ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 1, pp. 92-96. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 1, pp. 117-123.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-207, October 12, 2008.

Replication Protein A Modulates the Activity of Human Telomerase *in vitro*

M. P. Rubtsova[1], D. A. Skvortsov[1], I. O. Petruseva[2], O. I. Lavrik[2], P. V. Spirin[3], V. S. Prasolov[3], F. L. Kisseljov[4], and O. A. Dontsova[1]*

[1]*Chemical Faculty, Lomonosov Moscow State University, 119899 Moscow, Russia; E-mail: dontsova@genebee.msu.su*
[2]*Institute of Chemical Biology and Fundamental Medicine, Siberian Division of the Russian Academy of Sciences, pr. Akademika Lavrentieva 8, 630090 Novosibirsk, Russia*
[3]*Institute of Molecular Biology, Russian Academy of Sciences, ul. Vavilova 32, 119991 Moscow, Russia*
[4]*Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Kashirskoe Shosse 24, 115478 Moscow, Russia*

Received June 18, 2008
Revision received June 30, 2008

Abstract—Our aim was to investigate how replication protein A (RPA) in a wide range of concentration can regulate the activity of human telomerase. We used an *in vitro* system based on human cell extracts with or without RPA. It has been shown that removal of RPA leads to loss of telomerase activity and addition of RPA restores telomerase activity and at the same time promotes telomerase processivity. However, high excess of RPA inhibited telomerase processivity and caused the synthesis of relatively short DNA fragments (about 50-100 nucleotides). We assume that, together with other telomere-binding proteins, RPA may take part in activation of telomere overhang elongation by telomerase at a certain stage of a cell cycle as well as in regulation of telomere length.

DOI: 10.1134/S0006297909010143

Key words: telomerase, RPA, telomeres, regulation

Replication protein A (RPA) is a stable complex of three subunits—RPA70, RPA32, and RPA14 [1]—with very conservative primary structure in eukaryotes [2]. RPA forms very stable complex with single-strand DNA (ssDNA) [3] and is required for DNA replication, reparation, recombination, and cellular response to DNA damage and other cellular processes [2, 4]. RPA contains six OB-fold domains, a typical domain for ssDNA-binding proteins [5]. A fragment of ssDNA not shorter than 30 nucleotides (nt) is required for polar binding of one RPA molecule with high (up to 0.1 nM) affinity. In a complex, RPA heterotrimer has an elongated conformation [1, 2]. Binding of RPA in globular conformation to 8-10 nt ssDNA fragments is also known [1, 2]. RPA has a transition conformation when it forms a complex with 13-22 nt fragment of ssDNA [3]. Binding of the globular form of RPA to DNA is cooperative, but this cooperativity is not strong [3]. Also, RPA has some preference to pyrimidine-rich DNA sequences and may interact with non-canonical structures, like G-quadruplexes that are formed by telomeric sequences [6]. RPA interacts with many proteins that take part in DNA metabolic processes [2]. Regulation of RPA binding to DNA and dissociation of the protein from various complexes in the cell, as well as RPA interactions with other DNA-binding proteins are investigated very intensively [2].

Data concerning the role of RPA in telomere length maintenance started to accumulate recently. It was shown that RPA from *Saccharomyces cerevisiae* interacts with proteins associated with telomerase complex and recruits telomerase to telomeres [5], and in *Shizosaccharomyces pombe* yeast it participates in telomere length maintenance [7]. RPA is able to bind human telomere DNA *in vitro* [2], and furthermore it could disrupt G-quartet structure at least in *in vitro* experiments [6]. RPA co-localizes with telomeres *in vivo* in *Leishmania* [8]. Addition of RPA to an *in vitro* telomerase activity system from *Thermus thermophila* has a negative effect [9]. Data about the participation of RPA in regulation of telomerase activity in humans are lacking. In our work, we investigated the influence of RPA on human telomerase activity over a large range of protein concentration.

