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Analysis of Nucleic acid Binding by a Recombinant Translin-Trax Complex[1]

Matthew Lluis, **Warren Hoe**, **Jennifer Schleit**, and **Jon Robertus**
Institute for Cellular and Molecular Biology, Department of Chemistry and Biochemistry, 1 University Station A5300, University of Texas, Austin TX, 78712

Abstract

Translin is a highly conserved mammalian RNA and DNA-binding protein involved in DNA recombination and RNA trafficking. Crystal structures of mouse and human translin have been solved, but do not provide information about nucleic acid binding or recognition. Translin has a partner protein, translin-associated factor x (trax), which is believed to regulate translin's subcellular locale and affinity for certain RNA and DNA sequences. Here we present a comparative study of recombinant translin and translin•trax complex binding to specific RNA and DNA sequences. It was observed that translin preferentially binds to G-rich RNA sequences whereas translin•trax preferentially binds G-rich DNA sequences. Translin can bind mRNA sequences with submicromolar K_d values, and the complex with trax can bind G rich DNA with similar affinity. We conclude that trax acts to regulate translin's RNA and DNA binding affinities as part of a cellular RNA trafficking mechanism.

Keywords

translin; trax; nucleotide binding; polarization assay

Introduction

Translin, a 26 KDa human protein consisting of 228 amino acids, was named through its association with chromosomal translocations [1]. Translin bound to DNA sequences at breakpoint junctions of translocations associated with lymphoid malignancies. DNA binding was mediated by at least one of two conserved basic regions of the peptide chain [2]. Defining a consensus DNA recognition sequence for translin has proven difficult [3], although translin can bind poly single stranded d(GT) sequences with a K_d of 2 nM [4].

Hecht and coworkers' work led to the discovery of a protein, which they named Testis Brain RNA-Binding Protein (TB-RBP). TB-RBP proved to be the mouse ortholog of translin; in this paper we will refer to TB-RBP as translin. Translin appears to target specific protamine-2 mRNA sequences called Y and H elements [5].

Electron microscopy studies showed translin exists as a cyclic octamer, with a large central cavity that can by filled with either RNA or DNA [6]. Analytical ultracentrifuge data confirmed that translin exists as an octamer in solution [7]. These data also revealed that the

Correspondence to: Jon Robertus.

octamer bound a single 24-base ssDNA fragment with a K_d of 84 nM [7]. Our laboratory solved the structure of mouse translin (TB-RBP) to 2.65 Å resolution [8]. The protein assembled around a pseudo four-fold axis and then dimerized to an octamer in the shape of a prolate ellipsoid. The crystal form had the putative RNA binding residues inside and therefore appeared to be an intermediate assembly of translin not capable of binding extended nucleic acids. An X-ray structure was subsequently solved for human translin and was found to be essentially identical to the mouse protein structure [9].

A yeast two-hybrid study led to the discovery of a heteromeric protein complex formed by translin and another partner protein, translin-associated factor x (trax), a 33 KDa translin homolog [10]. Immunoprecipitation experiments showed that the translin•trax heteromeric complex, and not the translin homomeric complex, is the predominant cellular species [11]. Transgenic experiments in mice showed that deletion of the translin gene is coupled to the absence of trax protein from cellular extracts, even though northern blot analysis confirmed normal levels of trax mRNA [12]. This suggests that translin serves as a chaperone to stabilize trax in the cell [13].

Trax contains a nuclear localization signal (NLS) but cannot bind DNA or RNA on its own [13]. In this study, our laboratory set out to compare the nucleic acid-binding properties of recombinant translin homomeric complex and translin•trax heteromeric complex in an attempt to determine what role trax has in regulating translin's RNA and DNA binding activity.

Materials and Methods

Expression and purification of translin

The mouse (*M. musculus*) translin gene was previously cloned into pGEX-4T-1 (GE Healthcare, Piscataway, NJ), a glutathione-S-transferase fusion vector containing a thrombin cleavage site for removal of GST [14]. This construct was transformed into *E. coli* strain BL21(DE3) under the selection of ampicillin. Protein was purified essentiallly as described earlier [14].

