Telomerase

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Regulation of Telomerase Activity by the Thermodynamic Stability of a DNA·RNA Hybrid**

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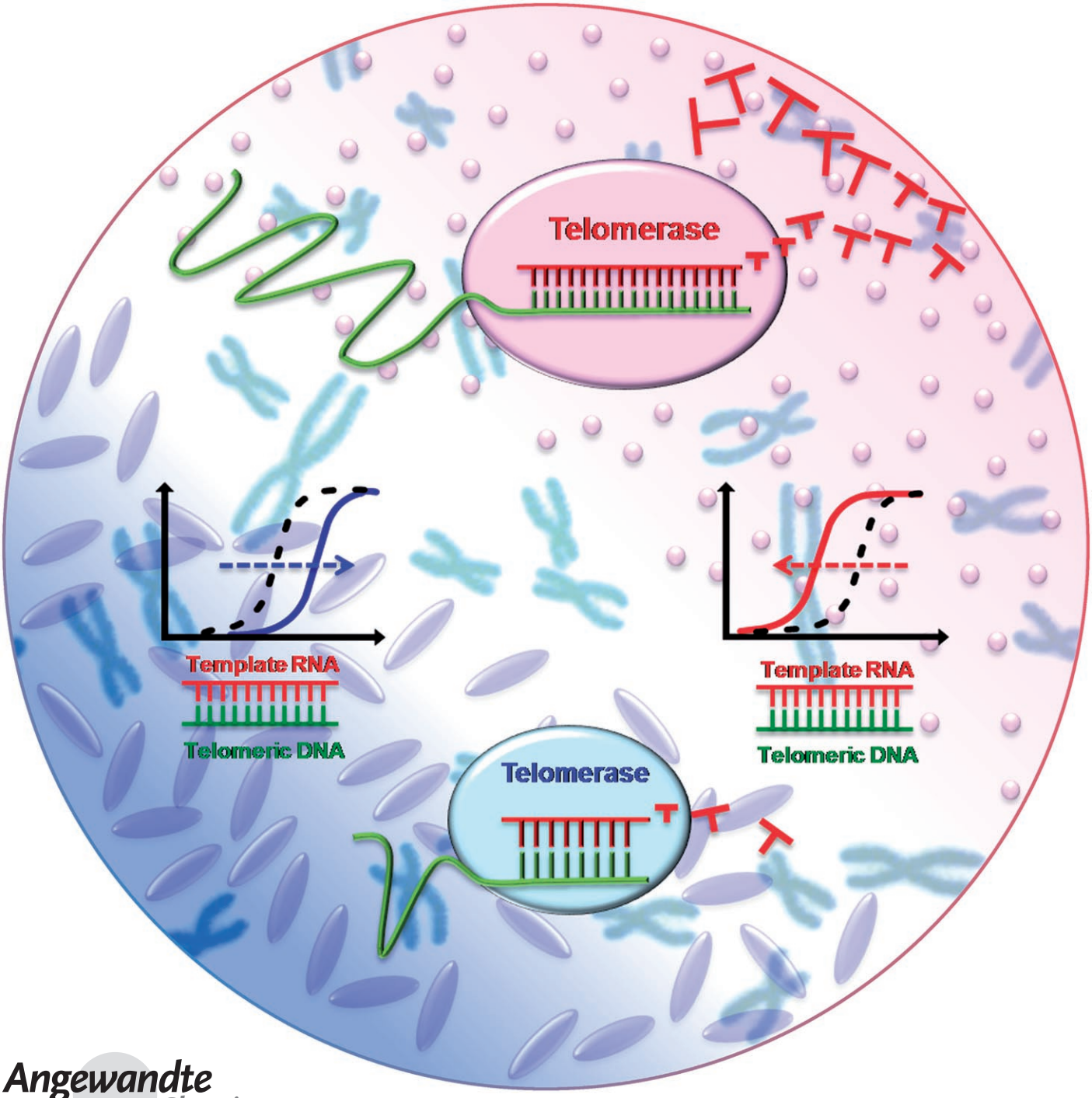

