Synthesis of the Mammalian Telomere Lagging Strand in Vitro*

(Received for publication, January 8, 1997, and in revised form, February 18, 1997)

Phillip M. Revel, Karen M. Henkels, and John J. Turchi‡

From the Department of Biochemistry and Molecular Biology, Wright State University School of Medicine, Dayton, Ohio 45435

Using a synthetic telomere DNA template and whole cell extracts, we have identified proteins capable of synthesizing the telomere complementary strand. Synthesis of the complementary strand required a DNA template consisting of 10 repeats of the human telomeric sequence d(TTAGGG) and deoxy- and ribonucleosidetriphosphates and was inhibited by neutralizing antibodies to DNA polymerase α. No evidence for RNA-independent synthesis of the lagging strand was observed, suggesting that a stable DNA secondary structure capable of priming the lagging strand is unlikely. Purified DNA polymerase α/primase was capable of catalyzing synthesis of the lagging strand with the same requirements as those observed in crude cell extracts. A ladder of products was observed with an interval of six bases, suggesting a unique RNA priming site and site-specific pausing or dissociation of polymerase α on the d(TTAGGG)₁₀ template. Removal of the RNA primers was observed upon the addition of purified RNase H. By varying the input rNTP, the RNA priming site was determined to be opposite the 3′ thymidine nucleotide generating a five-base RNA primer with the sequence 5′-AACCC. The addition of UTP did not increase the efficiency of priming and extension, suggesting that the five-base RNA primer is sufficient for extension with dNTPs by DNA polymerase α. This represents the first experimental evidence for RNA priming and DNA extension as the mechanism of mammalian telomeric lagging strand replication.

Semiconservative discontinuous bidirectional DNA replication presents the “end problem” for replication of linear genomes (1). Mammalian chromosomes contain short repeated DNA sequences complexed with specific proteins at each terminus (telomeres) and, in conjunction with telomerase, can circumvent this problem. Telomerase has been extensively studied and catalyzes the 5′ to 3′ extension of the terminal 3′-OH using an internal ribonucleotide template (reviewed in Ref. 2). To completely replicate telomeres, the strand complementary to the telomeric leading strand must be synthesized to generate a double strand DNA product.

The mechanism by which the DNA strand complementary to the telomerase catalyzed leading strand is synthesized has not been addressed experimentally. Two models have been proposed for synthesis of the telomeric lagging strand. The first involves RNA priming and DNA extension followed by removal of RNA primers and ligation of the Okazaki-like fragments (3, 4), presumably using the same enzymatic machinery employed for lagging strand synthesis at a replication fork (5, 6). The alternative hypothesis is based upon in vitro analyses of telomeric DNA sequences and the propensity to form higher ordered structures (7). The structure generated must present a 3′-hydroxyl for DNA polymerase (pol) catalyzed extension for synthesis of the lagging strand followed by a nucleolytic processing reaction to generate a terminal 3′-hydroxyl to enable telomerase extension in the next round of replication. Experimental evidence for either of these hypotheses or other alternative mechanisms has not been obtained. The goal of this study was to identify proteins in mammalian whole cell extracts and determine the mechanism for copying a synthetic mammalian telomeric substrate.

EXPERIMENTAL PROCEDURES

Unlabeled nucleotides were from Pharmacia Biotech Inc., and radiolabeled nucleotides were from DuPont NEN. HeLa whole cell extracts were prepared essentially according to Wood et al. (8). Calf thymus DNA pol α/primase was purified by immunoaffinity chromatography according to Nasheuer and Grosje (9). Calf thymus RNase HI was generously provided by R. Bambara (University of Rochester). Calf DNA ligase I and FEN-1 were purified as described previously (10). The TS10, consisting of 10 repeats of the sequence d(TTAGGG) was synthesized on a Molecular Biosystems 390 DNA synthesizer and purified by 15% polyacrylamide DNA sequencing gels. Products were separated by electrophoresis through 15% polyacrylamide/7M urea DNA sequencing gels. Gels were dried, and products were visualized by autoradiography.