Cloning, expression and purification of the translin•trax complex

Plasmids containing the mouse (*M. musculus*) translin gene, pGEX-4T-1-translin, and the mouse (*M. musculus*) trax gene, pET28a-trax, were generously provided by the Hecht laboratory (University of Pennsylvania Medical Center, Philadelphia, PA). We cloned both the translin and trax genes, under separate promoters, into the pETDuet-1 (Novagen, Madison, WI) co expression plasmid. Translin was first inserted into pETDUET-1, and then the 873 bp trax *BamHI* and *HindIII* fragment was ligated into the *BamHI* and *HindIII* restriction sites of the pETDUET-1-translin vector. Both translin and trax inserts were completely sequenced at the DNA Sequencing at the University of Texas DNA Analysis Core Facility (Austin, TX) to confirm that no PCR-induced mutations had occurred.

The pETDUET-1-translin•trax vector was transformed into *E. coli* strain Rosetta 2 (DE3) under the dual selection of ampicillin and chloramphenicol. Fresh transformants were grown in Terrific Broth (Difco) media at 37 °C until reaching an optical density of 0.6 and induced with 1 mM IPTG (Gold Biotechnology, St. Louis, MO). The cultures were grown for an additional 3 hours post induction at 25 °C. Cells were harvested by centrifugation for 10 min at 4000g, resuspended in lysis buffer (25 mM Tris-HCl pH 8, 500 mM KCl,10 mM Imidazole), and run through a French Pressure cell three times at 1000 psi. The cell lysate was then centrifuged at 100,000g for 1 hour and passed through a 2 μM filter before being passed over a column of NTA His Bind resin (Novagen, Madison, WI) that was equilibrated with lysis buffer. The translin•trax complex was eluted with a linear gradient from 10 mM to

250 mM Imidazole and eluted fractions were analyzed by SDS page. Protein containing fractions were pooled together and dialyzed against storage buffer, concentrated, and stored at 4 °C.

Gel filtration analysis of the translin•trax complex

Gel filtration experiments of the recombinant translin•trax complex were carried out using a HiPrep™ 26/60 Sephacryl S-300 High Resolution column and an AKTA FPLC system (GE Healthcare, Piscataway, NJ). A 1 mL sample of the translin•trax complex was concentrated to 20 mg/mL and run through the gel filtration system in 25 mM Tris, pH 7.5, 150 mM NaCl buffer at a flow rate of 1mL/min. The molecular weight of the native complex was calculated from the linear regression analysis of a calibration curve of K_{av} vs. log[molecular weight], where K_{av} = (protein elution volume – column void volume)/(column bed volume – column void volume), that was constructed using the elution profiles of the following protein standards and their respective molecular weights: Blue Dextran (2000 KDa), thyroglobulin (669 KDa), ferritin (440 KDa), aldolase (158 KDa), conalbumin (75 KDa), and ovalbumin (43 KDa). The majority of the translin•trax complex eluted at a volume that correlates to an apparent molecular weight of ~ 340 KDa.

Fluorescence polarization assays

Fluorescent-labeled polynucleotides were titrated with binding proteins (translin or translin•trax); as binding increases, fluorescence polarization (FP) increases. Serial dilutions of stock protein samples were prepared using storage buffer as the diluent. For each 30 μL sample, 3 μL of 10X binding buffer (250 mM Tris-HCl, pH 8, 1 M NaCl, 10 mM $MgCl_2$) was combined with a stock fluorescent nucleotide solution and a balancing volume of stock protein sample to give a final nucleotide concentration of 5 nM. The eight nucleic acid ligands used are defined in Table 1. All eight of these ligands had 5′ fluorescein modifications. The 39nt PRM-2 sequence was taken from the 3′ UTR of the protamine-2 mRNA and represents an *in vivo* target of translin. Prior to addition of the nucleotide to the protein, sample nucleotide solutions were heated to 70 °C and cooled at 25 °C for 3 minutes. 20 μL of each protein-nucleotide sample was added to a 384 well plate (Thermo Fisher Scientific, Rochester, NY), and fluorescence polarization was read using the Perkin Elmer Envision plate reader. Scatter plots of translin concentration and translin•trax concentration vs. polarization were constructed using Sigma Plot (Systat Scientific, San Jose, CA) and fit to a hyperbola.