Abbreviations: nt, nucleotides; RPA, replication protein A; SSB, single-strand binding protein; ssDNA, single-strand DNA.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Preparation of S100 cellular extracts. Approximately $2 \cdot 10^8$ cells were used for each extract. Cells growing in monolayer were harvested by scrapping with a rubber policeman and centrifuged for 10 min at 500*g* at 4°C. The pellets were rinsed twice with cold PBS (1.7 mM KH_2PO_4, 5.2 mM Na_2HPO_4, 150 mM NaCl) followed by centrifugation for 3 min at 700*g* at 4°C. The resulting pellet was rinsed in cold 2.3× Hypo-buffer (1× Hypo-buffer: 10 mM Hepes, pH 8.0, 3 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 10 units/ml RNasin (Helicon, Russia), Complete Protease inhibitor cocktail tablets (Roche, England)), centrifuged for 5 min at 700*g* at 4°C, and resuspended in 0.75 volume of 2.3× Hypo-buffer. After incubation on ice for 10 min, the sample was transferred to an ice-cold 7 ml Dounce homogenizer and homogenized on ice (0°C) using a B pestle.

After further 30-min incubation on ice, the sample was centrifuged for 10 min at 10,000 rpm at 4°C in a 5415R table-top centrifuge (Eppendorf, Germany). One-fiftieth volume of 5 M NaCl was added to the clarified extract and the sample was centrifuged for 1 h at 100,000*g* in a Beckman (USA) L8-50 M/E centrifuge (SW50.1 rotor) at 4°C. Glycerol was added to a final concentration of 20%, and the extract was aliquoted (50-100 µl) and stored at −70°C [10]. The final volume of the extract preparation was 1 ml. Thus, 1 µl of S100 preparation contains extract from $2 \cdot 10^5$ cells. Protein concentration determined by the Bradford method was 3-4 mg/ml.

Preparation and characterization of RPA protein. Recombinant human RPA protein was purified from producing *Escherichia coli* strain as in [11] with modifications. It did not contain nucleases. Protein concentration was measured using a Bradford method kit (Bio-Rad, USA) and BSA solution as a standard for calibration. RPA concentration was ~2.7 mg/ml. More than 95% of the protein was active as it was determined by polarization anisotropy of fluorescence [12]. All experiments were done taking in consideration these data.

Immunoprecipitation of RPA. Polyclonal anti-RPA antibodies from serum of an immunized rabbit was purified by affinity chromatography and given to us by P. P. Laktionov (Institute of Chemical Biology and Fundamental Medicine, Siberian Division of the Russian Academy of Sciences). Extract was depleted of RPA using a modified immunoprecipitation method [13]. S100 extract was diluted by PBS to protein concentration ~1 mg/ml. Anti-RPA antibodies (5-10 µl) were added to 400 µl of diluted extract, and the mixture was incubated for 1-2 h at 4°C with rotation. A mixture of 200 µl of protein G-Sepharose (Protein G Sepharose 4 Fast Flow; Amersham Biosciences, USA) and 50 µl of S100 extract was incubated for 10 min at 4°C and centrifuged at 1000*g*. Then 400 µl of the mixture of extract and antibodies was added to protein G-Sepharose and incubated for 1-2 h at 4°C with rotation and then centrifuged at 1000*g* for 5 min. The supernatant was collected, aliquoted, and stored at −70°C.

Western-blotting. RPA-depleted and native S100 extracts were subjected to 15% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell, Germany). Visualization was done using antibodies against corresponding proteins (anti-RPA, anti-actin) and a set of reagents for Western-blot (Amersham ECL Plus; GE Healthcare, USA). We used commercial preparation of actin (DPC Biermann) and anti-actin antibodies (serum from rabbit with reactivity to human actin; Sigma, USA).

TRAP-assay. TRAP-assay was done as described previously [14] with some modifications [15]. Reaction mixture of 1× TRAP-buffer (5× TRAP-buffer: 20 mM Hepes-KOH, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.1 mg/ml BSA, 0.005% Tween-20 (v/v)), 20 µM dNTPs except for dATP, 4 µM dATP, 1.6 µM oligonucleotide TS (aatccgtcgagcagagtt), ≤1 µl S100 extract, and RPA or SSB (single-strand binding protein) (USB Co., USA) in various quantities was incubated for 30 min at 30°C in a 45 µl volume. (For the negative control, 1 µl (10 mg/ml) of RNase A (Helicon) was added to 10 µl of native extract. The mixture was incubated for 20 min at room temperature prior to telomerase reaction.) Further 2 units of Taq DNA-polymerase (Helicon), 0.1 µg of oligonucleotide ACX (gcgcggcttaccctaccctaccctaaccc), 2 µCi [α-^{32}P]dATP (3000 Ci/mmol; GE Healthcare), and $MgCl_2$ up to concentration 1 mM was added to the reaction mixture. PCR was done using following parameters: 35 sec at 94°C, 35 sec at 50°C, 90 sec at 72°C (30 cycles) (amplifier Mastercycler (Eppendorf) was used). PCR products were purified by phenol extraction followed by separation in 10% denaturing PAGE. Radioactive products were detected using a phosphoimager (Fuji, Japan).

