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A high-throughput assay for a human telomerase protein–human telomerase RNA interaction

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Abstract

The rapid rate at which cancer cells divide necessitates a mechanism for telomere maintenance, and in approximately 90% of all cancer types the enzyme telomerase is used to maintain the length of telomeric DNA. Telomerase is a multi-subunit enzyme that minimally contains a catalytic protein subunit, hTERT, and an RNA subunit, hTR. Proper assembly of telomerase is critical for its enzymatic activity and therefore is a requirement for the proliferation of most cancer cells. We have developed the first high-throughput screen capable of identifying small molecules that specifically perturb human telomerase assemblage. The screen uses a scintillation proximity assay to identify compounds that prevent a specific and required interaction between hTR and hTERT. Rather than attempting to disrupt all of the individual hTR–hTERT interactions, we focused the screen on the interaction of the CR4–CR5 domain of hTR with hTERT. The screen employs a biotin-labeled derivative of the CR4–CR5 domain of hTR that independently binds [^{35}S]hTERT in a functionally relevant manner. The complex between hTERT and biotin-labeled RNA can be captured on streptavidin-coated scintillation proximity beads. Use of 96-well filter plates and a vacuum manifold enables rapid purification of the beads. After optimization, statistical evaluation of the screen generated a Z' factor of 0.6, demonstrating the high precision of the assay.

Keywords: Telomerase; Telomerase RNA; Telomerase assemblage; Telomere; High-throughput screen; Scintillation proximity assay

The ends of human chromosomes are capped by telomeres, which are complexes composed of hexanucleotide repeats of DNA (TTAGGG/CCCTAA) and specific telomere-associated proteins. Telomeres function in part to protect chromosome ends from exonuclease activity and atypical recombination by preventing the DNA repair machinery from mistaking the chromosome ends for double-strand breaks [1]. The telomeres of most human cells shorten after each cell division as a result of the end replication problem [2,3]. However, some normal human cells (e.g., germline cells, activated lymphocytes, and proliferative cells of renewal tissues), as well as most cancer cells, are able to maintain the length of telomeric DNA even after multiple cell divisions [4–7]. Telomere extension is achieved by an RNA-dependent DNA polymerase called telomerase [8]. Telomerase is a ribonucleoprotein that is responsible for the synthesis of the G-rich strand of telomeric DNA. The telomerase holoenzyme is composed of a number of subunits; however, the minimal requirements of an active complex are the catalytic protein subunit (hTERT) and the RNA subunit (hTR) [9–11]. Using a discrete portion of hTR as a template, hTERT uses its reverse transcriptase activity to extend the G-rich 3′ strand of telomeres. Aside from providing the template, hTR also contains a number of distinct structural regions that are conserved among all vertebrates [12]. One such region is the highly conserved CR4–CR5 domain that interacts independently with hTERT in a functional manner [13–16] (Fig. 1A). Important to the function of the CR4–CR5 domain is the short p6.1 stem–loop that was identified by Chen and co-workers [13] and subsequently characterized by NMR [17] (Fig. 1A, inset).

Although active telomerase is naturally present in only a few specific nonsomatic cells, the vast majority of cancer

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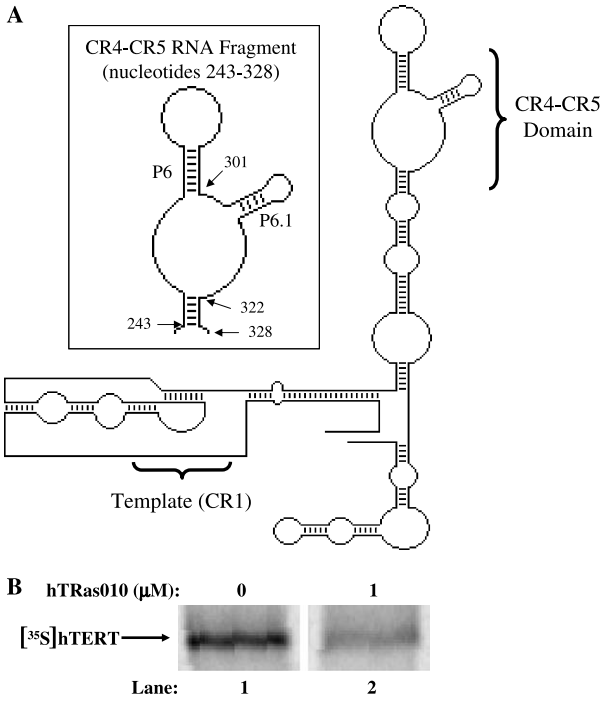

