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Inhibition of the DNA binding by the TCF-1 binding RNA aptamer

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Abstract

The DNA binding architectural protein, TCF, and the transcriptional activator, β-catenin, form a complex that regulates the expression of diverse target genes during early development and carcinogenesis. As an approach to modulating transcription by this complex, we selected an RNA aptamer that binds to the DNA binding domain of TCF-1. The aptamer interfered with the binding of TCF-1 to its specific DNA recognition sequences in vitro and also inhibited DNA binding of cellular TCF-1. We also developed the truncated version of the aptamer for efficient delivery to the cells. Structural analysis of the truncated aptamer revealed that a stem–loop with an internal loop was responsible for the binding to TCF-1. Similar approach may well be applicable to other proteins, especially DNA binding transcription factors, in order to modulate their DNA binding and transcriptional activity in the cells.

Keywords: TCF-1; RNA aptamer; HMG domain; DNA binding

T-cell factor-1 (TCF-1) was originally identified as a T-cell specific transcription factor that bound to specific DNA through its high mobility group-1 (HMG-1) DNA binding domain [1–3]. Even though transgenic and knockout approaches suggested that TCF-1 was likely to be involved in the expansion of T-lymphocytes, exact functions of the TCF-1 protein in T-cell development need to be understood [4–8]. TCF family proteins bind to DNA in a sequence-specific manner and they seem to act as architectural proteins for the assembly of other transcription factors [9]. Identification of β-catenin as a potent transcriptional co-activator of TCF family proteins led to a greater understanding of their function [10,11]. Since it is highly expressed in various cancer cells, it seemed possible that the formation of a transcriptional complex by an oncogenic β-catenin with TCF might be a central event in cancer cell development [12–14]. The TCF/β-catenin protein complex is also a critical regulator of early developmental events such as axis formation in the *Xenopus* embryo and Wingless signaling in *Drosophila* [15–17]. In addition, it was recently shown that the TCF/β-catenin complex mediating Wnt signaling seems to be an important pathway in immature thymocyte development [18–20]. Interestingly, the specific isoform of TCF-1 that bound to β-catenin was the only protein effective in ensuring the survival of immature thymocytes [21]. These findings began to point to the role of TCF family proteins as critical modulators of the expression of genes that control the decision between proliferation and apoptosis [22,23]. For example, TCF/β-catenin transcribes genes implicated in cancer development, such as cyclin D1 and c-myc [24,25].

A specific tool for modulating such transcription, either by inhibiting DNA binding or by disrupting protein–protein interactions, would be useful for understanding such complex phenomena.

High affinity molecules, such as nucleic acid ligands, can modulate the transcriptional activity of transcription factors. Reiterated in vitro selection procedures

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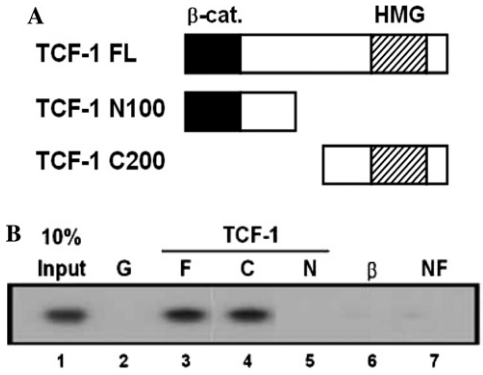

Fig. 1. Binding of RNA aptamer #10 to the DNA binding domain of TCF-1 protein. (A) Recombinant GST TCF-1 proteins used in this study. (B) Protein specificity of RNA aptamer #10. GST pull-down analysis was performed with labeled RNA aptamer #10 (100 pM) and diverse GST fusion proteins (500 nM). G, GST protein; F, full-length TCF-1 protein; C, TCF-1 C200; N, TCF-1 N100; β, β-catenin; and NF, NFAT protein.

are able to select specific RNA molecules from random RNA library, and nucleic acids selected by this procedure are generally referred to as 'aptamers' [26,27]. Because of the large size of RNA libraries (10^{14}–10^{15} molecules) and the ease of generating RNA molecules by in vitro enzymatic reactions, RNA libraries are superior to other biological or synthetic libraries for selecting high affinity aptamers [28], especially for those recognizing nucleic acid binding proteins such as TCF-1 transcription factor.