Results

The recombinant translin•trax complex was successfully expressed in *E. coli* using a dual expression vector that contained two open reading frames (ORFs). Because only trax encodes an N-terminal 6x-His tag, translin can only be purified from the Ni resin through its interaction with trax. On a 12% SDS PAGE gel, the trax and translin bands appear to be present in a roughly 1:1 ratio, given the greater mass of trax (Figure 1).

Gel filtration of the translin•trax complex suggests a molecular weight for the complex of ~340 KDa, while that for pure translin was ~280 kDa (data not shown). This appears consistent with the notion that the complex is a heterodecamer of the form $translin_5$•$trax_5$ and that translin exists as a decamer. However, if both the heteromeric complex and translin form cyclic disks, like those seen by EM, then their hydrodynamic volumes would be much larger than a sphere of the same mass, and would run aberrantly heavy. We believe this is the case and the true composition of the complex is most likely a heteroctamer of the form $translin_4$•$trax_4$. Translin most likely associates as an octamer as seen by electron microscopy [6] and validated by ultracentrifuge work [7].

To quantitatively investigate the binding of nucleic acids to translin and the translin•trax complex, eight RNA and DNA ligands with 5′ fluorescein modifications were used: RNA ligands were PRM-2, rGA, rGAG, and rCA, and DNA ligands d(PRM-2), dGA, dGAG and dCA, (as defined in Table 1). Figure 2 shows the binding of polynucleotides to recombinant translin as observed through the change in fluorescence polarization (FP). For this titration, the concentration of translin corresponds to its octameric form. Table 1 summarizes the binding constants. Translin was observed to bind the YH element containing PRM-2 mRNA with a K_d of 200 nM. It bound the synthetic rGA with an even higher affinity, K_d = 20 nM. The rCA and rGAG polymers bound more weakly, with a K_d of 0.9 and 0.5 μM, respectively. In general, translin bound deoxyribonucleotides more poorly than ribonucleotides. For example, the dPRM-2 and dGA oligonucleotides each bound about 50 times worse than the corresponding RNA sequence.

Figure 3 shows the oligonucleotide binding data for the translin•trax heterocomplex. The concentrations shown correspond to the hetero-octamer $translin_4$•$trax_4$. The overall scale of the protein concentration is increased 10-fold compared with Figure 2, indicating generally poorer binding. DNA sequences tend to bind more tightly than RNA, the reverse of the trend shown in Figure 2. The physiological RNA substrate, the YH from PRM-2, has a K_d of 40 μM, about 100 times poorer than the binding of the same ligand to translin alone. The binding experiments suggested that, in general, translin and translin•trax prefer to bind to GA rich sequences,. although translin binds stronger to RNA. The translin•trax complex binds stronger to DNA. This led us to further explore the role of these nucleotides in binding PRM-2 RNA.

We mutated two separate GAG trimers to CAC, and also made a double mutant in which both trimers were altered. These sequences, derived from PRM-2, were designated PRM-2 variants 1, 2 and 3, and are shown in Table 2. The bases that were mutated in the variants are underlined. We also made a truncation variant in which the last 9 bases were removed from the 3′ end of the oligonucleotide (labeled PRM-$2_{(1–30)}$). Table 2 shows the K_d values for the binding of these variants to translin. Translin was observed to bind to PRM-2 Variant 1 with a K_d of 1 μM, a slight reduction in affinity from the wt PRM-2 sequence (K_d = 0.2 μM). It bound the other two mutant ligands, variants 2 and 3, and the truncation variant, with no significant decrease in affinity (K_d of 0.2, 0.3, and 0.2 μM, respectively).

