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An RNA Mapping Strategy to Identify Ribozyme-Accessible Sites on the Catalytic Subunit of Mouse Telomerase

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Abstract

Telomerase reverse transcriptase (TERT) is an enzymatic ribonucleoprotein that prolongs the replicative life span of cells by maintaining protective structures at the ends of eukaryotic chromosomes. Telomerase activity is highly up-regulated in 85-90% of human cancers, and is predominately regulated by hTERT expression. In contrast, most normal somatic tissues in humans express low or undetectable levels of telomerase activity. This expression profile identifies TERT as a potential anticancer target. By using an RNA mapping strategy based on a *trans*-splicing ribozyme library, we identified the regions of mouse TERT (mTERT) RNA that were accessible to ribozymes. We found that particularly accessible sites were present downstream of the AUG start codon. This mTERT-specific ribozyme will be useful for validation of the RNA replacement as cancer gene therapy approach in mouse model with syngeneic tumors.

Keywords: cancer, gene therapy, group I intron, mTERT, RNA replacement, *trans*-splicing ribozyme, RNA mapping

A comparison with normal somatic tissues has shown that telomerase activity is up-regulated in the vast majority of human tumors. Particularly, expression of the catalytic subunit of telomerase, TERT, in cultured human primary cells reconstitutes telomerase activity and facilitates immortal cell growth (Bodnar *et al*., 1998). Furthermore, TERT-driven cell proliferation results in the activation of the c-*myc* oncogene (Wang *et al*., 2000), and TERT-activated telomerase can cooperate with other oncogenes to transform primary human cultured cells into neoplastic cells (Hahn *et al*., 1999). The effects observed in cultured cells suggested that telomerase upregulation, which occurs in >90% of all human tumors (reviewed in Shay and Bacchetti, 1997), may actively contribute to tumor growth (reviewed in Weitzman and Yaniv, 1999). Therefore, telomerase activity inhibition in tumor cells has been proposed as a potential approach for cancer therapy.

Recently, we described a new approach to human cancer gene therapy that relies on the *Tetrahymena* group I-based *trans*-splicing ribozyme. This ribozyme can specifically mediate RNA replacement of human TERT (hTERT) RNAs with new transcripts that exert cytotoxic effects, or cause up-regulation of suicide gene activity in the hTERT RNA-expressing cancer cells. These attributes enable the ribozyme to selectively impede the growth of telomerase-positive human cancer cells in cell cultures, and in xenotransplants in mice (Kwon *et al*., 2005).

A preclinical index which includes therapeutic effects, toxicities, and pharmacokinetics must be determined to evaluate the therapeutic potential of the hTERT-specific ribozyme in a clinical setting. An established animal model with syngeneic tumors would be useful in evaluating the ribozyme using standard immunological surveillance methods (Shi *et al*., 2001). To this end, we have developed mouse TERT (mTERT)-specific *trans*-splicing ribozymes by utilizing an RNA mapping method that identifies the most accessible ribozyme sites.

Mapping of the *trans*-splicing ribozyme

There is considerable flexibility in the selection of specific sites in target RNA for the *trans*-splicing ribozyme. This is because virtually any uridine (U) residue in a 5'exon can be targeted for splicing by altering the nucleotide composition of the 5' exon binding site on the ribozyme. This region is called the internal guide sequence (IGS), and it can be altered to make it complementary to a target sequence that is present on the substrate RNA (Sullenger, 1995; Long *et al*., 2003). However, only a limited number of uridines on the target RNA may actually be accessible to the ribozyme due to the complex, but stable tertiary configuration of the substrate RNA (Lan *et al*., 2000). Therefore, an RNA mapping strategy was performed to identify regions of the mTERT transcript that were accessible to ribozymes. The applied mapping methodology relied on RNA tagging (Jones *et al*.,1996) and a *trans*-splicing ribozyme library which has been described in detail elsewhere (Fig.1) (Lan *et al*., 1998; Lan *et al*., 2000; Ryu *et al*., 2003).

