

A three-state model for the regulation of telomerase by TERRA and hnRNPA1

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ABSTRACT

Telomeres, the physical ends of eukaryotic chromosomes, are transcribed into telomeric repeat-containing RNA (TERRA), a large non-coding RNA, which forms an integral part of telomeric heterochromatin. *In vitro*, naked TERRA molecules are efficient inhibitors of human telomerase, base-pairing via their 5'-UUAGGG-3' repeats with the template sequence of telomerase RNA, in addition to contacting the telomerase reverse transcriptase protein subunit. *In vivo*, however, TERRA-mediated inhibition of telomerase can be prevented by unknown mechanisms. Also, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) has been implicated in telomere length control. *In vivo*, TERRA is partially associated with hnRNPA1, and hnRNPA1 is also detected at telomeres. We demonstrate that on binding of TERRA, hnRNPA1 can alleviate the TERRA-mediated inhibition of telomerase. However, when in excess over TERRA, hnRNPA1 becomes itself an inhibitor of telomere extension, on binding of the telomeric DNA substrate. Yet, hnRNPA1 has no notable direct effects on the telomerase catalysis. Our *in vitro* results suggest that TERRA-mediated telomerase inhibition may be prevented by hnRNPA1 *in vivo*. Telomere extension by telomerase may require balanced levels of TERRA and hnRNPA1 at telomeres. Thus, TERRA and hnRNPA1 can function as a bimolecular regulator to turn telomerase and the telomere on and off.

INTRODUCTION

Telomeres protect chromosome ends from DNA repair activities that reseal chromosome internal DNA breaks that occur during DNA damage (1). Telomeric DNA shortens with every round of semiconservative DNA replication due to the end replication problem and nucleolytic

processing. Short telomeres induce cellular senescence. The telomerase enzyme can solve the end replication problem, re-extending telomere 3' ends by reverse transcribing the template region of its tightly associated RNA moiety into telomeric repeats (2–4). The regulation of telomerase at chromosome ends is not well understood and is the subject of intensive investigations in several laboratories (5,6).

Telomeres are transcribed in most or all eukaryotes into the long non-coding RNA TERRA (7). TERRA transcription starts in the subtelomeric region and proceeds toward chromosome ends. Thus, TERRA contains near its 3' end telomeric repeats in the form of RNA. The UUAGGG-repeat containing RNA molecules act as potent mixed-type inhibitors of human telomerase *in vitro* (8,9). However, it is unclear under which circumstances TERRA may regulate telomerase *in vivo*. Supporting a role of TERRA in telomerase control, it was found that in human cells TERRA is displaced or degraded at telomeres by factors involved in nonsense mediated RNA decay (NMD). Although more abundant in the cytoplasm, NMD factors are detected at low levels in the nucleus, physically interacting with the telomeric chromatin (10) and also with telomerase (11–14). On the other hand, elevated TERRA transcription at human telomeres did not prevent telomerase-mediated telomere extension (15). In *Saccharomyces cerevisiae*, induction of TERRA leads to telomere shortening. However, this involves activation of the chromosome end trimming exonuclease 1 at chromosome ends rather than inhibition of telomerase (16).

HnRNPA1 is a multifunctional RNA-binding protein involved in the regulation of RNA biogenesis. Interestingly, hnRNPA1 is also involved in telomere maintenance (17). Mouse cells deficient for hnRNPA1 have short telomeres, and this phenotype was rescued upon expression of hnRNPA1 from a cDNA. HnRNPA1 binds single-stranded human d(TTAGGG)_n telomeric DNA repeats *in vitro* (18,19) as well as r(UUAGGG)_n telomeric RNA repeats that are contained within TERRA (8,19,20). HnRNPA1 (21,22) or fragments thereof (17) also associate with telomerase activity. Finally, it has been proposed

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that a switch at telomeres from replication protein A (RP-A) for DNA replication in S phase to protection of telomeres 1 (POT1)/TPP1 after S phase is triggered by hnRNPA1 and TERRA (23). *In vitro*, hnRNPA1 can compete with RP-A for the binding of the single-stranded G-strand (23), and an increase in TERRA abundance after S phase may sequester hnRNPA1 from telomeric DNA. Binding of hnRNPA1 to TERRA may in turn free the telomeric 3' overhang for binding by POT1-TPP1. Proteins with sequence homology to hnRNPA1 and roles at telomeres have also been reported in *S. cerevisiae*, but the involved mechanisms are not known (24).

Here, we show that TERRA and hnRNPA1 cooperate in telomerase control *in vitro*. The telomerase-inhibitory effects of TERRA are alleviated on hnRNPA1 association with TERRA. On the other hand, we find that excess of hnRNPA1 also inhibits telomerase through binding and sequestration of the DNA primer, although hnRNPA1 has no notable direct effects on the telomerase catalytic activity. Our data demonstrate that *in vitro* telomerase activity is maintained as long as the levels of TERRA and hnRNPA1 are balanced. Thus, TERRA and hnRNPA1 may provide a bimolecular switch to fine-tune telomerase activity at chromosome ends.

MATERIALS AND METHODS

Plasmids and oligonucleotides

Plasmids for transient transfection of human telomerase reverse transcriptase (hTERT) (pcDNA6-ZZ-3xFlag-hTERT) and hTR (pBS-U1-hTR) were described previously (25). For bacterial expression of hnRNPA1, pGEX-hnRNPA1 was generated by PCR amplification of the hnRNPA1 open reading frame from pcDNA6-hnRNPA1 using BamHI-A1_F and A1-EcoRI_R primers (Supplementary Table S1) and subcloned into the BamHI and EcoRI sites of pGEX-6P-1 vector (GE Healthcare). pcDNA6-hnRNPA1 was generated by PCR amplification of the hnRNPA1 open reading frame from pCMV6-XL5-hnRNPA1 (Origene, clone NM_002136) using HindIII-A1_F and EcoRI-A1_R primers. cDNAs were subcloned into retroviral-based pCL vectors for expression of ZZ or hnRNPA1-ZZ proteins in HT1080 cells. Oligonucleotides were purchased from Microsynth and are listed in Supplementary Table S1.

Antibodies

Antibodies against Flag (F1804) and tubulin (T9026, for western blotting) were obtained from Sigma. The 9B11 antibody against Myc was purchased from Cell Signaling. Antibodies against hnRNPA1 (SC-32B01), tubulin (SC-8035, for immunoprecipitation), cyclin E (SC-247) and cyclin B1 (SC-245) were from Santa Cruz, and antibodies against GST (27457701) were from GE Healthcare.

Stable cell line generation

HT1080 stable cell lines were generated by viral transduction and puromycin selection (1 µg/ml).

hnRNPA1 depletion

The 293T cells were transfected for 48 h with siRNAs against GFP or hnRNPA1 (sequences in Supplementary Table S1) using Interferin (Polyplus).