We previously reported the in vitro selection of RNA aptamers that bind to TCF-1, without giving further details of their function [29]. Here we have extended our study and shown that one of the selected RNA aptamers (RNA aptamer #10) binds to the TCF-1 protein containing HMG domain, thereby interfering with its binding to DNA. Further refinement of this aptamer could provide a way for cancer cell gene therapy.

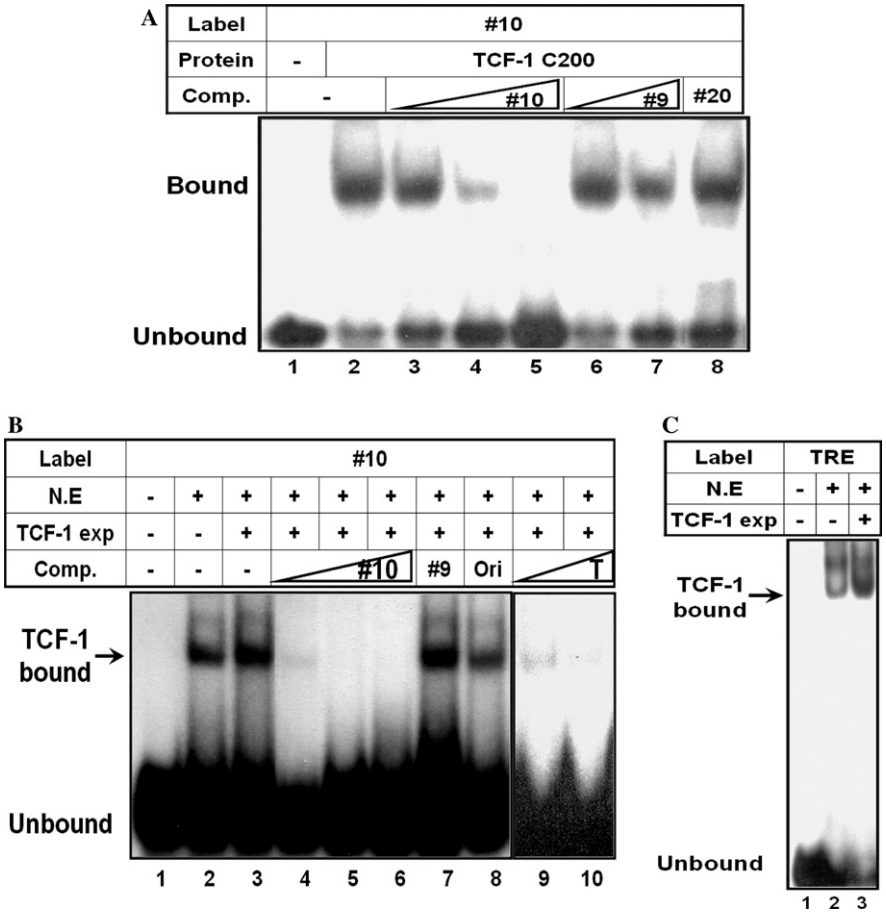

Fig. 2. Specific binding of RNA aptamer #10 to TCF-1 protein. (A) RNA-EMSAs were performed with labeled RNA aptamer #10 (500 pM) and TCF-1 C200 protein (500 nM) on its own, and in the presence of excess unlabeled RNA aptamer #10 (5, 50, and 500 nM), RNA #9 (50 and 500 nM), and RNA #20 (500 nM) as competitors. (B) Specific binding of RNA aptamer #10 to the cellular TCF-1 protein. RNA-EMSA was performed with labeled RNA aptamer #10 (100 pM) and 293T nuclear extract (N.E, 7 μg) in the presence of competitors. Lane 1, no extract; lane 2, vector transfected cells; lane 3, TCF-1 cDNA transfected cells; and lanes 4–8, with non-labeled RNA competitors, RNA aptamer #10 (20, 200, and 500 nM), RNA #9 (500 nM), and original RNA (Ori, 500 nM). The identity of the bound band is confirmed by the competition with the TCF-1 binding oligonucleotide, TRE (T, 100 nM and 500 nM, lanes 9–10). (C) Confirmation of the identity of the bound protein. EMSAs were performed with the TCF-1 binding oligonucleotide, TRE (20 nM), and nuclear extract (N.E) from 293T cells. Lane 1, no extract; lane 2, vector transfected cells; and lane 3, TCF-1 cDNA transfected cells. The TCF-1 bound band is indicated.