Telomerase can recognize chromosome ends and add telomeric DNA repeats onto them with the template RNA carried by this enzyme. This process prevents the replication-dependent loss of telomeres and cellular senescence in highly proliferative cells of the germline and in the majority of cancers.[1] As telomerase was unable to extend a potassium-stabilized intramolecular G-quadruplex in vitro,[2] it has been widely believed that telomeric DNA that forms a G-quadruplex is an ineffective substrate for telomerase and leads to the inhibition of telomerase activity.[3] This conclusion promoted many investigations of G-quadruplexes[4] and the regulation of telomerase activity by designing ligands that can induce G-quadruplex formation and increase G-quadruplex stability.[5] Great progress has been made in these efforts to prevent the binding of telomerase to telomeric DNA. On the other hand, few studies have examined the regulation of telomerase activity in a translocation state (Figure 1), in which telomerase binds to linear telomeric DNA and propagates the length of the telomeric DNA. Herein, we report that the translocation of telomerase along the telomeric DNA is critical for telomere elongation. Therefore, the activity of telomerase bound to telomeric DNA may be regulated by controlling the stability of a hybrid between the template RNA and its complementary telomeric (substrate) DNA.

To study the relationship between the stability of the DNA–RNA hybrid and telomerase activity, we utilized an improved telomerase repeat amplification protocol (TRAP) assay in which the telomerase reaction for the evaluation of telomerase activity and the polymerase chain reaction (PCR) for the amplification of the elongated product were carried out separately. Moreover, as there are large amounts of metabolites in cells, the presence of which leads to conditions of molecular crowding, we utilized cosolutes with different chemical properties, such as glycerol, ethylene glycol (EG), ethanol (EtOH), and poly(ethylene glycol) (PEG), with various molecular weights (MWs) to induce molecular-crowding conditions. First, we studied the structure and thermodynamics of the hybrid formed between the template RNA, CUAACCCUAAC, and the substrate DNA, GTTAGGGTTAG. This system was used as our model

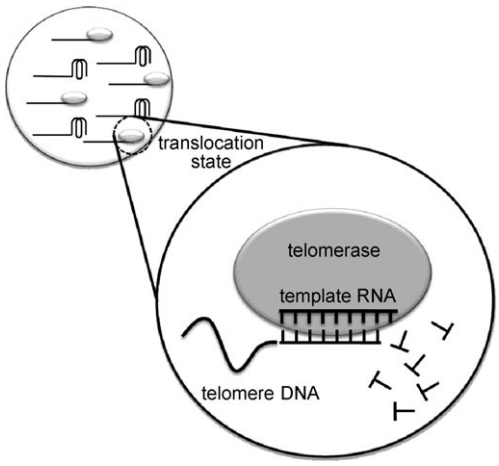

Figure 1. Schematic illustration of the translocation state in which telomerase binds to linear telomeric DNA and propagates the length of the telomeric DNA.

hybrid.[6] In the presence and in the absence of the cosolutes, circular dichroism (CD) spectra of the 1:1 mixture of DNA and RNA had large positive and small negative peaks at 275 and 240 nm, respectively, which indicated that the mixture folded into the A-form duplex, a typical structure of the DNA/RNA hybrid,[7] but not a G-quadruplex[8] (Figure 2 A).