Protein expression and purification

Rosetta pLysS competent bacteria (Novagen) were transformed with pGEX-hnRNPA1. For expression of recombinant GST-hnRNPA1, 21 of 2YT containing 34 µg/ml chloramphenicol, 50 µg/ml ampicillin and 50 µg/ml carbenicillin were inoculated with 50 ml of overnight culture and incubated at 37°C until the OD₆₀₀ reached 0.8. Protein expression was induced for 3 h at 37°C by adding 0.4 mM IPTG. After centrifugation, the bacteria were washed once with ice-cold 1x PBS. The pellet was resuspended in 60 ml lysis buffer (1x PBS, 1 mM DTT and 1x protease inhibitor cocktail EDTA-free from Roche). The lysate was sonicated five times for 10 s (Branson sonifier 250, setting 3, constant). Glycerol and Triton X-100 were added to final concentrations of 10 (v/v) and 1% (v/v), respectively. The extract was incubated on a rotating wheel for 15 min at room temperature and then centrifuged for 15 min at 12 000 g at 4°C. The supernatant was incubated with 2 ml 50% slurry of GSH-coupled beads (GE Healthcare). After binding for 2 h at 4°C, bead-bound proteins were washed once with 1x PBS, 10% (v/v) glycerol and three times with 1x PBS. GST-hnRNPA1 was eluted for 30 min at 4°C with 2 ml 20 mM NaCl, 20 mM GSH and 200 mM Tris-HCl (pH 9.5). Glycerol was added to a final concentration of 10% (v/v). To remove the remaining beads, the eluate was passed through a 35 µm filter (Mobicel). The same protocol was applied to express and purify GST alone using pGEX-6P-1 vector with the only exception that the elution steps were performed with 20 mM GSH and 200 mM Tris-HCl (pH 7.5). The second purification step was performed on a HiTrapQ HP 1 ml column using the Akta purifier system and the Unicorn software (GE Healthcare). Before loading, each eluate from the first purification step was diluted 10x in 20 mM Tris-HCl (pH 8.0) and 25 mM NaCl. A 20 ml NaCl gradient was applied from 25 mM to 1 M. 500 µl fractions were collected, glycerol was added to 10% (v/v) final concentration and samples were quick-frozen.

Telomerase purification

For telomerase overexpression, Human Embryonic Kidney (HEK) 293E cells were grown in suspension culture and transiently transfected with pcDNA6-ZZ-3xFLAG-hTERT and pBS-U1-hTR at the Protein Expression Core Facility of EPFL (<http://pecf.epfl.ch/>). Forty-eight hours post-transfection, cells were harvested by centrifugation. In all, 1.5×10^8 cells were resuspended in 12 ml 20 mM HEPES-KOH (pH 7.9), 2 mM MgCl₂, 200 mM KCl, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA containing protease inhibitor cocktail (Roche) and lysed in a dounce homogenizer using 12 strokes with a type B pestle. Triton X-100 was added to a final concentration of 0.1% (v/v), and the lysate was incubated

on a rotating wheel at 4°C for 30 min. The lysate was cleared by centrifugation at 20 000 *g* for 15 min at 4°C. Telomerase was bound to IgG Sepharose 6 Fast Flow (GE Healthcare; 50 µl beads per extract corresponding to 1.5 mg total protein) blocked with 0.5 mg/ml BSA and 0.05 mg/ml yeast RNA, and equilibrated in 20 mM HEPES-KOH (pH 7.9), 2 mM MgCl₂, 300 mM KCl, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM PMSF. After overnight binding at 4°C, beads were washed twice in the same buffer, once with 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM MgCl₂, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, and once with 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM MgCl₂, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol. Telomerase was eluted for 2 h at RT in 45 µl of the same buffer containing 0.11 U/µl AcTEV protease (Invitrogen). The eluate was passed through a 35 µm filter (Mobitec) by centrifugation, and samples were quick-frozen.

Gel mobility shift assay

PAGE-purified RNA and DNA oligonucleotides were resuspended in RNase-free water. Oligonucleotides were 5' end-labeled with [³²P]-γ-ATP (3000 Ci/mmol) using T4 polynucleotide kinase (NEB), and unincorporated nucleotides were removed using mini Quick Spin RNA Columns (Roche). RNA- or DNA-protein complexes were separated on native 8% polyacrylamide gels that were run in 0.5x TBE buffer at 4°C (350 V for 1 h). The gels were pre-run for 30 min at 350 V. The gels were dried and exposed to a PhosphorImager screen. Signals were quantified by Image Quant. For protein binding to (UUAGGG)₃, 2 nM [³²P] 5' end-labeled (UUAGGG)₃ was incubated for 30 min at 30°C in 1x EMSA buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM spermidine, 1 mM MgCl₂, 0.8 µg/µl deacetylated-BSA, 0.5 mM DTT and 10% (v/v) glycerol] with the indicated amounts of GST or GST-hnRNPA1 or 1 µl of the protein dilution buffer [20 mM Tris-HCl (pH 8.0), 215 mM NaCl, 10% glycerol], ±200 ng α-hnRNPA1 antibody. For the determination of the K_D of GST-hnRNPA1 for (UUAGGG)₃ or (TTAGGG)₃, GST-hnRNPA1 (12.5, 25, 37.5, 62.5, 75, 87.5, 112.5 or 137.5 nM) was incubated with 2 nM [³²P] 5' end-labeled (UUAGGG)₃ in EMSA 1x buffer for 45 min at 30°C. Reactions were placed on ice and loaded on native 8% polyacrylamide gels in 0.5x TBE at 4°C. For the competition experiments, competitor RNAs were added as indicated before the addition of GST-hnRNPA1. Moreover, rA₁₈ was always added to reach the same final oligonucleotide concentration in each reaction.

Direct telomerase activity assay and rescue experiment

To test effects of hnRNPA1 on telomerase activity, direct telomerase assays (DTA) were performed using enriched telomerase fraction and the 5' end biotinylated telomerase substrates B-(TTAGGG)₃ and B-TS (Supplementary Table S1). DTA was performed as previously described (25). Reactions were assembled on ice in 20 µl final volumes. They contained 35 nM biotinylated substrate

DNA oligonucleotide, 175 nM RNA oligonucleotides (mixture of (UUAGGG)₃ and rA₁₈ as indicated), 40 µM dATP, 80 µM dTTP, 2 µM dGTP, 20 µCi of α-[³²P]-dGTP (3000 Ci/mmol), 53 mM Tris-HCl (pH 8.0), 50 mM KCl, 45 mM NaCl, 1 mM MgCl₂, 1 mM spermidine, 1.7% glycerol, 5 mM β-mercaptoethanol, 0.8 µg/µl deacetylated-BSA and 5U SUPERaseIn (Ambion). Telomerase and dNTPs were added last to the reactions. Buffer composition was maintained constant in all the reactions. The reaction was incubated for 45 min at 30°C and stopped by addition of EDTA to 25 mM, mixed with 3' end biotinylated and [³²P] 5' end-labeled 10-mer (used as a recovery and loading control) before the purification step of the elongated products on paramagnetic M-270 streptavidin beads (Invitrogen). After washing, bead-bound products were resuspended in 12 µl of sample loading buffer and denatured at 95°C for 3 min; 6 µl was separated on denaturing (7 M urea) 1x TBE 12% polyacrylamide gels. Dried gels were exposed to a PhosphorImager screen. Signals were quantified by Image Quant.