Materials and methods

Plasmids, proteins, and reagents. We introduced the complete coding region of mouse TCF-1 into the *Eco*RI site of the vector pcDNA3.1 [30]. Bacterial expression vectors for recombinant TCF-1 proteins were obtained by cloning TCF-1 by PCR (amino acids 188–388 for C200; amino acids 5–100 for N100; and amino acids 5–388 for full-length TCF-1 protein) with primers containing *Eco*RI (5′ primer) and SalI (3′ primer) restriction sites. PCR products were digested with *Eco*RI and *Sal*I, and cloned into the pGEX 4T-1 plasmid. The procedures for purifying GST-TCF-1 proteins, GST-β-catenin and $(His)_6$-NFAT-1, have been described previously [29,31].

RNA binding assays. In vitro selection procedure for selecting RNA molecules binding to the HMG domain of TCF-1 has been previously described [29]. To test for RNA binding, the RNA was radiolabeled, incubated with GST-fused proteins, and assayed by GST pull-down. RNA–protein complex was precipitated with glutathione–Sepharose 4B bead, bound RNA was eluted, and electrophoresed onto 6% polyacrylamide/7 M urea gel and autoradiography. RNA-EMSA was also performed with radiolabeled RNA. RNA–protein complexes were separated on 5% native polyacrylamide gel in 1× TBE buffer at 150 V for 4 h. The gels were dried and subjected to autoradiography.

Preparation of nuclear extracts. Human embryonic kidney 293T cells and murine immature thymoma cell line S49.1 (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics. S49.1 cells (1×10^8 cells) and 293T cells (1×10^7 cells) were harvested and washed twice with ice-cold PBS. Cell pellets were resuspended in 200 μl ice-cold buffer A supplemented with the protease inhibitor cocktail and the phosphatase inhibitor cocktail (Sigma–Aldrich). After incubating for 15 min on ice, cells were lysed with 0.5% NP-40 and gently vortexed. Cell lysates were centrifuged at 4000 rpm at 4 °C for 30 s. Pellets were resuspended in 50 ml ice-cold buffer B. After incubating for 30 min on ice, debris was pelleted by centrifugation. Protein concentrations in the cleared nuclear extracts were measured by the Bradford assay (Bio-Rad).

Electrophoretic mobility shift assay. Double-stranded TRE oligonucleotide (5′-GGTAAGATCAAAGGG-3′) was synthesized by Bioneer. DNA was end-labeled with 20 U of T4 polynucleotide kinase and 50 μCi [γ-^{32}P]ATP, and purified on a G-50 Sephadex spin column, followed by phenol extraction and ethanol precipitation. To test for TCF-1 binding, nuclear extracts (5.25 μg of S49.1) were incubated with radiolabeled RNA or DNA at room temperature for 30 min in the presence of 0.2 μg poly(dI–dC). DNA–protein complexes were