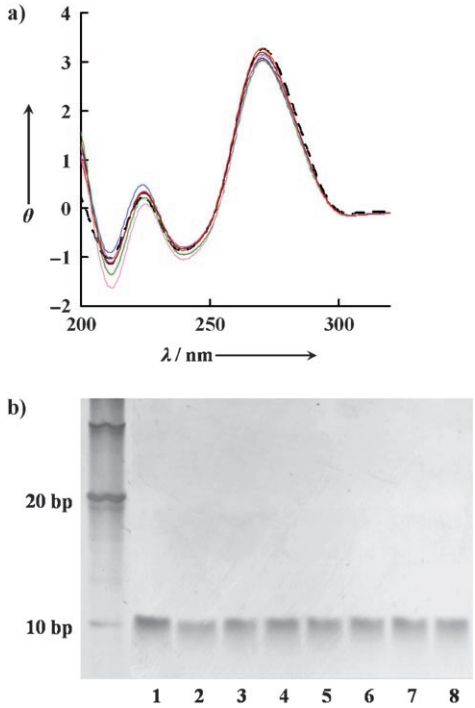

Figure 2. a) CD spectra (θ in 10^5 deg cm^2 dmol^{-1}) of the DNA·RNA hybrid in the presence or absence of a cosolute (30 wt%) at 4 °C (pale pink: PEG 20000; green: PEG 6000; brown: PEG 1000; red: PEG 200; light blue: glycerol; dark blue: ethanol; pink: ethylene glycol; dashed line: without cosolute). b) Native 20% PAGE of the DNA·RNA hybrid in a TRAP buffer at 4 °C without a cosolute (lane 1) or with 30 wt% EtOH (lane 2), EG (lane 3), glycerol (lane 4), PEG 200 (lane 5), PEG 1000 (lane 6), PEG 6000 (lane 7), or PEG 20000 (lane 8).

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The structures in the mixture were studied further by native polyacrylamide gel electrophoresis (PAGE; Figure 2B). The mixture and the 10-base-pair-duplex marker migrated a similar distance under these conditions, which confirmed the formation of an 11-mer duplex. Furthermore, hyperchromicity was observed in the UV melting curves of the mixture at 260 nm (Figure 3A), whereas hypochromicity was not observed at 295 nm (see Figure S1 in the Supporting Information); these results also support duplex formation. These structural studies demonstrate that the duplex is the main structure in the mixture under dilute and molecular-crowding conditions.

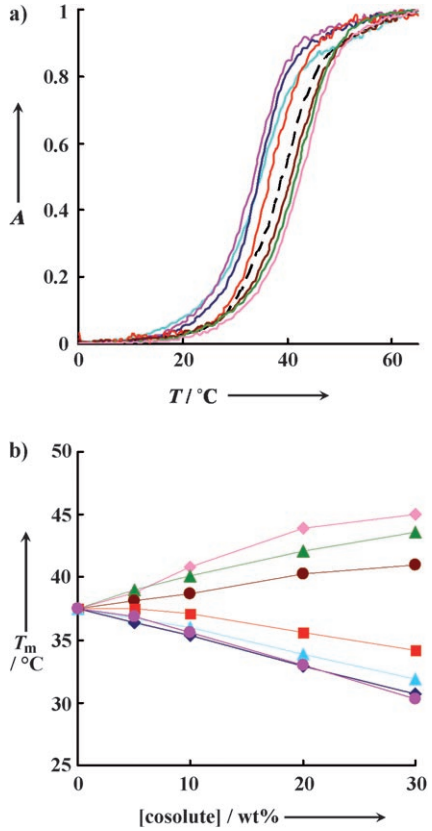

Figure 3. a) Normalized absorbance at 260 nm of the DNA·RNA hybrid in the presence or absence of a cosolute (30 wt%) versus temperature. b) T_m value of the DNA·RNA hybrid versus concentration of the cosolute. (In both (a) and (b), pale pink: PEG 20000; green: PEG 6000; brown: PEG 1000; red: PEG 200; light blue: glycerol; dark blue: ethanol; pink: ethylene glycol; dashed line: without cosolute.)

bilized and stabilized by cosolutes with low and high MWs, respectively.[9]

The effects of molecular crowding on telomerase activity were investigated further. Figure 4A shows the results of the improved TRAP assay with various concentrations of EG. Surprisingly, higher concentrations of EG enhanced the telomerase activity. Moreover, it was found that cosolutes