Immunoprecipitation from cell extracts and RNA extraction

All steps were performed at 4°C unless stated otherwise. For Figure 1, immunoprecipitations from enriched nuclear extracts were performed as described before (8), with slight modifications: for the washing steps after the overnight immunoprecipitation, the beads were first washed three times with B1/2 NP40 [10 mM HEPES (pH 7.5), 200 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA, 0.5 mM DTT, 0.25 mM PMSF and protease inhibitor cocktail (Roche), 0.6% (v/v) NP40] and then washed twice with buffer B1/2 without NP40. Beads were resuspended in 200 µl buffer B1/2 NP40 containing 10% (v/v) glycerol. Protein fractions were loaded on a 4–20% gradient Tris-HCl Precast Gel (Bio-Rad). RNA was extracted with Trizol-LS (Invitrogen). After precipitation and washes, the RNA was resuspended in 8 µl of H₂O and DNase-treated following the Nucleospin Macherey Nagel kit protocol. After DNase treatment at 37°C for 10 min, 1.2 µl 10 mM EDTA was added and DNase was heat inactivated at 75°C for 20 min.

RNA chromatin immunoprecipitations

RNA chromatin immunoprecipitations (RNA-ChIP) assays were performed as described (http://www.protocol-online.org/cgi-bin/prot/view_cache.cgi?ID=3241) with minor modifications. Briefly, 10⁶ cells/ml in 1x PBS were cross-linked for 10 min with 1% formaldehyde. Glycine was added to a final concentration of 125 mM to quench cross-linking, and the cells were collected at 500 *g* for 5 min. The cell pellet was washed with cold PBS, resuspended in 200 µl of buffer A [5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP40, protease inhibitors cocktail (Roche), 50 U/ml SUPERase•in] and placed on ice for 10 min. Nuclei were collected by centrifugation at 2 400 *g* for 5 min at 4°C, washed in buffer A without NP40 and resuspended in 500 µl of 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), protease inhibitors cocktail

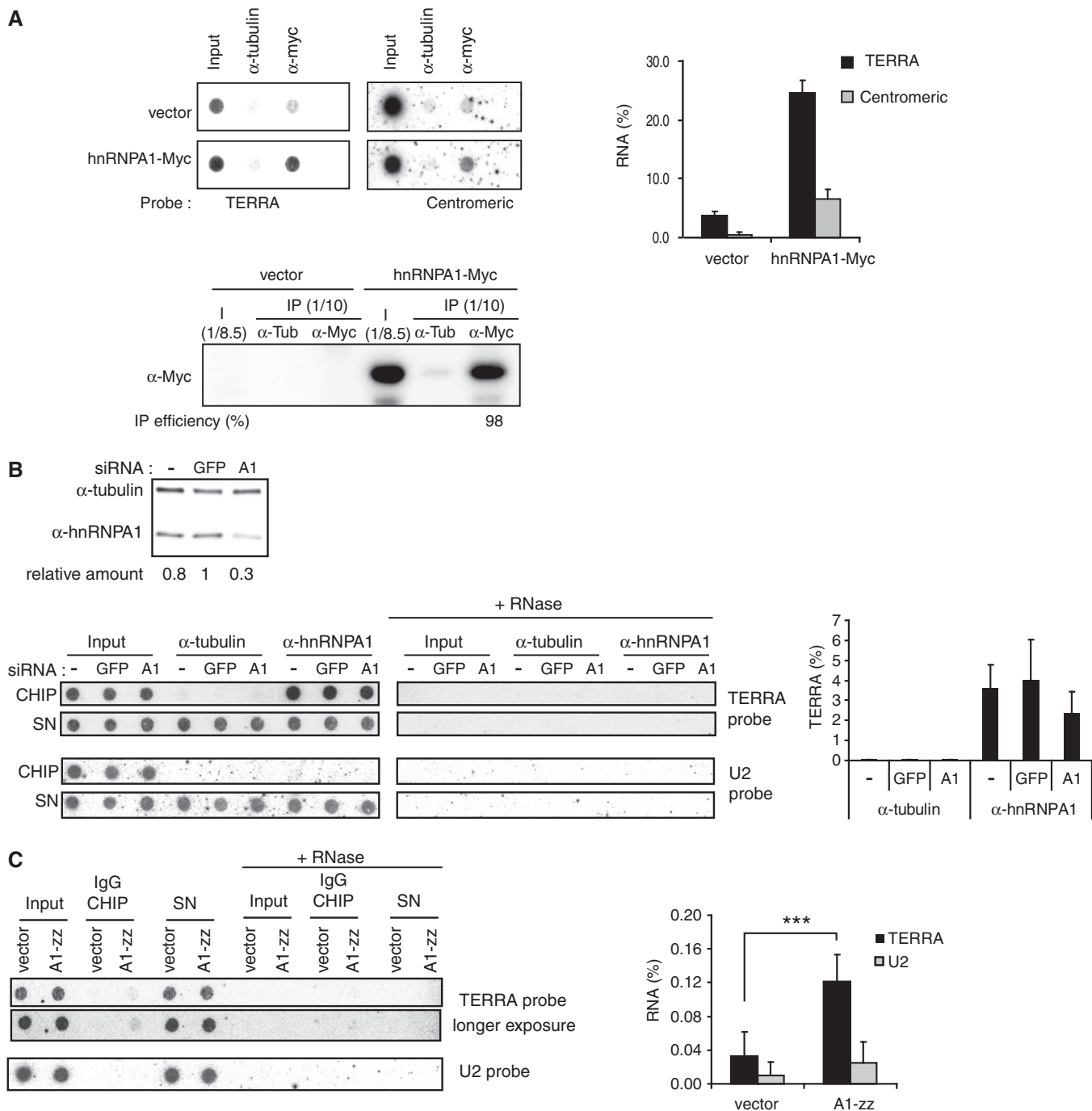


Figure 1. Endogenous TERRA is bound to hnRNPA1. (A) Immunoprecipitation of hnRNPA1-myc in 293T nuclear extract. TERRA and centromeric RNA were detected by dot blot hybridization. Immunoprecipitation efficiency was determined by western blotting. Twenty-five percent of the RNA extracted from the input (I) and all RNA from the immunoprecipitates were spotted on the membrane and quantified. Bars represent the means \pm SD of three independent experiments. (B) RNA-ChIP of TERRA associated with endogenous hnRNPA1 in 293T cells untreated (–) or transfected with siRNA against GFP or hnRNPA1. RNA was detected by dot blot hybridization. One percent of the input RNA or supernatant and half of the RNA from the immunoprecipitates was loaded. The same samples were treated with RNase A before loading (+ RNase). Bars represent the means \pm SD of three independent experiments. (C) RNA-ChIP of TERRA associated with hnRNPA1-ZZ in HT1080 cells. RNA was detected as in (B). One percent of the input RNA or supernatant and half of the RNA from the immunoprecipitates was loaded. *** $P = 0.0002$. Bars represent the means \pm SD of six independent experiments.