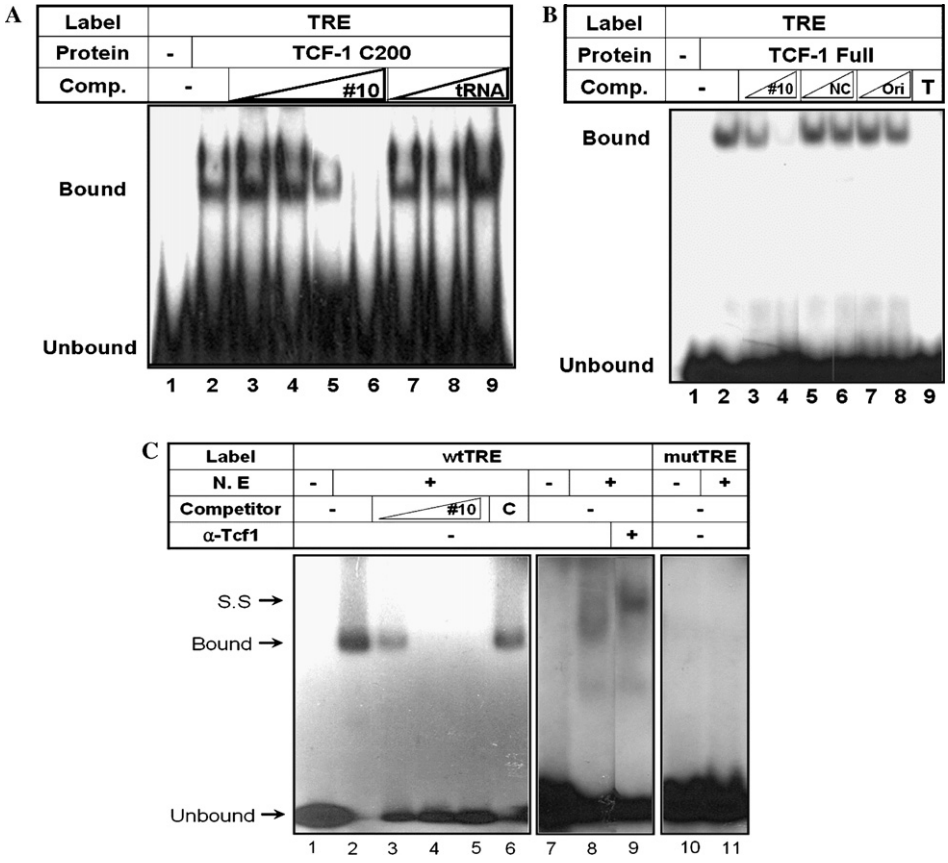

Fig. 3. Inhibition of DNA binding by RNA aptamer #10. (A) Inhibition of DNA binding to TCF-1 C200 protein. TRE (20 nM) was end-labeled and incubated with TCF-1 C200 protein (200 nM). Unlabeled RNA aptamer #10 or tRNA was added to the mixtures and resolved on a 5% polyacrylamide gel. Lane 1, labeled DNA only; lane 2, binding in the presence of TCF-1 C200 protein; lanes 3–6, with addition of 2, 20, 200, and 1000 nM of unlabeled #10; and lanes 7–9, with 20, 200, and 1000 nM tRNA. (B) Specific inhibition of DNA binding to TCF-1 full-length protein. TRE (20 nM) was end-labeled and incubated with full-length TCF-1 protein (400 nM) in the presence of various competitors. Unlabeled RNA aptamer #10, RNA aptamer for nucleocapsid protein (NC), and original RNA library (Ori) were used as competitors. Lanes 3–8, 100 and 1000 nM of competitors, respectively. Unlabeled TRE (T) was also used to show the band specificity. (C) Inhibition of the DNA binding activity of nuclear TCF-1 protein of immature S49.1 immature thymocytes. Wild-type (lanes 1–9) and mutant (lanes 10–11) TRE DNA (20 nM) was end-labeled and incubated with S49.1 nuclear extract (7 μg). Various concentrations of non-labeled RNA aptamer #10 (lanes 3–5; 50, 500, and 1000 nM) and Ori RNA (lane 6, 1000 nM) were added to the DNA–protein complex. Supershift with an anti-TCF-1 antibody was also performed (lane 9).

separated on a 5% native polyacrylamide gel in 1× TBE at 150 V for 4 h and visualized by autoradiography.

RNase footprinting analysis. In vitro transcribed RNA was dephosphorylated with calf intestine phosphatase (New England Biolabs) and labeled with [γ-^{32}P]ATP (Amersham). Labeled RNA (62 nM) was denatured and renatured in binding buffer and various concentrations of TCF-1 protein were added and incubated at 37 °C for 10 min. The RNA–protein complexes were incubated with RNase T1 (0.02 U) or RNase S1 (3 U) at 37 °C for 10 min, or with RNase V1 (0.01 U) at room temperature for 15 min. Nucleotide ladders were generated by alkaline hydrolysis in 50 mM $NaHCO_3$ (pH 9.0), 1 mM EDTA, and 0.25 mg/ml tRNA for 15 min at 90 °C. After ethanol precipitation of samples, the pellets were resuspended in loading dye, denatured for 5 min at 65 °C, and immediately placed on ice. Samples were loaded on 15% polyacrylamide/7 M urea gels in 1× TBE for 4 h at 1500 V. The gels were dried, exposed to X-ray film, and analyzed with a PhosphorImager (FUJIX Bio Image Analyzer System). The radioactivity of each band was measured with Image Gauge 3.3 software, and a percentage of the total radioactivity was assigned to each band. Differential cleavage was calculated as log(fa) − log(fc), where fa is cleavage in the presence of TCF-1 protein, and fc, cleavage in its absence.