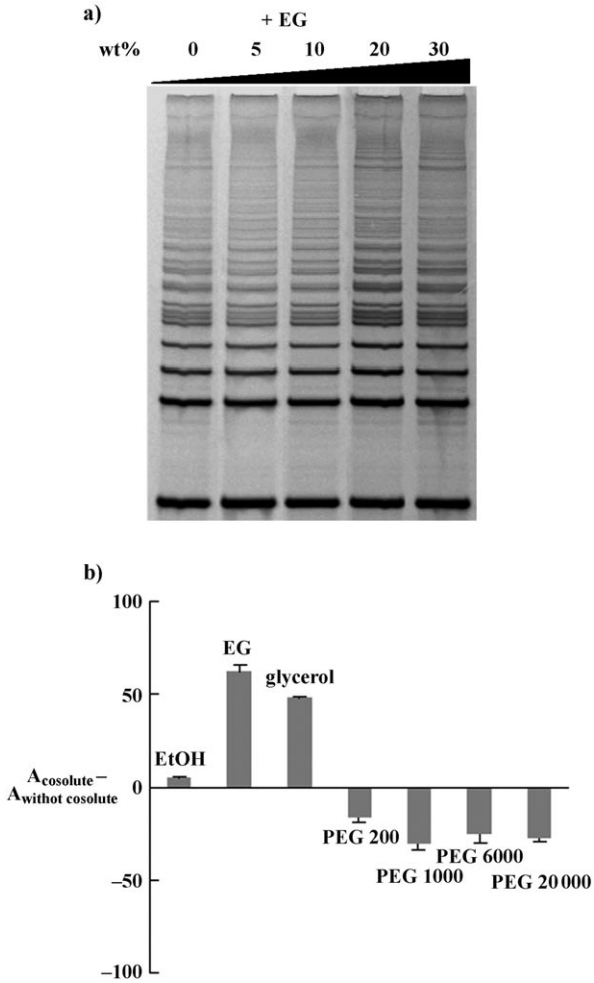

Figure 4. a) Results of the TRAP assay (PAGE) with various concentrations of EG. b) Difference between telomerase activity in the presence of various cosolutes (20 wt%; $A_{cosolute}$) and telomerase activity in the absence of a cosolute ($A_{without\ cosolute}$). Here A stands for relative activity. A value of 100 corresponds to the positive control, namely without cosolute. For details of the calculation of the relative activity, see the Supporting Information.

Next, we evaluated the effect of molecular crowding by the cosolutes on the stability of the DNA·RNA hybrid from UV melting curves at 260 nm (Figure 3A and Figure S2 in the Supporting Information). The results showed that low-MW cosolutes, such as glycerol, EG, EtOH, and PEG 200 (PEG with an average MW of 200), decreased the melting temperature (T_m), and high-MW cosolutes, such as PEG 1000, PEG 6000, and PEG 20000, stabilized the hybrid (Figure 3B). These results are consistent with those of previous studies, which showed that a DNA·DNA duplex was destabilized with low MWs enhanced the telomerase activity, and cosolutes with high MWs decreased the telomerase activity (Figure 4B and Figure S3 in the Supporting Information). In the two-step TRAP assay, the product of the telomerase reaction was transferred to a PCR tube with a 50-fold dilution, and the amplification of the product by PCR was carried out under dilute conditions; thus, molecular crowding affected the telomerase reaction, but not the amplification.[10] The results of the TRAP assay and the thermodynamic stabilities of the DNA·RNA hybrid under the molecular-

crowding conditions demonstrate that the destabilization and stabilization of the hybrid are favorable and unfavorable for telomerase activity, respectively. We concluded that molecular crowding is able to enhance or decrease the activity of the enzyme, depending on the molecular weight of the cosolutes. The effects of molecular crowding on telomerase activity may be due to alterations in the stability of the DNA·RNA hybrid. The cosolute PEG 200 forms an exception to this relationship, as it destabilized the hybrid but reduced the enzyme activity slightly, as discussed later.

Telomerase, which includes the template RNA, initially binds to the telomeric DNA and adds bases according to the sequence of the template RNA. The translocation of telomerase then proceeds with the sequential addition of the repeat units.[10,11] Therefore, changes in the stability of the DNA·RNA hybrid may affect both binding and translocation steps. If the stability of the hybrid affects the binding step, the destabilization and stabilization of the hybrid would decrease and enhance telomerase activity, respectively, by changing the binding affinity of telomerase to the substrate DNA. On the other hand, if the stability affects the translocation step, the destabilization of the hybrid would make the dissociation of the substrate DNA and template RNA easier, which would lead to an enhancement in activity, whereas the stabilization of the hybrid would lead to a decrease in activity. As our results demonstrated that the destabilization and stabilization of the hybrid enhanced and diminished telomerase activity, respectively, an alteration in the thermodynamic stability of the hybrid should mainly affect the translocation step. This conclusion is consistent with a previous study, which showed the critical role of the translocation step in the regulation of telomerase activity by small ligands.[10]