(Roche), 50 U/ml SUPERaseⁱⁿ; they were then incubated on ice for 10 min. The Diagenode bioruptor was used for sonication (settings high, five cycles of 30 sec on and 30 sec off in ice-cooled water). The lysates were cleared by

centrifugation at 12 000 g for 15 min at 8°C, and diluted 5-fold in IP buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, protease inhibitors cocktail (Roche), 50 U/ml

SUPERase•in; $\sim 4 \times 10^6$ cells per IP]. Before the immunoprecipitation, a pre-clearing step was performed for 1 h on a rotating wheel at 4°C using Sepharose 6 Beads (Sigma). The pre-cleared extracts were incubated with 5 μ g of antibody for 2.5 h at 4°C on a rotating wheel and Sepharose 6 protein-G beads were added and tubes were placed at 4°C overnight on a rotating wheel. Beads were washed five times (1 ml wash, 5 min each) with the following buffers. First, in 0.1% SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. Second, in the same buffer containing 500 mM NaCl. Third, with 250 mM LiCl, 1% (v/v) NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0). Fourth and fifth, with 1 mM EDTA, 10 mM Tris-HCl (pH 8.0). Elution was done with 100 μ l of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA and 1% SDS. After 2 h incubation at 70°C to reverse the cross-link, RNA was isolated using the RNeasy kit (Qiagen) including a DNaseI treatment on the column for 30 min at RT. Before loading onto positively charged nylon membrane (GE Healthcare), all samples were denatured for 5 min at 65°C in RNA loading buffer (Qiagen RNeasy Kit). TERRA was detected with a TERRA-specific probe prepared as described (10). For detection of U2 snRNA or centromeric RNA, oligonucleotide probes (Supplementary Table S1) were 5' end-labeled with 32 P.

Cell cycle synchronization

HeLa cells were synchronized in early S-phase using a double-thymidine block. Cells were seeded at 10% confluency. After 24 h, thymidine was added to the medium to a final concentration of 2 mM. After 18 h, cells were released by washing three times with PBS and addition of fresh medium. Nine hours after release, thymidine was added again to a final concentration of 2 mM. After further 17 h, cells were collected (sample '0 h') or released as after the first block, followed by collection at 2-h intervals. For cell collection, cells were detached by trypsinization and washed with PBS. Aliquots of cells were removed and resuspended in SDS-PAGE sample buffer for subsequent western blot analysis of protein content, or fixed with cold 70% ethanol for fluorescence-activated cell sorting analysis of synchronization.

RESULTS

TERRA ribonucleoprotein particles contain hnRNPA1

Immunoprecipitation assays with antibodies against endogenous hnRNPA1 protein indicated association of TERRA with hnRNPA1 (8,20). To confirm the specificity of these results, we expressed myc-tagged hnRNPA1 from transiently transfected plasmids in (HEK) 293T cells. Two days post-transfection, we prepared nuclear extract as described (8) and immunoprecipitated hnRNPA1 with anti-myc monoclonal antibody (Figure 1A). Immunoprecipitation efficiency of hnRNPA1 was determined on a western blot, and TERRA was quantified by dot blotting (Figure 1A). Twenty-five percent of total TERRA was immunopurified with myc-tagged hnRNPA1, whereas only 3% of TERRA was immunopurified in extracts

derived from empty vector control cells (Figure 1A). Therefore, TERRA is associated with hnRNPA1 in nuclear extracts.

To determine whether hnRNPA1 association with TERRA exists in live cells as opposed to an association occurring post-lysis in extracts, we carried out RNA-ChIP experiment. We cross-linked nucleic acids and proteins in cells with formaldehyde, lysed the cells in presence of 1% SDS, solubilized complexes by sonication and immunoprecipitated endogenous hnRNPA1 and tubulin as a negative control (Figure 1B). After reversal of the cross-link, TERRA was quantified by dot blot and U2 snRNA was quantified as a negative control (Figure 1B). Approximately 4% of TERRA was immunoprecipitated with hnRNPA1 antibody in these experiments, whereas TERRA did not immunoprecipitate with tubulin. U2 snRNA was not detected in the immunoprecipitates. siRNA-mediated depletion of hnRNPA1 led to a reduction of hnRNPA1 to 30% and the fraction of TERRA in the immunoprecipitates dropped slightly, from ~ 4 to 2.5%. However, the difference was not statistically significant, possibly because of insufficient depletion of the abundant hnRNPA1 protein. Therefore, we repeated the RNA-ChIP experiment with HT1080 cells that expressed Protein A-tagged (ZZ) hnRNPA1 from stably transduced vector (Figure 1C). HnRNPA1-ZZ was purified over IgG beads, and TERRA and U2 were quantified as in Figure 1B. Although the recovery of TERRA was low in this experiment, significantly more TERRA was purified from cells transduced with hnRNPA1-ZZ vector as compared with empty vector transduced cells ($P = 0.0002$; Figure 1C). The low recovery rate in the RNA-ChIP experiments in comparison with the native immunoprecipitation may be due to epitope-tag damaging that may occur on cross-linking with formaldehyde. Together, these experiments indicate that TERRA is associated with hnRNPA1 *in vivo*.

hnRNPA1 binds with high affinity to UUAGGG and TTAGGG-repeats

We studied the effects of hnRNPA1 in conjunction with TERRA on telomerase control. Therefore, we expressed recombinant GST-tagged hnRNPA1 in *Escherichia coli* and purified it via the tag on glutathione (GSH)-beads. As a second purification step, GST-hnRNPA1 was fractionated by anion chromatography on a HiTrap Q HP column. The purified protein (Figure 2A) was, as expected, recognized by anti-GST and anti-hnRNPA1 antibodies. The biochemical properties of recombinant GST-hnRNPA1 were characterized in gel mobility shift assays (Figure 2B–E), giving results that extend previously published data (17–19,26,27). GST-hnRNPA1 shifted a 5' end-labeled 5'-(UUAGGG)₃-3' oligonucleotide, and the shifted species was further retarded in the gel on addition of the hnRNPA1 antibody (Figure 2B, compare lanes 4 and 5). The GST-hnRNPA1 bound telomeric RNA sequence 5'-(UUAGGG)₃-3' with a slightly higher affinity ($K_d = 27 \pm 8$ nM) than the same sequence (5'-(TTAGGG)₃-3') in form of DNA ($K_d = 37 \pm 9$ nM). However, hnRNPA1 did not bind the non-telomeric