Results

Specific binding of RNA aptamer #10 to the DNA binding domain of TCF-1 protein

We have previously described our scheme for selecting RNA aptamers (100 nucleotides) for the C-terminal 200 amino acids of TCF-1 protein (TCF-1 C200 in Fig. 1A) which contains the DNA binding HMG domain [29]. We chose to study one of the selected RNA aptamers, #10 (K_d was around 100 nM). Since RNA aptamer #10 was selected with a fragment of the TCF-1 protein (C200), we tested whether it also bound to full-length TCF-1 (TCF-1 FL). As shown in Fig. 1B, it indeed bound to full-length TCF-1 (FL, F) as well as to its C-terminal region (C200, C), but did not bind to its N-terminal β-catenin-binding domain (N100, N). Moreover, it did not bind to other proteins, such as β-catenin or the DNA binding domain of NFAT (NF).

We performed competition experiment to examine the binding specificity of RNA aptamer #10 for TCF-1 protein. We observed a gradual decrease in the strength of the bound band as the amount of unlabeled self RNA was increased; at the same time, non-binding RNA molecules (#9 and #20) did not compete for binding of RNA aptamer #10. We next showed that the aptamer bound to a cellular protein from 293T cells that had been transfected with TCF-1 cDNA (Fig. 2B). The RNA-bound protein band was shown to be TCF-1 by two methods. First, a competition experiment was performed with the DNA oligonucleotide (TRE, TCF responsive element) containing the TCF-1 binding consensus sequences (A/T A/T CAAAG). This showed that, as expected, the RNA-bound band was competed by TRE. Second, we performed an EMSA with the TRE. When TCF-1 cDNA was introduced into 293T cells, the intensity of the bound band increased (compare lanes 2 and 3, Fig. 2C). A similar pattern of band enrichment was also shown in an EMSA with RNA aptamer #10 (Fig. 2B), and the binding was specific since it was competed by unlabeled aptamer #10, but not by the non-binding RNA #9 or the original RNA pool (Ori).

Inhibition of DNA binding by TCF-1

Since RNA aptamer #10 bound to TCF-1 C200 protein which contained the DNA binding domain, we tested whether the aptamer interfered with DNA binding by TCF-1. We labeled the TRE oligonucleotide and performed EMSA with TCF-1 proteins. Binding of TRE to TCF-1 C200 protein was gradually competed by increasing concentration of unlabeled RNA aptamer #10, but not by non-specific tRNA (Fig. 3A). We also observed the specific inhibition of TRE binding to TCF-1 full-length protein by RNA aptamer #10 (Fig. 3B). Other RNAs, such as HIV-1 nucleocapsid (NC) protein binding RNA aptamer [31] or original RNA library, did not compete for the binding. It was also noted that order of incubation did not affect the inhibition.

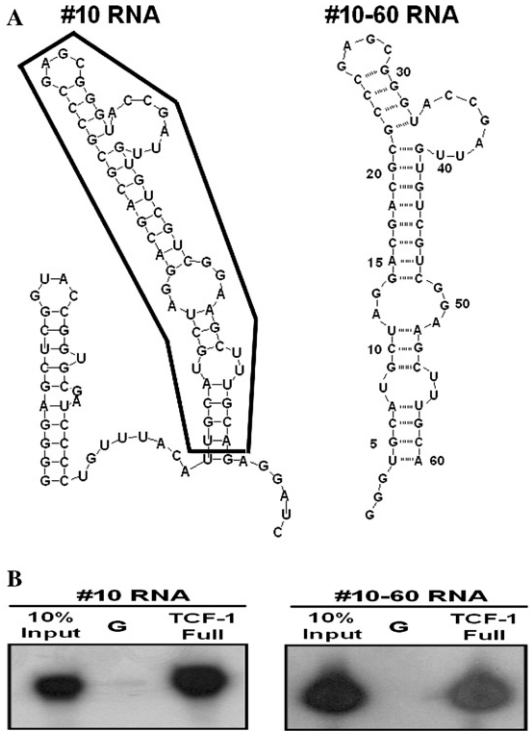

Fig. 4. Truncation of RNA aptamer #10 to #10–60 RNA. (A) Predicted secondary structures of RNA aptamer #10 and truncated aptamer #10–60. RNA #10–60 was designed to fold into the same structure as boxed in the structure of RNA aptamer #10. (B) GST pull-down-assay to estimate the binding of RNA aptamer #10 and truncated RNA #10–60 molecules to full-length TCF-1. 180 pM of each labeled RNA was incubated with GST (G, 500 nM) or full-length GST TCF-1 (TCF-1 Full, 300 nM). The RNA was eluted and subjected to 6% polyacrylamide/7 M urea gel electrophoresis.