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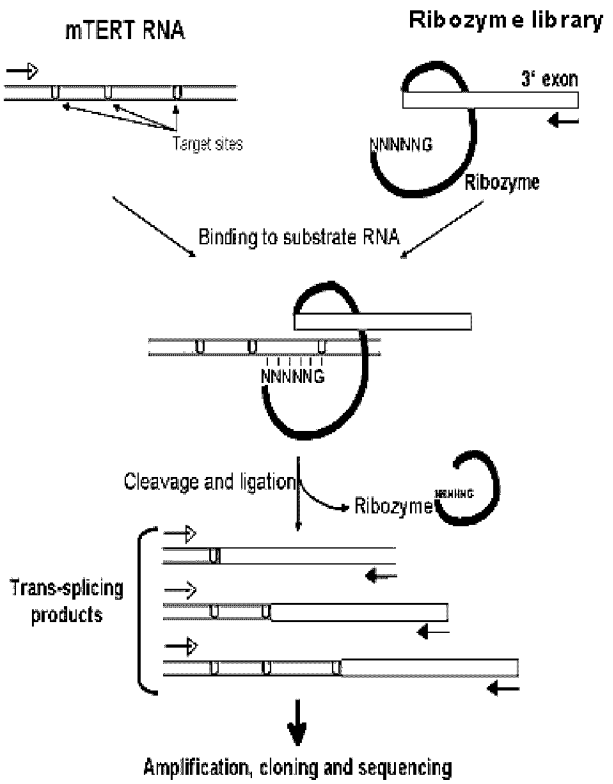

Fig. 1. A schematic representation demonstrating the methodology used for the mapping of accessible sites in mTERT RNA *in vitro* using a *trans*-splicing ribozyme library.

The construction of the GN5 ribozyme library used in this study was based on the *Tetrahymena* group I intron and contains a randomized IGS. Ribozymes in the GN5 library can bind to the substrate RNA and cleave at any accessible U residue, followed by the transference of a 3' exon to the end of the 5' target cleavage product. Part of the *lacZ* gene was used as a 3' exon molecular tag in the GN5 library that could be spliced onto the accessible target RNA's. In order to map the mTERT RNA, 10 nM of the GN5 library was incubated at 37°C for 3 h under splicing conditions (50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$) in the presence of guanosine (200 μM) and 100 nM of *in vitro* generated mTERT RNA. The *trans*-splicing reaction products were reverse-transcribed at 37°C for 30 min in the presence of argininamide (10 mM) with a 3' tagged primer that was specific for the ribozyme's 3' exon *lacZ* sequence (5'-ATGTGCTGCAAGGCGATT-3') (Jones *et al*. 1996). Following reverse transcription, the cDNAs were amplified by PCR for 30 cycles using the same 3' primer and a 5' primer that was specific for the 5' end of the target mTERT RNA (5'-GGGGAATTCGTCTAGAG CCACCGTCCA-3'). The amplified *trans*-splicing products were then cloned into the pUC19 vector and several clones were sequenced.

Ribozyme-accessible uridines in mTERT

Sequence analysis of the splicing junction sites revealed that several uridines that were present downstream of the AUG start codon (position 30 nt) appeared to be particularly accessible (Fig. 2). The most accessible site was located in the uridines at position 67 on the mTERT RNA. With the exception of position 67, most of the accessible sites identified using this mapping strategy were generally present in the stem region of the RNA's secondary structure, as predicted by computer modeling (Fig. 2). The relative *trans*-splicing efficiency at several sites on the target RNA in cells and *in vitro* has previously been shown to be closely correlated with the predicted accessibility determined by mapping studies (Ryu *et al*., 2003; Kwon *et al*., 2005). Therefore, we cannot anticipate ribozyme-accessible sites on the substrate solely by using the computer-predicted RNA structure.

A specific ribozyme targeting position 67 on the mTERT RNA (Rib67) was constructed by *in vitro* transcription of cDNA template. The pT7L-21 plasmid, which encodes a slightly shortened version of the natural group I intron from *Tetrahymena*, was used to create the template by PCR. The 5' primer contained the T7 promoter and the ribozyme's IGS, while the 3' primer was specific for the 3' exon sequence (Sullenger and Cech, 1994; Jones *et al*.,