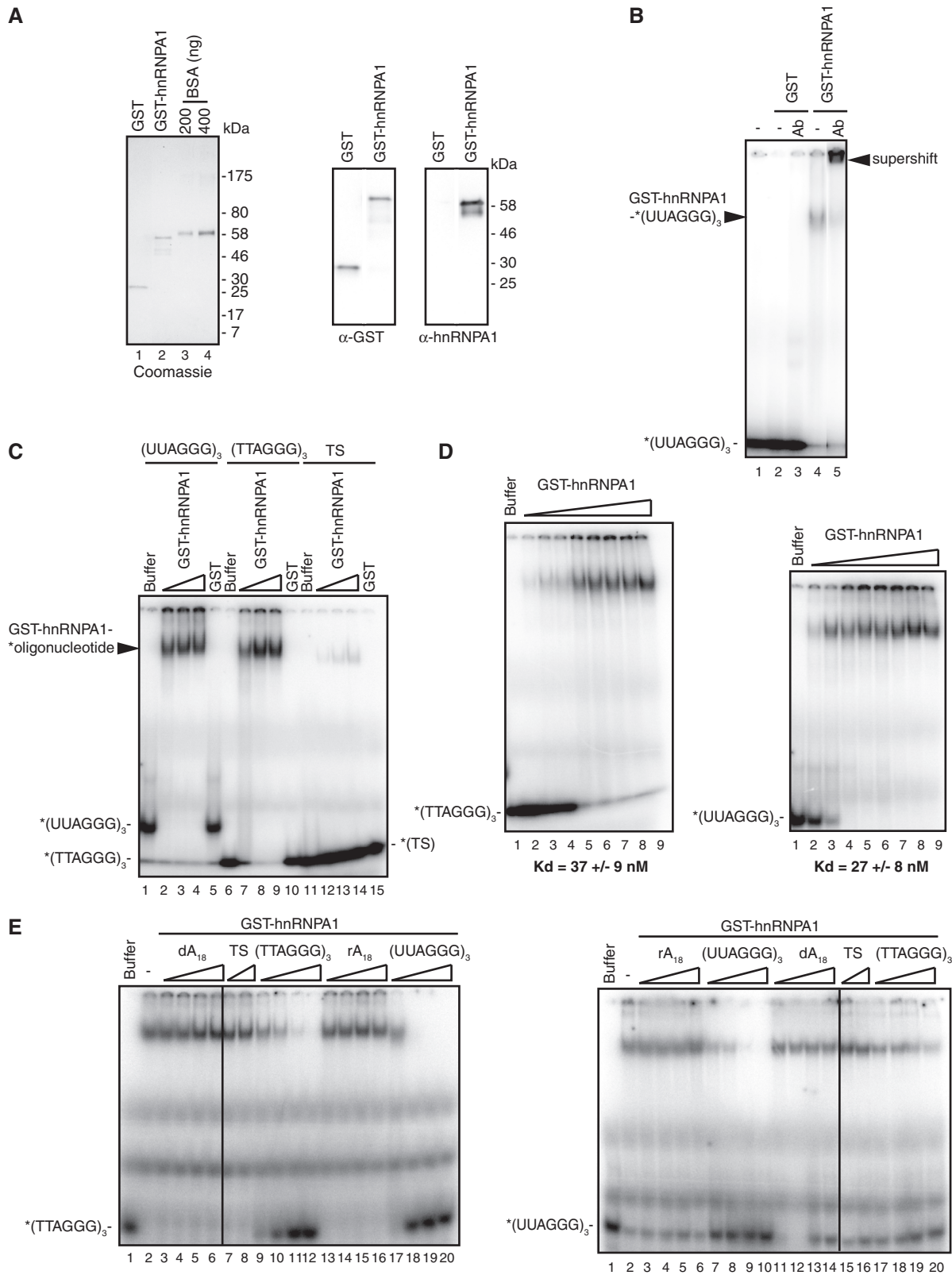


Figure 2. Purification and characterization of recombinant GST-hnRNPA1. **(A)** GST and GST-hnRNPA1 fractions used for *in vitro* assays after the second purification step on the Mono Q sepharose column. The left SDS polyacrylamide gel was stained with Coomassie brilliant blue. Lane 1: GST, lane 2: GST-hnRNPA1, lanes 3 and 4: 200 and 400 ng of BSA. On the right are the corresponding western blots. Western blots were probed with α -GST antibody and α -hnRNPA1 antibody as indicated. **(B)** Recombinant GST-hnRNPA1 but not GST binds (UUAGGG)₃. Electromobility shift assay and supershift of [³²P] 5' end-labeled (UUAGGG)₃ (2 nM) with purified GST or GST-hnRNPA1 (180 nM) with antibody (Ab) or without (–)200 ng α -hnRNPA1 antibody. Lane 1: (UUAGGG)₃ alone. Lane 2: (UUAGGG)₃ incubated with GST. Lane 3: (UUAGGG)₃ incubated with GST and α -hnRNPA1 Ab. Lane 4: (UUAGGG)₃ incubated with GST-hnRNPA1. Lane 5: (UUAGGG)₃ incubated with GST-hnRNPA1

(continued)

sequence containing TS oligonucleotide primer (Supplementary Table S1, Figures 2C and D) that was used in telomerase assays further below. Competition experiments in which unlabeled oligonucleotides were titrated into the gel mobility shift assays with labeled telomeric DNA and RNA (Figure 2E) confirmed the remarkable specific binding of single-stranded telomeric DNA and RNA sequences by hnRNPA1. Competition was only achieved when adding unlabeled 5'-(TTAGGG)₃-3' or 5'-(UUAGGG)₃-3'. Consistent with the K_d-measurements, 5'-(UUA GGG)₃-3' was a better competitor than the corresponding DNA oligonucleotide.

hnRNPA1 inhibits telomerase through sequestration of the primer

We went on to study the effects of hnRNPA1 on telomerase activity. Telomerase was obtained from HEK293E cells that overexpressed hTERT and hTR (28). The hTERT subunit carried at the N-terminus tandem protein A-tags (ZZ) followed by a cleavage site for the TEV protease and a FLAG-tag. Telomerase was purified on IgG-sepharose and eluted by TEV cleavage (Figure 3A). The TERT protein was detected by western blotting with an anti-FLAG antibody. Western analysis of the fractions with an anti-hnRNPA1 antibody revealed that endogenous hnRNPA1 was removed during the purification (Figure 3A).