The inhibitory effect of RNA aptamer #10 on DNA binding was also tested with nuclear TCF-1. We prepared the nuclear extract from the S49.1 T lymphoma cell line, which has characteristics of CD4/CD8 double negative immature thymocytes, has a high level of TCF-1, and may be regulated by TCF signaling [32]. The extract was incubated with radiolabeled TRE and analyzed by an EMSA (Fig. 3C). Excess RNA aptamer #10 inhibited binding to TRE DNA, whereas the non-binding RNA molecules did not. The specificity of the bound band was confirmed by the supershift with anti-TCF-1 antibody and by the absence of mutant TRE bound band. Evidently the selected RNA aptamer #10 inhibits DNA binding by TCF-1 both in vitro and in the cells.

Structure of the truncated RNA aptamer

To understand the molecular basis of the interaction between TCF-1 and the RNA aptamer, we used the program mfold to predict the secondary structure of the

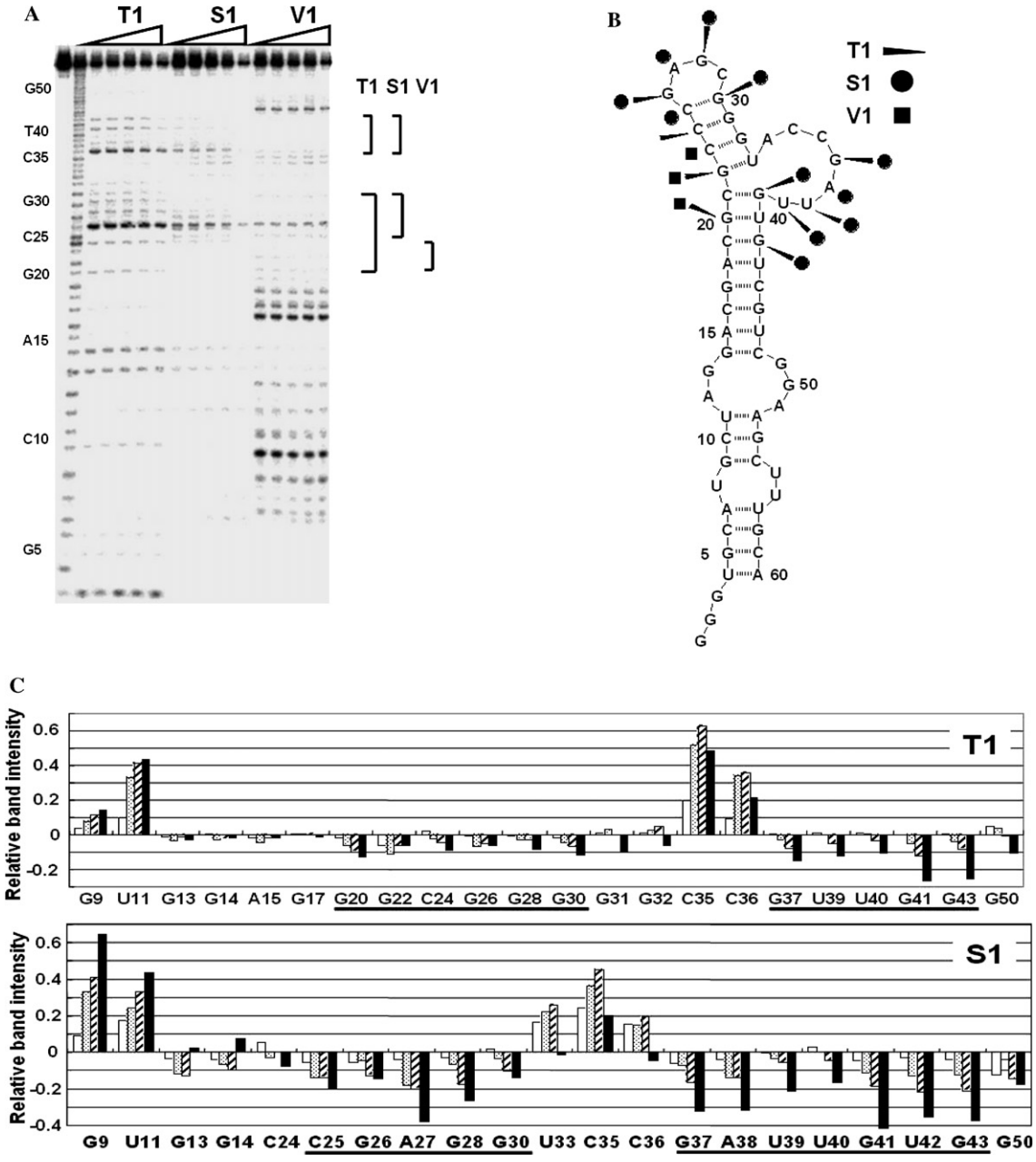

Fig. 5. Determination of the TCF-1 binding sites of RNA #10–60. (A) #10–60 RNA was end-labeled and incubated with various concentrations of TCF-1 C200, followed by digestion with RNase T1 (T1), RNase S1 (S1) or RNase V1 (V1). Lane 1, no treatment; lane 2, alkaline hydrolysis; and lanes 3–7, RNA: protein ratios for the RNase treated samples were 1:0, 1:1, 1:2, 1:5, and 1:10. Nucleotide positions are indicated on the left side of the gel. RNase-protected nucleotides are marked by solid lines. (B) Diagram of the secondary structure of RNA #10–60. The protected bases are indicated by triangles (T1), circles (S1), or squares (V1). (C) Relative differential cleavage by RNase is shown for each nucleotide. The RNase-protected region is denoted by solid lines.

aptamer (Fig. 4A). Although RNA aptamer #10 has defined sequences at its ends (at positions 1–15 and 84–100), the predicted structure consisted of a long stem–loop with an internal loop and two small bulges that involved most of the selected sequence as well as the defined 3′ sequence. We therefore decided to generate a truncated version of the RNA aptamer (#10–60) with 60 nucleotides that contained the same stem–loop structure (spanning nucleotides 37–93). This truncated aptamer retained the ability to bind to TCF-1, as shown by the GST pull-down assay in Fig. 4B.