RESULTS

Preparation of RPA-depleted cellular extracts. Cellular extracts depleted of RPA were prepared by immunoprecipitation [13]. S100 extract from HeLa cells was treated with polyclonal antibodies against RPA and complexes of protein and antibody were precipitated by Protein G-Sepharose. The amount of RPA was substantially reduced in the immunodepleted extract. Completeness of RPA removal was checked by Western-blot analysis shown in Fig. 1. RPA is readily detected in a native extract, and it is lacking in the immunodepleted extract (Fig. 1a, lanes *1* and *2*). Other proteins are present in the same amount in the depleted extract. This is shown by Western-blot analysis using antibodies against actin (Fig. 1b, lanes *1* and *2*).

Detection of telomerase activity. Telomerase activity was detected by a standard method—TRAP-test [14]. The first step of this method is elongation of the primer by cel-

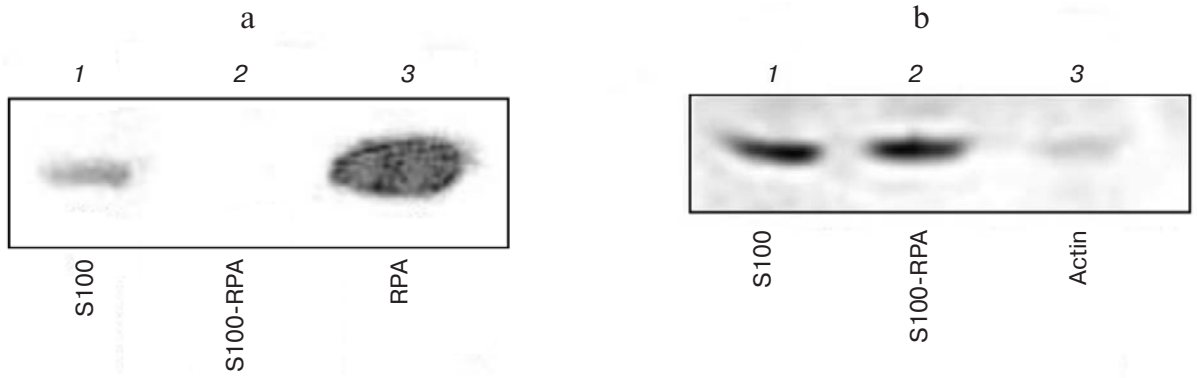

Fig. 1. Immunodepletion of RPA from HeLa cells extract. a) Western-blot analysis of presence of RPA in extracts: *1*) extract before immunoprecipitation; *2*) after immunoprecipitation; *3*) purified RPA. b) Western-blot analysis of presence of actin in extracts: *1*) extract before immunoprecipitation; *2*) after immunoprecipitation; *3*) purified actin.

lular telomerase that is present in an extract. The products of telomerase activity are amplified by PCR in a second step. We used an equal amount of DNA-primer and different dilutions of native and RPA-depleted cellular extracts. The amount of extract in each experiment is normalized as number of cells, which was varied from five to 1000. The telomerase activity is substantially decreased in extracts from 100 and lower numbers of cells. At the same time, telomerase activity is detected in native extracts even from five cells (Fig. 2).