Telomerase activity was measured in direct telomerase assays in which the DNA substrates were extended in presence of α -³²P-labeled dGTP and unlabeled dATP and dTTP. As DNA substrates we used 5'-(TTAGGG)₃-3' (Figure 3B, left panel), which can be bound by hnRNPA1 or the TS-primer (Figure 3B, right panel), which cannot be bound by hnRNPA1 in its unextended form (see Figure 2). The TS-primer has been extensively used in TRAP-telomerase assays (29). Despite its non-telomeric sequence (5'-AATCCGTCGAGCAGAGTT-3'; Supplementary Table S1), TS is a good telomerase substrate, and its three most 3' terminal bases are complementary to the telomerase RNA template. We used the TS-primer below as a telomerase substrate, as it cannot be bound by hnRNPA1 in its unextended form. When increasing the amounts of hnRNPA1 in the assay with 5'-(TTAGGG)₃-3', we observed complete blocking of extension with primer-saturating amounts of GST-hnRNPA1 (150 nM). However, lower amounts of hnRNPA1 had no notable effects on the extension of

5'-(TTAGGG)₃-3'. A different result was obtained with the TS-primer. GST-hnRNPA1 did not inhibit addition of the first 20 nucleotide (nt). However, the +26nt product was reduced, and longer extension products were nearly absent with the highest concentration of hnRNPA1. This, therefore, indicates that hnRNPA1 prevents telomere elongation when pre-bound to the substrate (Figure 3B, left). In addition, hnRNPA1 inhibits telomerase during telomere elongation once sufficient TTAGGG-repeats are added by telomerase to allow the binding of hnRNPA1 (Figure 3B, right). However, the unperturbed addition of the first 4–5 telomeric repeats to the TS-primer indicates that hnRNPA1 has no effect on the enzymatic properties of telomerase, at least as long as hnRNPA1 cannot bind the DNA substrate. In a time course experiment with the TS-primer, hnRNPA1 had also no detectable effects on the kinetics of telomere extension for the addition of the first 4–5 repeats (data not shown).

hnRNPA1 and TERRA can alleviate their inhibitory effects on telomerase when balanced

TERRA inhibits telomere elongation on binding of the telomerase enzyme (8), whereas hnRNPA1 inhibits telomere elongation on binding of the DNA substrate (see earlier in text). As hnRNPA1 also binds TERRA, we investigated their combined effects on telomerase activity (Figure 4) using 5'-(TTAGGG)₃-3' or TS as primer substrates (Figure 4, left and right panels, respectively). As expected, with increasing concentration of TERRA-oligonucleotide, telomerase activity was inhibited with both DNA primers (Figures 4A and C, lanes 1–4; see Figures 4B and D for the quantification). Presence of purified GST in the absence of hnRNPA1 had no effect (Figures 4A and C, lanes 15–18). Addition of rA₁₈ to the reaction did also not influence the reaction. GST-hnRNPA1 again completely inhibited extension of the 5'-(TTAGGG)₃-3' primer at 150 nM, whereas for the TS-primer again only the longer extension products disappeared (Figure 4A, compare lanes 1–2 with 11).

Strikingly, the combined addition of hnRNPA1 and TERRA could partially or nearly completely suppress the negative effects on the reaction that we observed when each component was added individually. Suppression of inhibition depended on the relative concentration of hnRNPA1 and TERRA. For example, with the TS-primer and hnRNPA1 at 150 nM, the long

Figure 2. Continued

and α -hnRNPA1 Ab. GST-hnRNPA1- (UUAGGG)₃ and Ab-GST-hnRNPA1- (UUAGGG)₃ complexes (supershift) are indicated. (C) GST-hnRNPA1 interacts with TERRA RNA and telomeric DNA oligonucleotides but not with the TS primer. EMSA analysis of [³²P] 5' end-labeled (UUAGGG)₃, (TTAGGG)₃ or TS (2 nM) with purified GST-hnRNPA1 (35, 105 or 315 nM) or 315 nM GST. (D) GST-hnRNPA1 has similar affinity for TERRA and telomeric DNA oligonucleotides. EMSA analysis of [³²P] 5' end-labeled (UUAGGG)₃ or (TTAGGG)₃ (2 nM) with purified GST-hnRNPA1 (12.5, 25, 37.5, 62.5, 75, 87.5, 112.5 or 137.5 nM). K_ds are given and were determined from four independent experiments. (E) EMSA competition experiments to assess specificity of hnRNPA1 for TERRA and telomeric DNA oligonucleotides. On the left gel, GST-hnRNPA1 (60 nM) was incubated with [³²P] 5' end-labeled (TTAGGG)₃ (2 nM) mixed with H₂O or 17.5, 35, 175 or 350 nM of non-labeled dA₁₈; 175 or 350 nM TS; 17.5, 35, 175 or 350 nM of (TTAGGG)₃; 17.5, 35, 175 or 350 nM of rA₁₈; 17.5, 35, 175 or 350 nM of (UUAGGG)₃. On the right gel, GST-hnRNPA1 (60 nM) was incubated with [³²P] 5' end-labeled (UUAGGG)₃ (2 nM) mixed with H₂O or 17.5, 35, 175 or 350 nM of rA₁₈; 17.5, 35, 175 or 350 nM of (UUAGGG)₃; 17.5, 35, 175 or 350 nM of non-labeled dA₁₈; 175 or 350 nM TS; 17.5, 35, 175 or 350 nM of (TTAGGG)₃. For both gels, in lane 1, the radiolabeled oligonucleotide was incubated with the protein buffer only. The vertical line between lanes indicates juxtaposition of separate parts of the same gel.