We next mapped the binding sites of aptamer #10–60 by RNase footprinting analysis (Fig. 5). The TCF-1 binding site was mapped by identifying sequences protected from RNases (G nucleotide-specific RNase T1, single strand-specific RNase S1, and double-strand-specific RNase V1) upon binding to TCF-1. In the event, nucleotides 20–43 that form the double-stranded stem and a internal loop were found to be the major binding site for TCF-1 protein (Fig. 5B). Quantitative analysis of each band showed that nucleotides 20–30 and 37–43 were strongly protected from RNase T1 and S1 (Fig. 5C). Interestingly, these nucleotides form a more complex tertiary structure, as shown above. We may conclude that the double-stranded stem–loop with an internal loop seems to be responsible for the binding to TCF-1.

Discussion

Transcription factors are known to be exceptionally useful targets for therapeutics [33]. Antisense oligonucleotides or small interfering RNAs can be used to reduce transcription of a given protein [34], but in order to selectively inhibit particular functions of a transcription factor, the protein itself must be the target. Nucleic acid aptamers can achieve this, because of their high affinity and specificity for the nucleic acid binding pockets of such proteins. In fact, an RNA library selection procedure has been previously used to select an RNA aptamer for NF-κB transcription factor [35,36]. We previously reported the selection of the RNA aptamer that disrupted the protein–protein interaction between TCF-1 and its activator β-catenin [37]. Here we report the first characterization of an RNA aptamer that inhibits the binding of the transcription factor TCF-1 to its cognate DNA sequences. We expect that these aptamers could inhibit the TCF-1 mediated signaling by modulating the expression of TCF-1 target genes in mammalian cells if they are efficiently delivered or expressed to the cells. It is also expected that the TCF-1 binding aptamers could shed light on the development of anti-cancer therapeutics as well as on the mechanistic understanding of the roles of TCF-1 in T-cell development.

Acknowledgments

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