We added purified recombinant RPA to restore its concentration in extract and to exclude other possibilities of telomerase activity reduction following RPA immunodepletion, such as telomerase inactivation or inactivation or co-precipitation with RPA of other proteins important for telomerase. We did not detect telomerase activity in RPA-depleted extracts from 20 cells up to RPA concentration 0.1 mM. The telomerase activity can be detected when concentration of RPA is 0.2 mM and higher. Extract prepared from 20 cells has high telomerase activity (Fig. 3a, lane *1*) and lacks it after immunoprecipitation (Fig. 3a, lane *2*). RPA (0.1-0.5 mM) restores telomerase activity (Fig. 3a, lanes *3-6*). Low concentration of RPA (0.1-0.3 mM) stimulates processive telomerase activity, and we can see longer DNA fragments (Fig. 3a, lanes *3* and *4*). Further increase of RPA concentration in the reaction mixture causes preferable synthesis of short length (50-60 nt) products (Fig. 3a, lanes *5* and *6*). In the native S100 extract, low concentration of RPA stimulates and high concentration inhibits telomere DNA synthesis (Fig. 3a, lanes *7-9*). Telomerase activity inhibition and preferable synthesis of short products could be detected at high excess of RPA (Fig. 3a, lanes *5*, *6*, and *9*). Nonspecific product of PCR reaction is also detected, as in the case of telomerase inactivation by RNase A (Fig. 3a, lane *2*).

To check cross-species complementation, we added *E. coli* SSB protein to the human cell extract in the same quantity as RPA. SSB is a ssDNA binding protein participating in replication, repair, and other activities like RPA

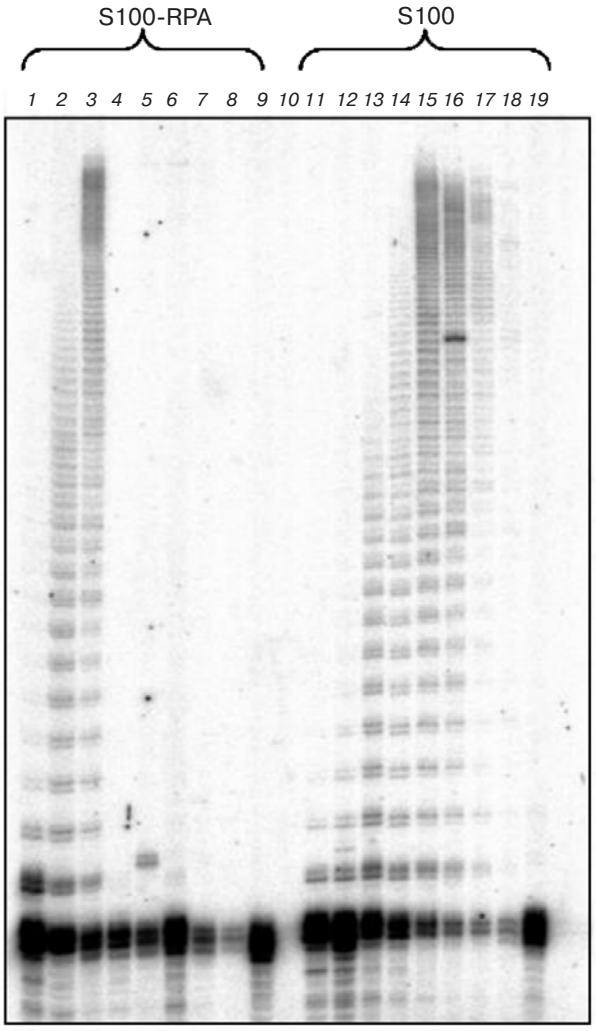

Fig. 2. TRAP-assay analysis of telomerase activity in extracts: *1-8*) telomerase activity of RPA-depleted extract corresponding to 1000, 500, 200, 100, 50, 20, 10, and 5 cells, respectively; *11-18*) the same for native extract; *9*, *19*) telomerase activity in native and immunodepleted extracts after RNase A treatment (control); *10*) reaction without extract (control for TRAP-test specificity).