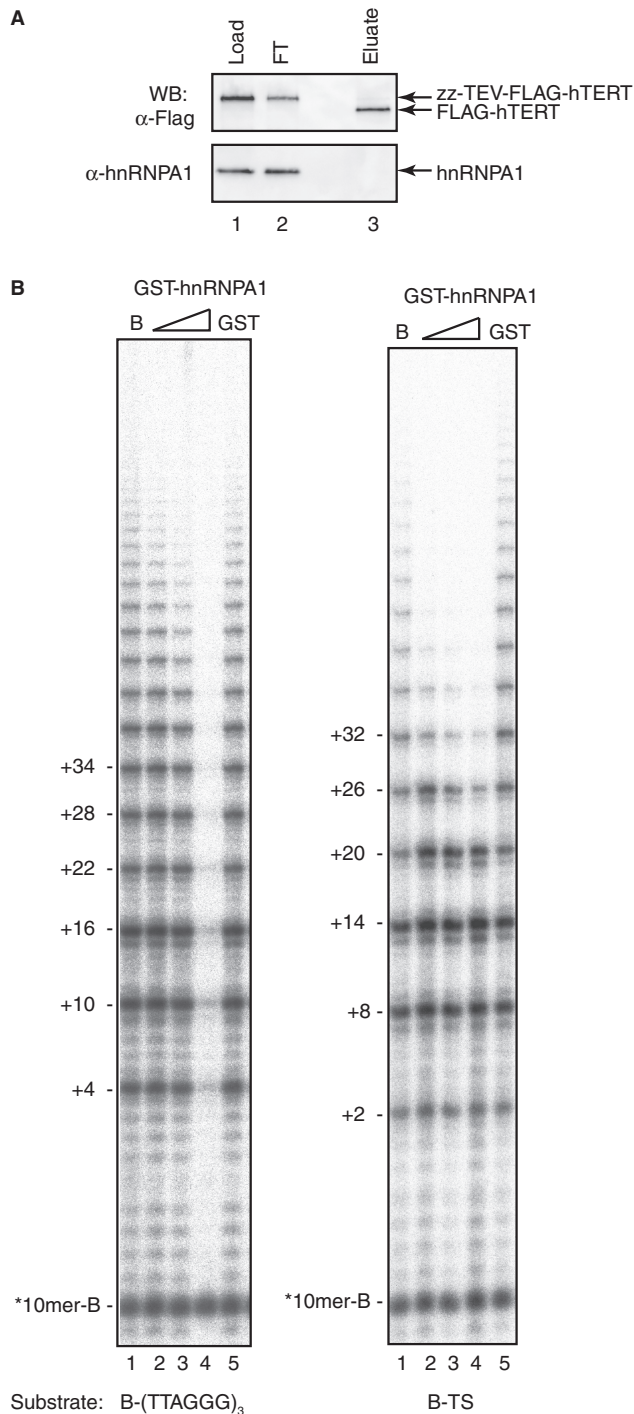


Figure 3. Purification of overexpressed telomerase and inhibiting effects of hnRNPA1 on telomerase activity by primer binding. **(A)** The enriched telomerase fraction used for direct telomerase assays does not contain detectable endogenous hnRNPA1 protein. Load, flow through and eluate from TEV cleavage were analyzed by western blotting using the indicated antibodies. **(B)** hnRNPA1 inhibits telomerase activity by binding to the primer. Direct telomerase assay with telomerase substrates B-(TTAGGG)₃ (left) and B-TS (right). The 3' end biotinylated 10-mer was [³²P] 5' end-labeled and used as a loading control (added during the purification step of the elongated products on streptavidin beads). The numbers on the left of the gels indicate the number of nucleotides added to the substrates. For both gels, lane 1: DTA performed in presence of the protein buffer, lanes 2, 3 and 4: DTA performed in presence of 17.5, 50 and 150 nM GST-hnRNPA1, lane 5: DTA performed with 150 nM GST.

extension products reappeared when TERRA was added to 175 nM, whereas TERRA alone completely inhibited telomerase and hnRNPA1 on its own prevented the accumulation of long products (Figure 4A, compare lanes 11 and 14 for the rescue by TERRA from inhibition by hnRNPA1; compare, for example, lane 4 with lanes 7 and 10 for the rescue by hnRNPA1 from inhibition by TERRA). Also with the (TTAGGG)₃ substrate primer, telomerase activity was partially rescued when combining TERRA and hnRNPA1, except for the highest concentration of hnRNPA1 at which apparently enough hnRNPA1 remained TERRA-free to bind and block extension of (TTAGGG)₃ substrate primer (Figure 4C, lanes 11–14). For example, with TERRA at 17.5 or 35 nM, addition of hnRNPA1 to 50 nM roughly doubled the activity (see Figure 4D for the quantification of the results). The most straightforward interpretation of these results is that hnRNPA1 and TERRA, when assembling together into an RNP complex, lose their inhibitory effects on DNA primer extension and telomerase activity, which they possess as individual components. The slight increase of longer reaction products in presence of TERRA and hnRNPA1 (Figure 4A, lanes 7 and 14) suggested stimulation of repeat addition processivity by TERRA-hnRNPA1 complexes, but further experiments will be required to understand the nature of this effect.

hnRNPA1 protein levels do not vary substantially during the cell cycle

The aforementioned experiments support a model in which the relative abundance of TERRA and hnRNPA1 determines whether telomerase can elongate chromosome ends. Therefore, we tested whether hnRNPA1 levels are regulated during the cell cycle. We synchronized HeLa cells with a double thymidine block at the G1/S boundary and collected cells at 2-h intervals after the release. Protein extracts were prepared, and cell cycle progression was monitored by following the abundance of cyclins E and B1 on a western blot (Figure 5A). Cyclin E peaks during S phase and cyclin B1 in M phase of the cell cycle. The cyclin analysis indicated that cell synchronization and release were successful and that the samples covered all stages of the cell cycle. The western blot analysis of hnRNPA1 indicates that its overall levels are not strongly regulated during the cell cycle. Thus, whereas TERRA levels peak in G1 and are low levels in S phase (30), we do not see corresponding changes in hnRNPA1.

DISCUSSION

In this article, we investigate the combined effects of hnRNPA1 and TERRA on telomerase activity. Our studies with hnRNPA1 reveal that this protein does not perturb the catalytic activity of telomerase as long as it does not bind the DNA substrate. On primer binding, however, telomerase extension is blocked. Interestingly, this mechanism even functions when hnRNPA1 primer-binding sites are only generated on primer extension by telomerase. Thus, it seems that hnRNPA1, on binding the 5' end of the extended substrate, can promote the