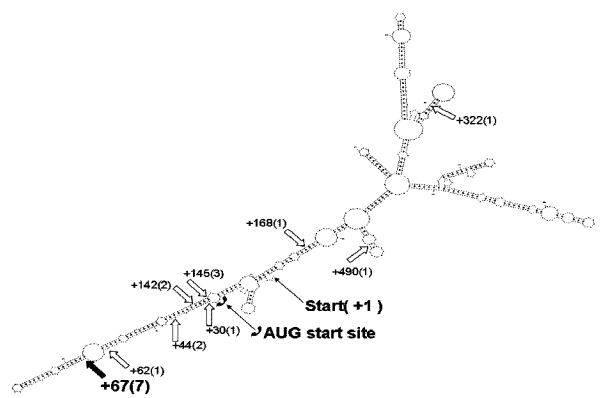

Fig. 2. The structure of the ribozyme-accessible sites in mTERT RNA. Nucleotide positions of the accessible uridines identified from *in vitro* mapping analysis are indicated by arrows and nucleotide numbers on the secondary structure of mTERT RNA as predicted by the mfold computer simulating program (Jager *et al*., 1989). The number of clones that contained a given uridine at the splice site is shown in parentheses. The AUG residue located at nt 30-32 denotes the initiator codon of the mTERT protein.

1996). The IGS on the L-21 *trans*-splicing ribozyme (5'-GGAGGG-3') was exchanged with 5'-GGAGAG-3' in Rib67 and the specific ribozyme (100 nM) was then incubated with the substrate mTERT RNA (10 nM) under splicing conditions. RT-PCR analyses were performed using a 3' primer specific for the 3' exon tag sequence and a 5' primer specific for mTERT RNA. The amplified fragment corresponded to the expected size of 165 bp using the mTERT RNA and Rib67 (data not shown). Sequence analyses of the amplified spliced products demonstrated that Rib67 correctly *trans*-spliced its 3' exon tag onto the mTERT target RNA at the predicted reaction site (Fig. 3). From these results we conclude that specific ribozyme targeting of predicted accessible sites can replace mTERT RNA with a 3' exon tagged at the 3' end of the ribozyme with high reliability by using targeted *trans*-splicing. This mTERT-specific ribozyme will be useful for validation of RNA replacement as an approach to cancer gene therapy in syngeneic mouse models.

There are major differences in the dynamics and regulation of telomerase in mouse and human cells. For example, the telomeres of laboratory mice are significantly longer (40-60 kb) than those in human (10 kb), and telomerase is widely expressed in adult mouse tissues (Newbold, 1997; Greeberg *et al*., 1998). The use of inhibitors such as *trans*-cleavage ribozymes, antisense oligonucleotides and siRNA to repress mouse telomerase expression may be limited in their use with mouse tumors. This is due to the lag phase that occurs between the time of telomerase inhibition and the time at which the telomeres actually become short enough to cause harmful effects associated with cancer growth. However, the *trans*-splicing approach will overcome the lag phase because it can reduce the TERT level and can simultaneously induce cytotoxicity in the TERT-expressing cancer cells. Moreover, cancer- or condition-specific expression of the mTERT-targeting ribozyme could improve the specificity of tumor cytotoxicity (Song and Lee, 2006).

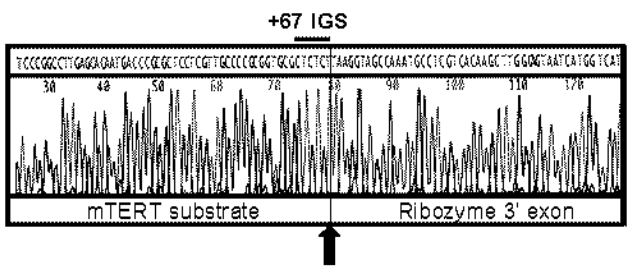

Fig. 3. A representative sequence of the *trans*-spliced transcripts of Rib67. The expected sequence around the splicing junction is indicated with an arrow, and the ribozyme recognition sequence in mTERT RNA indicated with a line.

Recently, many gene therapy protocols based on inhibitory RNA or short oligonucleotides have been described. A key factor that influences the efficiency of RNA-mediated cancer suppression is the accessibility of the substrate RNA for binding of inhibitory elements. Mapping studies, such as those described here, could be used generally in a wide range of applications for optimizing intracellular anti-TERT activity with various inhibitory RNAs. Furthermore, the mapping methodology that was developed in this study could potentially be used to identify the most accessible sites in other unique tumor-associated RNAs or viral RNAs for the development of therapeutic strategies for treating malignant or infectious diseases.

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