pairing of sufficient strength to provide protection of some thymidines from the osmium reagent at this low temperature. In contrast, radiolabeled $d(TTGGGG)_4$ was highly resistant to osmium-mediated strand scission at 0°C for 5 or 30 min (Fig. 1, lanes 7 and 8) when it was hybridized to a 10-fold excess of the complementary sequence $d(CCCCAA)_4$. As the duplex melted at 22 (Fig. 1, lanes 9 and 10) and at 37°C (Fig. 1, lanes 11 and 12), the radiolabeled DNA was completely cleaved by this treatment. These results demonstrated that the thymidine residues of $d(TTGGGG)_4$ in a Watson-Crick duplex are protected from osmium-mediated cleavage under these conditions.

***Tetrahymena* and *Didymium* rDNA telomeres have 3′ overhangs.** Purified *Tetrahymena* and *Didymium* rDNAs were radiolabeled at the 3′ end of the telomere with [α-^{32}P]ddATP and terminal transferase, gel purified, and treated with osmium tetraoxide as described for the controls, except that the OsO_4 concentration was 0.067%. Figure 2, lanes 2 through 6 and 8 through 12, shows chemical sequencing reactions (21) for the rDNAs of *Tetrahymena* and *Didymium* spp., respectively. Lanes 1 and 7 are the corresponding osmium reactions performed under single-strand-specific cleavage conditions. In both rDNAs, only the two terminal repeats were susceptible to modification by the osmium reagent, as assayed by subsequent piperidine cleavage. These results demonstrate that the two terminal repeats are single stranded and thus constitute telomeric 3′ G-strand

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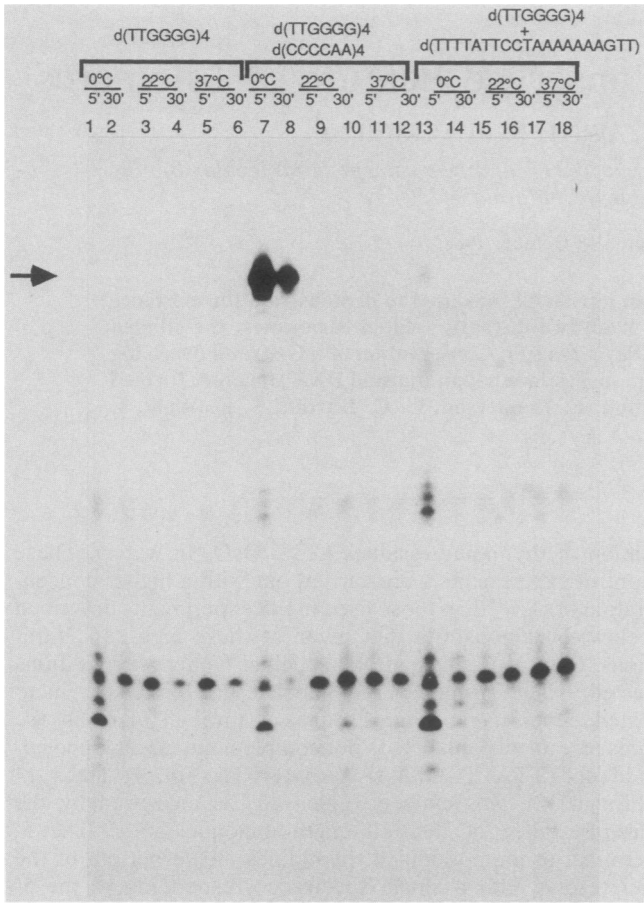