RESULTS AND DISCUSSION

We have initiated the study of how mammalian cells complete replication of linear chromosomes using an in vitro approach. A synthetic single-stranded DNA substrate, TS10, was constructed consisting of 10 repeats of the human telomeric DNA sequence d(TTAGGG). The TS10 DNA substrate was incubated with crude cell extracts prepared from exponentially growing HeLa cells. HeLa cells have been demonstrated to contain active telomerase and have stable telomere lengths (12). Therefore, these cells were used to identify proteins that are capable of copying the telomere leading strand template. Synthesis of the complementary strand requires only dATP, dTTP, and dCTP; therefore we employed conditions to measure the incorporation of [α-32P]dCTP into DNA, dependent upon

This work was supported by a grant from the Ohio Cancer Research Associates and National Institutes of Health Grant CA64374 (to J. J. T.).

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435. Tel.: 937-775-2853; Fax: 937-775-3730; E-mail: jturchi@sirius.wright.edu.

The abbreviations used are: pol, polymerase; FEN-1, flap endonuclease-1.

* This work was supported by a grant from the Ohio Cancer Research Associates and National Institutes of Health Grant CA64374 (to J. J. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435. Tel.: 937-775-2853; Fax: 937-775-3730; E-mail: jturchi@sirius.wright.edu.

1 The abbreviations used are: pol, polymerase; FEN-1, flap endonuclease-1.
from calf thymus by immunoaffinity chromatography (9). The reaction contained 40 μg of a HeLa cell crude extract, dATP, dGTP, dTTP, and [α-32P]dCTP. Reactions also contained rNTPs, rCTP, and rUTP without the TS10 substrate (lane 1); TS10 substrate without rNTPs (lane 2); both rNTPs and TS10 (lane 3); both rNTPs and TS10, except DNA pol α/primase was preincubated with antibody SJK 132-20 on ice for 30 min prior to initiating the reaction (lane 4). Reactions were terminated, and samples were processed by gel electrophoresis as described under “Experimental Procedures.” The gel was dried, and the products were visualized by autoradiography.

Because the synthetic activity associated with the telomeric template was dependent on DNA pol α and rNTPs, an RNA priming mechanism was likely. DNA pol α has a tightly associated primase activity capable of de novo RNA priming (reviewed in Ref. 14). Therefore, we purified DNA pol α/primase from calf thymus by immunoaffinity chromatography (9). The purified DNA pol α/primase was then assayed for the ability to perform lagging strand synthesis on the TS10 substrate. The results shown in Fig. 2A demonstrate that in a complete reaction containing TS10, rNTPs, and dNTPs, increasing concentrations of calf DNA pol α/primase were able to catalyze the synthesis of DNA using the telomeric DNA as a template. Quantification of these results demonstrate a linear increase in incorporation. The results presented in Fig. 2B demonstrate that DNA pol α/primase requires rNTPs and TS10 to generate DNA synthetic products (lane 1), because reactions performed without rNTPs (lane 2) or without TS10 (lane 3) show no synthesis. The products observed from DNA in the reaction with purified DNA pol α/primase synthesis occur in groups of three bases. These products are likely the first, second, and third dCMP label being incorporated. The low concentration of dCTP employed is necessary to achieve the specific activity required to efficiently detect synthesis and likely results in dissociation or pausing of DNA pol α/primase as a result of low dNTP concentration and an inherent low processivity of DNA pol α (15). The products are also observed at six-base intervals centered approximately at 16, 22, 28, and 34 bases, a similar length distribution to that observed in the cell extract (Fig. 1). This pattern suggests a unique primer initiation site and DNA synthesis termination site. The site-specific termination or pausing is likely in the run of three guanosine bases in the synthesis termination site. The site-specific termination or pausing observed in the three guanosine bases in the template strand, as discussed earlier. In addition, DNA synthetic products generated by calf DNA pol α/primase on the TS10 substrate could be extended further by the addition of the large fragment of Escherichia coli DNA pol I (data not shown). This result provides evidence that termination or pausing observed is not the result of complete synthesis over the entire template. The difference in small products observed in the cell extract compared with the purified DNA pol α is likely the result of contaminating nucleases present in the extract.