Discussion

Translin and trax play roles in very important cellular processes, such as DNA recombination and the spatial and temporal expression of mRNA. Translin's DNA binding properties have been quantitatively analyzed previously [4;7]; here we expand that work and extend it to the trans•trax complex. In the broadest terms, we show that translin alone prefers to bind GA-rich RNA sequences, while the translin•trax complex shifts the binding affinity to GA-rich DNA sequences (Table 1) Translin binds poly-GA with sub-micromolar affinity, while the translin•trax complex binds poly-dGA with sub-micromolar affinity. In contrast, translin binds the corresponding dGA nearly 1000 times more poorly, while the complex binds the ribonucleotide GA about 25 times more poorly. When the ligand is the GA-rich element from the PRM-2 message, or its deoxyribonucleotide variant, we see the same pattern between free translin and the complex, albeit with less dramatic differences. This observation is consistent with more qualitative work by others; Hecht and coworkers used gel shift assays to show that trax decreases translin's affinity for a specific RNA sequence but increases its affinity for specific a DNA sequence [15].

The switch in binding affinity between DNA and RNA may be important in understanding the physiological role of translin, trax, and their complex. Perhaps the significance of these

observations is most clearly seen by attending to the binding of the natural protamine mRNA sequence, PRM-2; this sequence has been identified as crucial to the recognition of mRNA functioning in sperm development [16]. We see that the mRNA motif binds at least 100 times more tightly to translin than to the translin•trax complex. It is known that trax has a nuclear localization signal (NLS) [10], while translin has a nuclear export signal (NES) [15]. At the simplest level one might imagine the translin•trax complex being taken into the nucleus using the trax NLS. The complex favors DNA binding in the nucleus. However, once translin is released from trax it favors RNA binding, and can recognize certain mRNAs, like PRM2, and carry them into the cytoplasm using its NES.

The natural translin substrate, PRM-2, contains four GAG sequences. If translin is an octameric disk [6], one might expect it to have eight binding sites and a polymer with the correct eight fold repeat might bind very tightly. In fact, as shown in Table 1, translin bound $(GAG)_8$ more weakly than the PRM-2 sequence, and binds only $1/20^{th}$ as well as $(GA)_{12}$. This suggests that the binding of RNA to translin is more complex than simply reading base sequences by eight symmetrically related active sites. It is interesting to note that both the $d(GA)_{12}$ and $d(GAG)_8$ oligonucleotides have four evenly spaced GAG sequences and they both bind to the complex with sub-micromolar affinities. Perhaps the complex, unlike translin, does indeed read the bases directly.

To further investigate the roles of GAG trimers in ligand recognition, two GAG trimers at positions 11–13 and 29–31 were separately and jointly mutated to CAC, and their binding affinities measured (Table 2). The mutations in the first PRM-2 variant sequence yielded a slight, but reproducible, reduction in translin's affinity for PRM-2 (K_d 1 μM and K_d 0.2 μM respectively). However, the mutations of the other two variant sequences did not perturb binding. This is somewhat difficult to rationalize since the double mutant includes the 11–13 mutation which does perturb binding. One possible explanation for this effect can be seen by examining Figure 4, which shows the predicted secondary structure of the PRM-2 variants [17]. The PRM-2 sequence and the tighter binding variants all exhibit a stem loop structure with two unpaired A bases in the stem. The weaker binding variant 1 is predicted to have a distinct secondary structure that lacks this feature. Consistent with this, a truncated 30-mer PRM-2 ($PRM\text{-}2_{(1–30)}$) ligand containing only the putative stem loop structures bound with a K_d of 0.2 μM, essentially the same as that determined for PRM-2 (data not shown).