FIG. 1. Treatment of radiolabeled $d(TTGGGG)_4$ with osmium tetraoxide as described in the text to determine conditions for cleavage at single-stranded thymidines. Cleavage reactions were analyzed by electrophoresis through a 12% acrylamide (1:20, bis-acrylamide)-7 M urea gel in 0.6× TBE (1× TBE is 0.89 M Tris base plus 0.89 M boric acid, pH 8.2). Cleavage of the 24-mer was monitored as the disappearance of the full-length oligonucleotide (indicated by arrow). Lanes 1 through 6 show cleavage of the single-stranded oligonucleotide at 0, 22, and 37°C for 5 and 30 min as indicated. Lanes 7 through 12 show the effect of hybridization of $d(TTGGGG)_4$ to a 10-fold excess of the complementary sequence $d(CCCCAA)_4$ prior to treatment with osmium. Lanes 13 through 18 are identical to the previous six lanes except that the noncomplementary oligonucleotide d(TTTTATTCCTAAAAAAAGTT) was substituted for $d(CCCCAA)_4$. Cleavage products at the bottom of the gel are 5′ terminal thymidines.

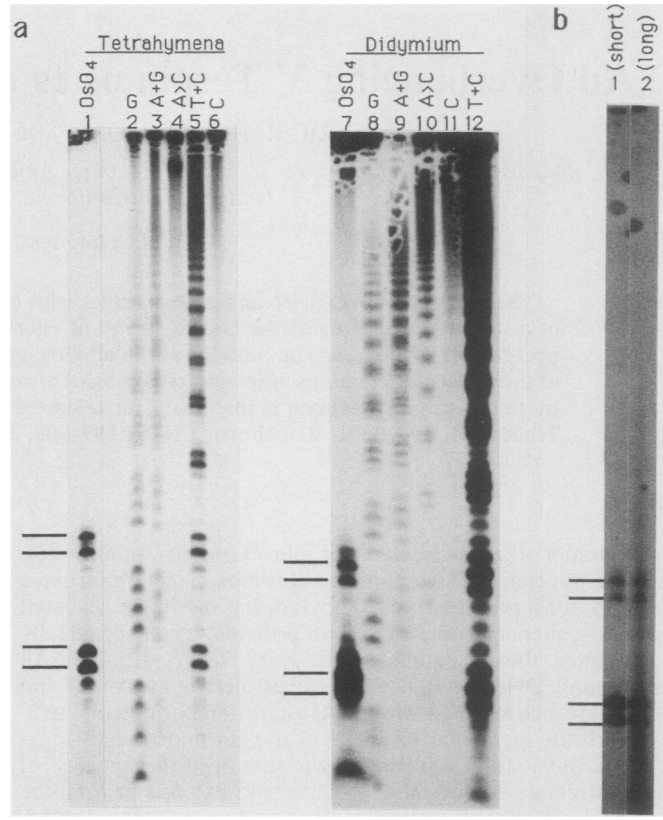

FIG. 2. (a) The effect of osmium treatment on *Tetrahymena* and *Didymium* rDNAs that have been radiolabeled at the 3′ position of the telomere with [^{32}P]ddATP by terminal transferase in the presence of Mg^{2+}. The conditions used are specific for scission at single-stranded thymidines. The osmium reactions are in lanes 1 and 7. Chemical sequencing (21) reactions are shown for comparison in lanes 2 through 6 and 8 through 12. (b) The effect of telomere growth on the single-stranded G-strand overhang. The osmium reacted similarly with long (approximately 300 to 400 base pairs extra length [lane 1]) and short (lane 2) telomeres, indicating that both types of telomeres have identical G-strand overhangs.

overhangs analogous to those previously reported for the telomeres of hypotrichous ciliates (19, 22).

The length of the overhang in both the *Tetrahymena* and *Didymium* telomeres is 12 to 16 bases. The precision of this number depends on whether or not there are single-stranded, nonthymidine nucleotides 5′ to the single-stranded thymidines. Since the osmium cleavage technique is specific for thymidine residues, a more precise number cannot be obtained by using this technique.