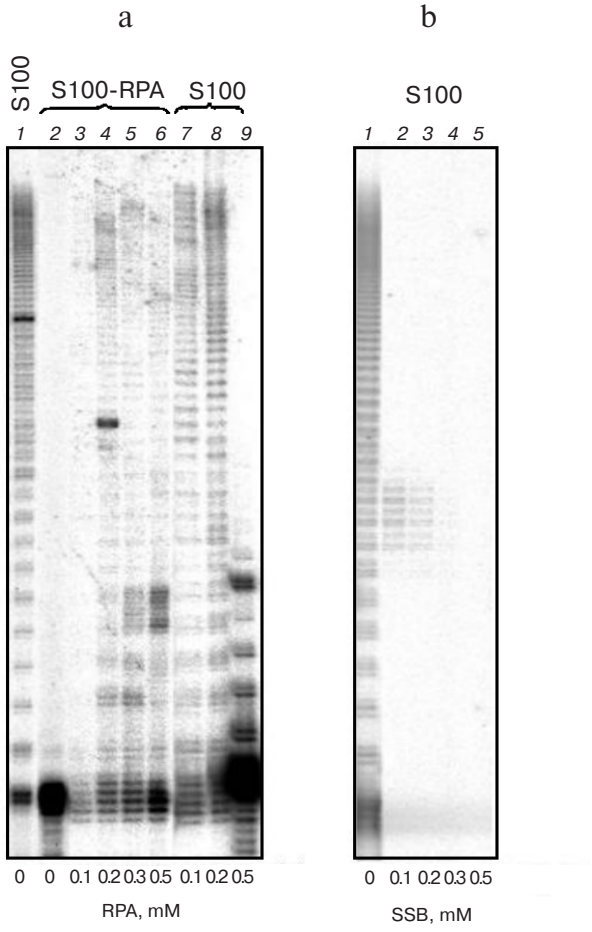

Fig. 3. TRAP-assay analysis of telomerase activity in extracts after RPA (a) and SSB (b) addition. a: *1*) telomerase activity in native extract from 20 cells; *2*) telomerase activity in immunodepleted extract from 20 cells; *3-6*) telomerase activity in immunodepleted extract after RPA addition up to concentration of 0.1, 0.2, 0.3, and 0.5 mM, correspondingly; *7-9*) telomerase activity in native extract after RPA addition up to concentration of 0.1, 0.2, and 0.5 mM, correspondingly. b: *1*) telomerase activity in native extract from 50 cells; *2-5*) telomerase activity in native extract from 50 cells after SSB addition up to concentration of 0.1, 0.2, 0.3, and 0.5 mM, correspondingly.

[16]. SSB blocked telomerase activity in extracts from 20 cells. Even small amounts of SSB changed the set of telomerase reaction products in a native extract from 50 cells, while excess of SSB blocked telomerase activity completely (Fig. 3b).

DISCUSSION

In our work, we have shown that lack of RPA has a negative effect on the ability of telomerase to elongate DNA-primer in extracts from HeLa cells. A small amount of RPA restores telomerase activity in processive form, but excess of RPA inhibits the telomerase reaction. Prokaryotic SSB protein does not stimulate telomerase activity like RPA, but in contrast inhibits it.

A set of proteins binds 3′-end of telomere in eukaryotes, and one of them is POT1. POT1 is a protein participating in telomere length maintenance *in vivo* [17]. This protein binds protruding 3′-end of telomere and protects it from degradation and recombination. It is able to facilitate [18] or prevent telomerase binding to telomeres [19]. A set of proteins is needed for telomerase binding to telomeres and its activation [17]. It is known that repeated G-rich sequences, such as human telomeres, are able to form stable G-quartet structure [6]. This was shown by fluorescent microscopy using antibody against G-quartet for some eukaryotic organisms [20]. Many proteins are known to interact with G-quartet structures [21], and with these structures on telomeres [20].

Consequently, processes regulated by binding of telomerase to telomeres might be very complicated and need a huge set of proteins. It was supposed that telomere lengthening occurs at the end of S-phase, at G2-checkpoint [22], when DNA replication is completed. RPA synthesis continues at this time, but the amount of ssDNA decreases and the amount of free RPA increases in the nucleus. It is possible that RPA participates in telomere replication at the stage of telomerase working as well as in a process of complement strand synthesis. It is known that RPA can bind DNA with telomere sequences and promote unfolding of G-quartet *in vitro*. It was proposed that for this process cooperative binding of several RPA molecules to DNA is needed [6]. RPA may promote processive synthesis of telomere DNA by telomerase because it may displace telomere-binding proteins from telomeres and promote disruption of non-canonical structure of telomeric DNA. Increase in telomerase activity caused by increase of the RPA-depleted extract concentration in the reaction mixture is much sharper than those for increase of native extract concentration. Addition of purified RPA protein to the RPA-depleted extract causes dramatic increase in telomerase activity, which could be explained by cooperative RPA binding to DNA. RPA binds DNA very strongly in case of large excess of protein and like SSB could displace telomerase from the complex with DNA. DNA fragments with distinct length are accumulated in our experiments because RPA binds DNA by an SSB-like mechanism. Consequently, the competition between RPA, POT1, G-quartet binding, and other proteins may be crucial for regulation of the length of ssDNA synthesized by telomerase, and regulation of length of newly synthesized telomere.