How does molecular crowding affect the telomerase activity physicochemically? It is well known that solution viscosity affects the activity of an enzyme.[12] However, we observed no clear relationship between telomerase activity and viscosity (see Figure S4A in the Supporting Information), which implies that viscosity is not a determinant factor for activity. Another possible factor in the regulation of telomerase activity is water activity, as many reports have indicated that water molecules participate in most enzymatic reactions and stabilize the protein.[13] All cosolutes used in this study decreased the water activity (see Figure S4B in the Supporting Information), which may be unfavorable for telomerase activity, as observed for DNA polymerase.[14] For glycerol, EG, and EtOH, the negative effect of the water activity and the positive effect of hybrid destabilization on telomerase activity may compete with each other to give a bell-shaped relationship between telomerase activity and cosolute concentration (see Figure S3 in the Supporting Information). On the other hand, for PEG 1000, PEG 6000, and PEG 20000, the negative effects of both water activity and hybrid stabilization on telomerase activity lead to a monotonous decrease in activity with an increasing cosolute concentration. Furthermore, the presence of small cosolutes[15] with fewer hydroxy groups to bind instead of water molecules to telomerase (and thus affect the affinity of the enzyme for the substrate by changing the enzyme conformation) leads to lower telomerase activity than that observed with small consolutes containing more hydroxy groups (Figure 5). This result demonstrates the critical role of water molecules in the regulation of telomerase activity.

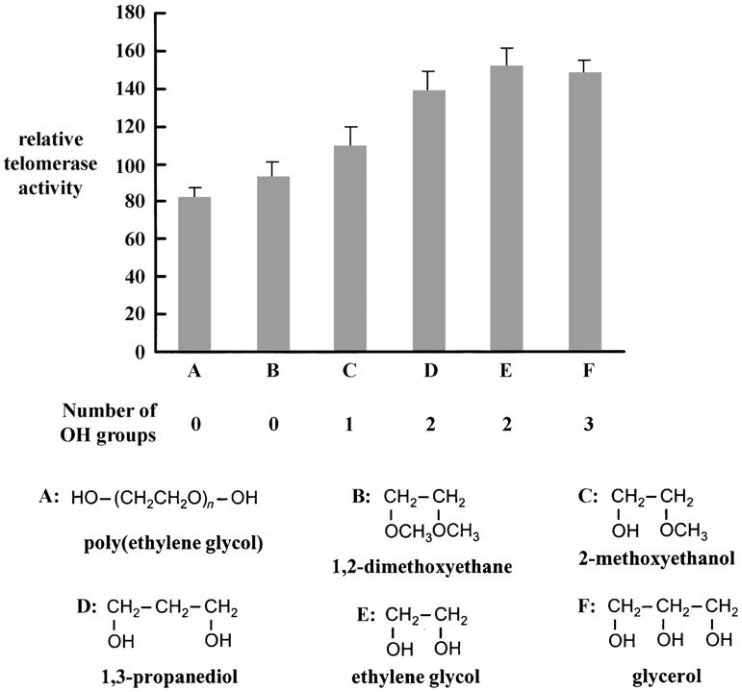

Figure 5. Relative telomerase activity in the presence of various small cosolutes (20 wt%).

In conclusion, we have found that molecular crowding can both decrease and enhance telomerase activity by changing the stability of the DNA·RNA hybrid. Our results show a new way to regulate the activity of telomerase bound to telomeric DNA. Furthermore, we reported previously that molecular-crowding conditions increase the stability of the G-quadruplex structure of guanine-rich sequences,[9a–c] which were able to prevent telomerase binding to telomeric DNA.[4a] If telomerase dissociates from the substrate DNA after four or more telomeric repeats have been added onto the primer, the substrate DNA has the opportunity to fold into a G-quadruplex. Telomerase difficulty elongates the substrate DNAs including G-quadruplexes, which eventually results in the ladder pattern of the TRAP assay. Therefore, molecular crowding can regulate both the binding and the elongation step of the telomerase. These regulation strategies may be general for enzymes whose substrates are nucleic acids.[14,16] Moreover, this method for the regulation of telomerase activity may be useful for various applications, such as the regulation and sensitive detection of immortalized cancer, germ, and stem cells.

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