Fig. 1. (A) Proposed secondary structure of hTR. The template and CR4–CR5 domain are indicated. Inset: p6.1 stem–loop-containing CR4–CR5 RNA fragment (nucleotides 243–328) used in the SPA. Structure modified from Chen and co-workers [13]. (B) Effect of 1 μM hTRas010 on the affinity purification of [^{35}S]hTERT using biotinylated CR4–CR5 RNA when added prior to protein–RNA association.

cell types express active telomerase so as to maintain genetic stability throughout their unlimited replication. The loss of telomerase activity in telomerase-dependent cancer cells adversely affects telomeric integrity and results in senescence or cell death, thereby validating telomerase as an anticancer drug target [18]. In fact, the telomerase inhibitor GRN163L has entered phase I/II clinical trials for the treatment of chronic lymphocytic leukemia [19]. Past approaches toward affecting telomerase activity include, but are not limited to, targeting hTERT, hTR, telomerase enzymatic activity, and the telomere [20,21]. The use of dominant negative mutants [18,22] and the use of ribozymes [23,24] are common methods of hTERT-targeted telomerase inhibition, and hTERT-targeted immunotherapy is a more recent approach that is showing great promise [25]. Targeting hTR is carried out mainly through antisense interactions using various modified oligonucleotides, including phosphorothioate [26,27], 2′-*O*-methyl [26], 2′-*O*-(2-methoxyethyl) (2′-MOE)[1][27], N3′ → P5′ *thio*-phosphoramidate (NPS) [28], and peptide nucleic acid (PNA) [29]. The most potent antisense oligonucleotide inhibitors found to date target the template portion of hTR [19,27,28,30]. Targeting the telomere as an anti-telomerase approach has been achieved using compounds that bind and stabilize G quadruplexes at the end of the telomere. This class of telomerase inhibitors, which includes various derivatives of anthraquinones [31–33], acridines [34], porphyrins [35,36], perylenes [37–39], and ethidium [40], inhibits telomerase activity by preventing its access to the telomere or by binding the nascent DNA product. Recently, our laboratory determined that preventing proper telomerase holoenzyme assemblage is a viable approach to inhibiting the primer extension reaction catalyzed by telomerase [41]. We have advanced this approach by confirming that small molecules such as known nucleic acid-binding compounds can also adversely affect telomerase assembly and thus its activity [42].

To facilitate the discovery of telomerase inhibitors, several high-throughput methods have been developed. Originally, telomerase activity was detected using a direct assay that required large amounts of reagents, including partially purified telomerase from cell extracts and radioactive substrates. Issues related to the minute amounts of telomerase available from cultured cells were partially overcome by a PCR-based telomerase assay called the telomeric repeat amplification protocol (TRAP) [43]. By amplifying the telomerase extension products, the TRAP assay allows detection of very small amounts of telomerase activity, although without the proper controls the TRAP assay produces false positives due to *Taq* DNA polymerase inhibition [44]. Both the direct telomerase assay and the TRAP assay require gel analysis to visualize and quantify activity. To bypass the requirement for gel electrophoresis, Francis and Friedman used a biotinylated primer that was bound to streptavidin-coated 96-well plates after extension by telomerase with radioactive nucleotides [45]. They further modified this assay to use the nucleic acid stain Pico Green to detect the PCR-amplified double-stranded DNA products [46]. The use of streptavidin-coated 96-well PCR plates allowed rapid screening and inhibitor removal before the amplification step, thereby preventing false positives from inhibition of *Taq* polymerase during PCR [46]. Kha and co-workers [47] developed a similar high-throughput screen using streptavidin-coated plates; however, this assay quantified activity using enzyme-linked immunosorbent assay (ELISA)-based chemiluminescence, precluding the use of radioactivity or electrophoresis. Atha and co-workers [48] developed a high-throughput method to detect telomerase activity using a fluorescent-labeled primer, PCR-amplified products, and capillary electrophoresis. Savoysky and co-workers [49,50] developed a screen combining both TRAP and scintillation proximity assay (SPA) technology. In this assay, a biotinylated primer was used to amplify telomerase extension products with [^{3}H]thymidine as one of the dNTPs. Following extension and amplification, the products were captured on fluorophore-containing streptavidin-coated beads [49]. Notably, each of these screens reports directly on the enzymatic activity of telomerase.

[1] *Abbreviations used:* 2′-MOE, 2′-*O*-(2-methoxyethyl); NPS, N3′ → P5′ *thio*-phosphoramidate; PNA, peptide nucleic acid; TRAP, telomeric repeat amplification protocol; ELISA, enzyme-linked immunosorbent assay; SPA, scintillation proximity assay; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IGEPAL, nonyl phenoxy polyoxyethylene ethanol; BSA, bovine serum albumin.