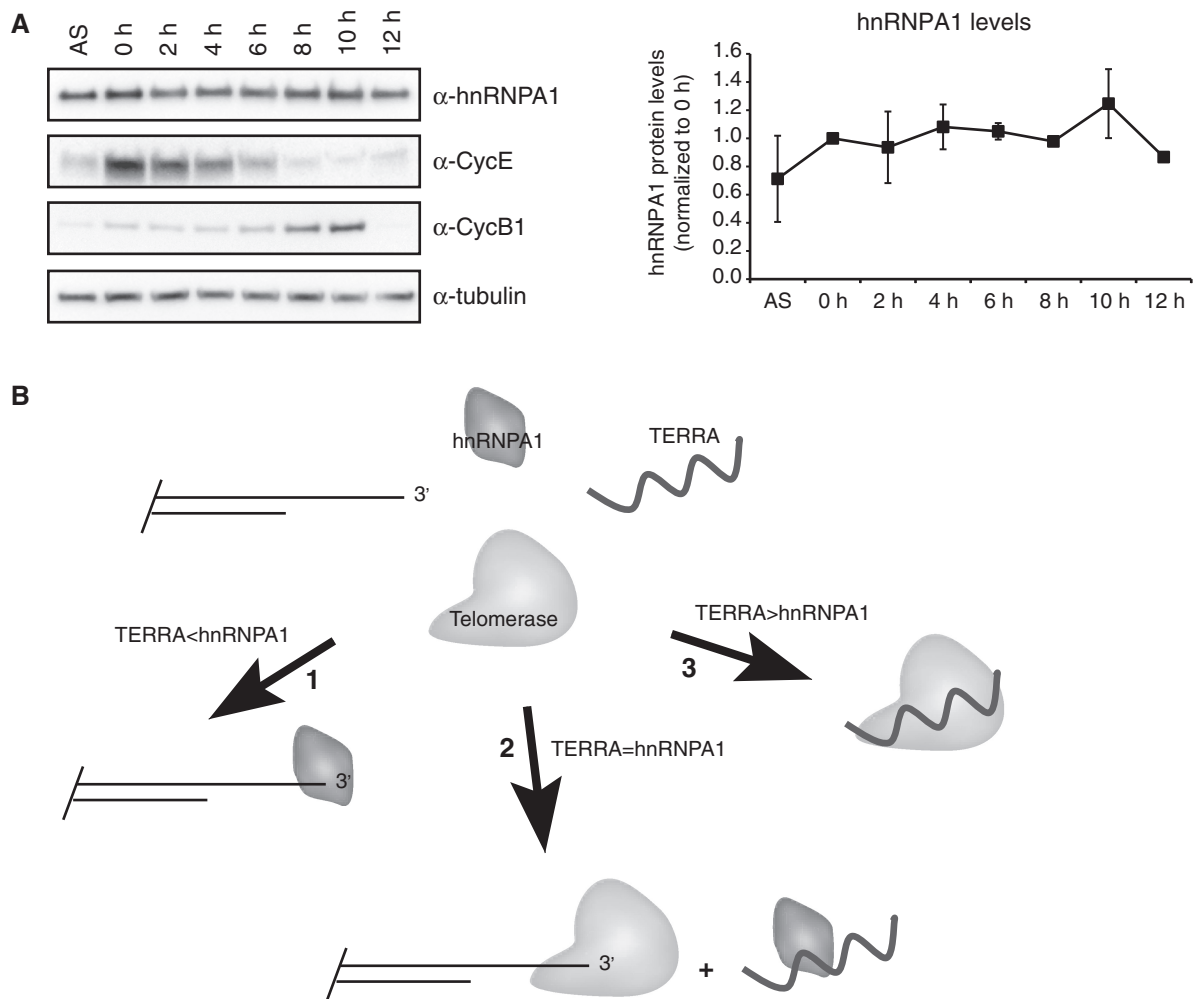


Figure 5. (A) hnRNPA1 levels do not vary throughout the cell cycle. Left panel: hnRNPA1 protein level in synchronized HeLa cells after release from a double thymidine block. AS: asynchronous cells, 0 h: before release. Cells were collected every 2 h (2, 4, 6, 8, 10 and 12 h). Protein content was analyzed by western blotting using the indicated antibodies. CyclinE and CyclinB1 were used as markers for cell synchronization. Right panel: hnRNPA1 levels were quantified and normalized to tubulin levels and to the 0 h sample. Error bars correspond to standard deviation of two independent experiments. (B) Model: telomerase is switched on or off depending on the local concentrations of telomerase, hnRNPA1, TERRA and free 3' telomeric single-stranded overhang. (1) TERRA < hnRNPA1: only a subset of hnRNPA1 is bound by TERRA and the remainder is bound to the 3' telomeric single-stranded overhang, inhibiting the access for telomerase. (2) TERRA = hnRNPA1: TERRA and hnRNPA1 form an inert RNP complex. Telomerase can act on the telomeric single-stranded 3' overhang. (3) TERRA > hnRNPA1: hnRNPA1-free TERRA will bind telomerase and inhibit its activity.

dissociation of telomerase from the 3' end which it is extending (Figure 3B). Alternatively, hnRNPA1 might not promote telomerase dissociation, but hold onto the elongating enzyme and prevent it from further extending the telomeric 3' end. Inhibition of telomerase at a distance has also been seen with the *S. cerevisiae* Cdc13p G-overhang telomere-binding protein (31). Human POT1, however, inhibits telomerase through sequestration of the 3' end of the substrate but it does not perturb telomere extension once telomerase has gained access to the DNA end (32,33). Our results on hnRNPA1 seem to rule out a previous proposal based on TRAP assays in extracts that hnRNPA1 would promote telomere elongation (26). However, our results do not address potential roles of hnRNPA1 in telomerase assembly (26).

We also find that hnRNPA1 and TERRA, which are partially co-assembled *in vivo*, can alleviate each other's effects on telomere elongation. Our *in vitro* results support

the existence of three states (Figure 5B). When hnRNPA1 is in excess over TERRA (state 1), it inhibits telomere elongation through binding of the DNA substrate (Figure 5B, reaction 1). On the other hand, when TERRA is more abundant than hnRNPA1 (state 3), TERRA can bind telomerase and thus inhibit its activity (Figure 5B, reaction 3). Only when both, hnRNPA1 and TERRA, are in equilibrium and form inert complexes (state 2), telomere extension by telomerase can occur (Figure 5B, reaction 2). It remains unclear under which conditions the proposed regulatory mechanisms may occur *in vivo*. We find here that hnRNPA1 is not cell cycle-regulated, whereas TERRA levels fluctuate strongly, being low during S phase (30). One could, therefore, argue that hnRNPA1 may restrict telomerase action by DNA end binding during S phase. However, the situation *in vivo* likely depends on the local concentrations of hnRNPA1 and TERRA at a particular telomere at a

particular time, factors for which we lack the resolution to experimentally address them. Further complexity is added to the *in vivo* situation, as hnRNPA1 may compete for telomere binding with several other single-stranded DNA-binding proteins like the telomere-specific POT1-TPP1 (34), the DNA replication protein RP-A (23,35) or the CTC1/STN1/TEN1 (CST) complex (36). Currently, it is not well understood how binding of these proteins occurs and how their exchange is regulated. TERRA seems well suited to promote the exchange of telomere-binding proteins that have affinity for both telomeric DNA and RNA (23), and TERRA may be regulated at individual telomeres to respond to local stress situations. In addition, it is possible that phosphorylation events or other post-translational modifications during the cell cycle or on DNA damage may regulate the recruitment or DNA-binding affinities of individual proteins. Whatever the exact mechanisms, our data demonstrate that hnRNPA1 can prevent TERRA-mediated inhibition of telomerase, providing a possible molecular explanation for why experimental overexpression of TERRA (15,16) may not be sufficient to inhibit telomerase *in vivo*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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