The G-strand overhang is a feature of *Tetrahymena* telomeres lengthened in vivo. The molecular ends of telomeres which had grown by 300 to 400 base pairs during prolonged vegetative cell divisions (2, 20) were compared with those from telomeres of standard length in *Tetrahymena* spp. The results of this comparison are shown in Fig. 2b. The ends of the short (lane 1) and long (lane 2) telomeres are indistinguishable in terms of their susceptibility to cleavage by the osmium reagent. Therefore, the 3′ overhang is maintained in telomeres actively involved in the lengthening process.

Natural *Tetrahymena* rDNA ends are substrates for telomerase in vitro. In vitro, telomerase, the enzyme most likely to be responsible for telomere elongation, utilizes telomeric G-strand primers as substrates for elongation. Duplex telomeric DNA and C-strand primers are not elongated in this assay (7, 13, 14, 17, 18, 25). We tested the ability of telomerase to recognize natural telomeres as substrates for elongation in vitro. *Tetrahymena* rDNA was incubated with telomerase as previously described (13, 14). After telomerase treatment, the rDNA was digested with *Pst*I and the reactions were assayed by monitoring the incorporation of [α-^{32}P]dGTP into a telomeric *Pst*I restriction fragment approximately 1 kilobase long. Since the rDNA is a palindrome, there are two similar *Pst*I restriction fragments, one at each end of the molecule. For size comparison, this telomeric fragment was labeled with Klenow fragment at discontinuities known to exist in the telomere repeats (5, 16)

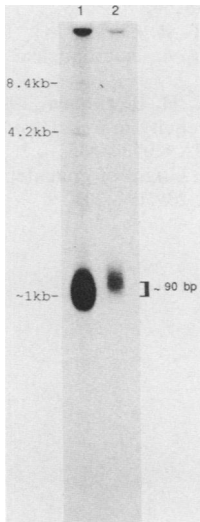

FIG. 3. Telomerase recognition of the natural *Tetrahymena* telomere structure. Telomeres were radiolabeled at G-strand 3′ discontinuities (5, 16) by Klenow fragment (lane 1) or at the molecular terminus by telomerase (lane 2). Telomerase recognized and labeled only the telomeric *Pst*I restriction fragment by addition of G and, presumably, T residues, indicating that labeling was at the terminus and not at internal discontinuities. Since telomerase does not recognize the C strand as a substrate for telomere sequence addition and there is no precedent for repetitive addition of nucleotides to the 5′ end of a DNA molecule by an enzyme, we concluded that addition was to the 3′ end, i.e., the G-strand overhang. An average of 90 nucleotides was added to the telomeres by telomerase.

(Fig. 3, lane 1). Labeling by this method does not result in telomere elongation. Telomerase mediated the incorporation of radiolabel exclusively into the telomeric restriction fragment (Fig. 3, lane 2), indicating addition of radiolabeled guanosine to the terminus rather than at internal nicks in nontelomeric sequences. Telomerase will not add telomeric repeats to recessed 3′ G-strand ends so it is unlikely that internal discontinuities in the G strand were labeled by this protocol. Furthermore, in contrast to the labeling by the Klenow fragment, the incorporation of label into the telomeric fragment by telomerase resulted in a decrease in its electrophoretic mobility, corresponding to an apparent increase of approximately 90 bases in length. These results show that the natural *Tetrahymena* rDNA telomere can function as a primer for addition of telomeric repeats by telomerase, presumably via the 3′ G-strand overhang.

We have shown here that an overhang of the telomeric G-rich strand is a conserved feature of the molecular terminus of telomeres. Therefore, a G-strand overhang may play a central role in telomere function. We had previously demonstrated that synthetic oligonucleotides corresponding to telomeric G-strand overhangs can assume novel higher order structures involving non-Watson-Crick base pairing interactions (15). This observation is consistent with the idea that the G-strand overhang at telomeres is not single stranded in vivo but rather assumes an unusual DNA conformation which is responsible for some of the attributes of the telomere, including its resistance to degradation and recombination, and recognition by telomere-specific proteins.

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