Removal of RNA primers from lagging strand Okazaki fragments is necessary for complete processing and ligation. The
eukaryotic pathway for removal of RNA primers requires both RNase H I and FEN-1 (5, 6). Therefore, we employed purified calf RNase H I in complete lagging strand synthetic reactions. The results shown in Fig. 3 demonstrate an increased mobility of products (lanes 2–4) compared with reactions performed without RNase H I (lane 1). The decrease in intensity of products observed at 26, 32, and 38 bases is accompanied by an increase in products at intermediate positions and low molecular weight. These results demonstrate that the RNA primers are at least partially processed by RNase H I. Complete processing requires FEN-1 and DNA ligase I in addition to RNase H I (5, 6). Reactions were performed with each of these components, and the results are also shown in Fig. 3. The addition of RNase H I and FEN-1 results in a further increase in mobility (lane 6) compared with reactions performed without RNase H. These results are consistent with the role of FEN-1 in removing the last ribonucleotide from an Okazaki fragment (6, 10). The addition of DNA ligase I to the reactions had essentially no effect (lane 7). The inability to observe ligation products likely results from not having multiple priming events on a single template. Each reaction contains 5 pmol of TS10 substrate, and we estimate that less than 5% of the substrates are utilized during the course of the reaction, thereby decreasing the likelihood of a single DNA molecule sustaining two priming events. Although RNase H I can degrade RNA primers from RNA-DNA hybrids, RNase H from Drosophila melanogaster alters its cognate DNA pol α/primase activity increasing primer synthesis (16). A similar interaction has also been observed with RNase H and DNA pol α from calf (17). Interestingly, using calf thymus RNase H I purified by a different method (18), we have observed the stimulation of DNA pol α/primase (data not shown). The relationship between these two forms of RNase H and their interaction with DNA pol α is currently under investigation.

The specific banding pattern observed in telomere lagging strand reactions suggests that primer initiation may be at a unique site on the telomeric DNA template. Previous studies have shown that DNA pol α/primase initiates primer synthesis by creating a dinucleotide complex with a single-stranded template (19). DNA pol α/primase acts by first adding the eventual second nucleotide of the RNA sequence, preferring this nucleotide to be a purine. More recently, a preferential priming sequence 5‘-d(GCTTTCTTCC) has been deduced in vitro (20). In vivo experiments mapping replication initiation sites identified a similar sequence that serves as a preferential priming site (21). The telomeric d(TTAGGG) repeat contains only two pyrimidines, and if sequence-specific initiation is occurring, it is likely to be opposite the two thymidine bases. To test this hypothesis, we performed an experiment varying the rNTPs added to the lagging strand reactions. The results are shown in Fig. 4 and demonstrate that DNA pol α/primase catalyzed priming and extension in reactions containing all four rNTPs (lane 1). In addition, reactions performed with only rATP, rCTP, and rUTP (lane 2) and rATP and rCTP (lane 5) resulted in a similar distribution of products and rate of incorporation to that observed with the full complement of rNTPs (lane 1). The synthetic activity observed in reactions performed with only rATP and rCTP (lane 5) was 50% greater than that observed in reactions performed with the full complement of rNTPs (lane 1). Interestingly, all reactions containing rCTP (lanes 3 and 6) supported approximately 10–20% of primer synthesis and extension compared with reactions performed with all four rNTPs. CTP also alone resulted in this low level of priming and extension (data not shown) and is likely the result of a minor rATP contamination in the rCTP preparation because the product distribution is unchanged. The maximum length of primers that can be synthesized with rCTP and rATP is five nucleotides and corresponds to primers having the sequence 5’-AACCC. This is significant because previous studies have demonstrated that elongation of RNA primers by DNA pol α/primase requires a minimal length of seven to ten nucleotides (19). Our results suggest that in fact DNA pol α can recognize and extend RNA primers as short as five nucleotides. Interestingly, the data presented in Figs. 3 and 4 reveal a distinct product migrating at greater than 60 bases in all lanes. This product is observed independent of rNTPs employed in the reaction and has also been observed in some reactions without the addition of rNTPs. However, this product was also observed in reactions employing a [α-32P]rCTP label, suggesting that it is not a result of DNA extension and labeling of the TS10 substrate via a stable secondary structure (data not shown).