We thank M. E. Zvereva, O. V. Schpanchenko, and A. A. Bogdanov for discussion of our results.

This work was supported by HFSP RGP0032/2005, RFBR 08-0401220-a and 08-0400204-a, HHMI 55005605, OFI-c 07-0413832, the Russian Academy of

Sciences Program for Molecular and Cellular Biology, and contracts 02.435.11.3016 and 02.435.11.2039 with Ministry of Science.

REFERENCES

1. Iftod, C., Daniely, Y., and Borowiec, J. A. (1999) *Crit. Rev. Biochem. Mol. Biol.*, **34**, 141-180.
2. Fanning, E., Klimovich, V., and Nager, A. R. (2006) *Nucleic Acids Res.*, **34**, 4126-4137.
3. Lavrik, O. I., Kolpashchikov, D. M., Weisshart, K., Nasheuer, H. P., Khodyreva, S. N., and Favre, A. (1999) *Nucleic Acids Res.*, **27**, 4235-4240.
4. Maga, G., Frouin, I., Spadari, S., and Hubscher, U. (2001) *J. Biol. Chem.*, **276**, 18235-18242.
5. Schramke, V., Luciano, P., Brevet, V., Guillot, S., Corda, Y., Longhese, M. P., Gilson, E., and Geli, V. (2004) *Nat. Genet.*, **36**, 46-54.
6. Salas, T. R., Petruseva, I., Lavrik, O., Bourdoncle, A., Mergny, J. L., Favre, A., and Saintome, C. (2006) *Nucleic Acids Res.*, **34**, 4857-4865.
7. Kibe, T., Ono, Y., Sato, K., and Ueno, M. (2007) *Mol. Biol. Cell.*, **18**, 2378-2387.
8. Neto, J. L., Lira, C. B., Giardini, M. A., Khater, L., Perez, A. M., Peroni, L. A., dos Reis, J. R., Freitas-Junior, L. H., Ramos, C. H., and Cano, M. I. (2007) *Biochem. Biophys. Res. Commun.*, **358**, 417-423.
9. Cohen, S., Jacob, E., and Manor, H. (2004) *Biochim. Biophys. Acta*, **1679**, 129-140.
10. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S. (1992) *EMBO J.*, **11**, 1921-1929.
11. Henricksen, L. A., Umbricht, C. B., and Wold, M. S. (1994) *J. Biol. Chem.*, **269**, 11121-11132.
12. Perrault, R., Cheong, N., Wang, H., Wang, H., and Iliakis, G. (2001) *Int. J. Radiat. Biol.*, **77**, 593-607.
13. Petruseva, I. O., Tikhanovich, I. S., Chelobanov, B. P., and Lavrik, O. I. (2008) *J. Mol. Recogn.*, **21**, 154-162.
14. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994) *Science*, **266**, 2011-2015.
15. Kim, N. W., and Wu, F. (1997) *Nucleic Acids Res.*, **25**, 2595-2597.
16. Meyer, R. R., and Laine, P. S. (1990) *Microbiol. Rev.*, **54**, 342-380.
17. Smogorzewska, A., and de Lange, T. (2004) *Annu. Rev. Biochem.*, **73**, 177-208.
18. Wang, F., Podell, E. R., Zaug, A. J., Yang, Y., Baciu, P., Cech, T. R., and Lei, M. (2007) *Nature*, **445**, 506-510.
19. Loayza, D., and de Lange, T. (2003) *Nature*, **423**, 1013-1018.
20. Paeschke, K., Juranek, S., Simonsson, T., Hempel, A., Rhodes, D., and Lipps, H. J. (2008) *Nat. Struct. Mol. Biol.*, **15**, 598-604.
21. Arthanari, H., and Bolton, P. H. (2001) *Chem. Biol.*, **8**, 221-230.
22. Sabourin, M., Tuzon, C. T., and Zakian, V. A. (2007) *Mol. Cell*, **27**, 550-561.