The results from this study identified a protein capable of synthesizing the mammalian telomeric lagging strand in vitro. The protein was identified as DNA pol α/primase by antibody inhibition experiments. Characterization of telomeric lagging strand synthesis demonstrated complete dependence on rNTPs, suggesting an RNA priming mechanism. Direct evidence of RNA priming of the TS10 substrate was obtained using a [α-32P]rCTP label (data not shown). A unique primer initiation site was established, and five-base-long ribonucleotide primers are capable of being extended with DNA by pol α. Degradation
of the RNA primers was accomplished by RNase HI and FEN-1 consistent with their role in Okazaki fragment processing.

The results presented suggest that DNA pol α/primase has a role in telomere maintenance. In a genetic screen to identify proteins that interact with the yeast PRI1 gene, encoding the DNA primase subunit of pol α/primase, the MEC3 was isolated (22). MEC3 has been found to participate in the G2 cell cycle checkpoint and, in conjunction with RAD24 and RAD17, degrade the C-rich strand of telomeric and subtelomeric DNA in response to DNA damage (23). The demonstration that mec3, pri1 double mutants are synthetically lethal (22) supports our hypothesis that primase is involved in mammalian telomere maintenance. Interestingly, a recent study has identified the Saccharomyces cerevisiae cdc13 gene product as a specific telomere DNA binding protein (24). The finding that arrest of cdc13 mutants requires RAD24 (25) and therefore MEC3 provides further evidence that primase is involved in mammalian telomere metabolism.

REFERENCES
1. Watson, J. D. (1972) Nat. New Biol. 239, 197–201
2. Blackburn, E. H. (1992) Annu. Rev. Biochem. 61, 113–129
3. Zahler, A. M., and Prescott, D. M. (1989) Nucleic Acids Res. 17, 6299–6317
4. Greider, C. W., and Blackburn, E. H. (1985) Cell 43, 405–413
5. Waga, S., Bauer, G., and Stillman, B. (1994) J. Biol. Chem. 269, 10923–10934
6. Turchi, J. J., Huang, L., Murante, R. S., Kim, Y., and Bambara, R. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9803–9807
7. Balagurumoorthy, P., and Brahmachari, S. K. (1994) J. Biol. Chem. 269, 21858–21869
8. Wood, R. D., Robins, P., and Lindahl, T. (1988) Cell 53, 97–106
9. Nasheuer, H. P., and Grosse, F. (1987) Biochemistry 26, 8458–8466
10. Turchi, J. J., and Bambara, R. A. (1993) J. Biol. Chem. 268, 15136–15141
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Morin, G. B. (1989) Cell 59, 521–529
13. Syroja, J., Suomensaari, S., Nishida, C., Goldsmith, J. S., Chui, G. S., Jain, S., and Linn, S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6664–6668
14. Kaguni, L. S., and Lehman, I. R. (1988) Biochim. Biophys. Acta 950, 87–101
15. Hohn, K. T., and Grosse, F. (1987) Biochemistry 26, 2870–2878
16. DiFrancesco, R. A., and Lehman, I. R. (1985) J. Biol. Chem. 260, 14764–14770
17. Hagemeier, A., and Grosse, F. (1989) Eur. J. Biochem. 185, 621–628
18. Eder, P. S., and Walder, J. A. (1991) J. Biol. Chem. 266, 6472–6479
19. Sheaff, R. J., and Kuchta, R. D. (1993) Biochemistry 32, 3027–3037
20. Harrington, C., and Perrino, F. W. (1995) Nucleic Acids Res. 23, 1003–1009
21. Waltz, S. E., Trivedi, A. A., and Leffak, M. (1996) Nucleic Acids Res. 24, 1887–1894
22. Longhese, M. P., Fraschini, R., Plevani, P., and Lucchini, G. (1996) Mol. Cell. Biol. 16, 3235–3244
23. Lydall, D., and Weinert, T. A. (1995) Science 270, 1488–1491
24. Nugent, C. I., Hughes, T. R., Lue, N. F., and Lundblad, V. (1996) Science 274, 249–252
25. Garvik, B., Carson, M., and Hartwell, L. (1995) Mol. Cell Biol. 15, 6128–6136