In discussing the roles for translin and the complex with trax it is important to note that trax has also been shown to interact with the nuclear protein C1D as part of a cellular DNA repair response [18]. Trax's ability to change protein partners between translin and C1D could act i*n vivo* to switch translin from a DNA-binding protein to an RNA-binding protein. This is generally consistent with, the model proposed by Hecht and coworkers that trax can regulate translin's subcellular locale [19]. Both trax and translin co-localize to the nucleus in meiotic pachytene spermatocytes and co-localize to the cytoplasm in post meiotic spermatids [19]. Once in the nucleus, tanslin•trax can interact with DNA, presumably functioning in DNA crossing over which occurs at this stage. Thereafter, trax dissociates, exchanging translin for C1D, and freeing translin to interact with mRNAs marked for export from the nucleus via translin's NES [18;19]. Once translin has re-entered the cytoplasm, it can remain bound to the mRNA until a cellular signal for release and subsequent translation of the message has been received [19]. The ability to act as a shuttling protein is a hallmark of the RNA-binding proteins that traffic mRNAs in neuronal cells [20].

Acknowledgments

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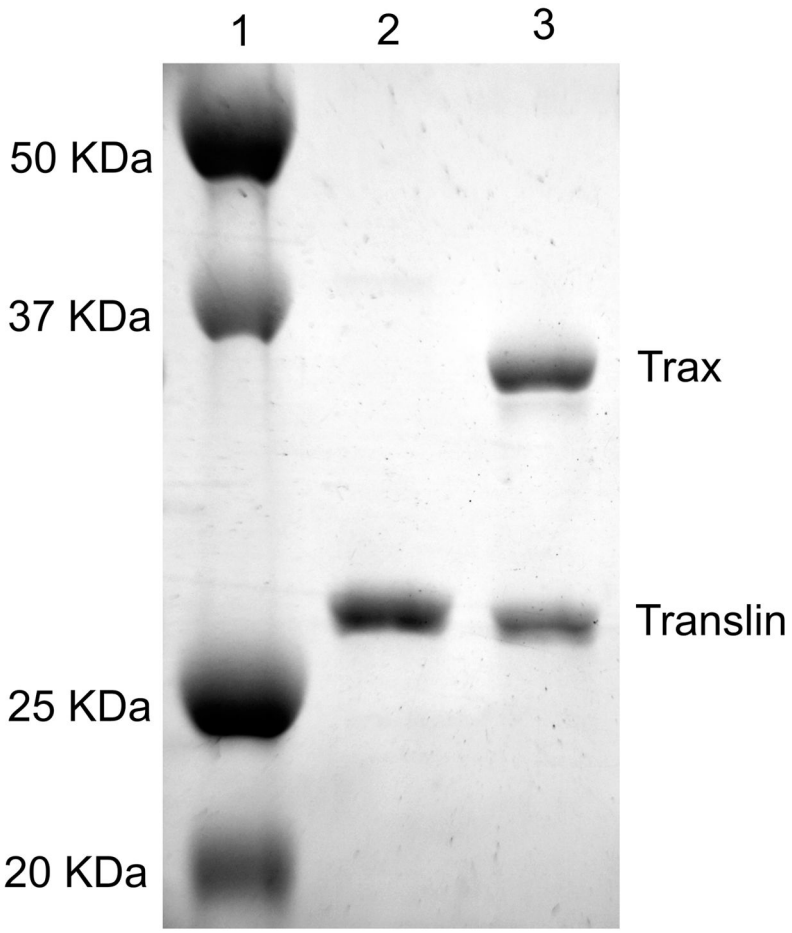

Figure 1.
A 12% SDS gel of translin and translin•trax. Lane 1: molecular weight standards,;Lane 2: recombinant mouse translin (26 KDa); Lane 3: recombinant mouse translin•trax complex (translin 26 KDa, trax 33 KDa).

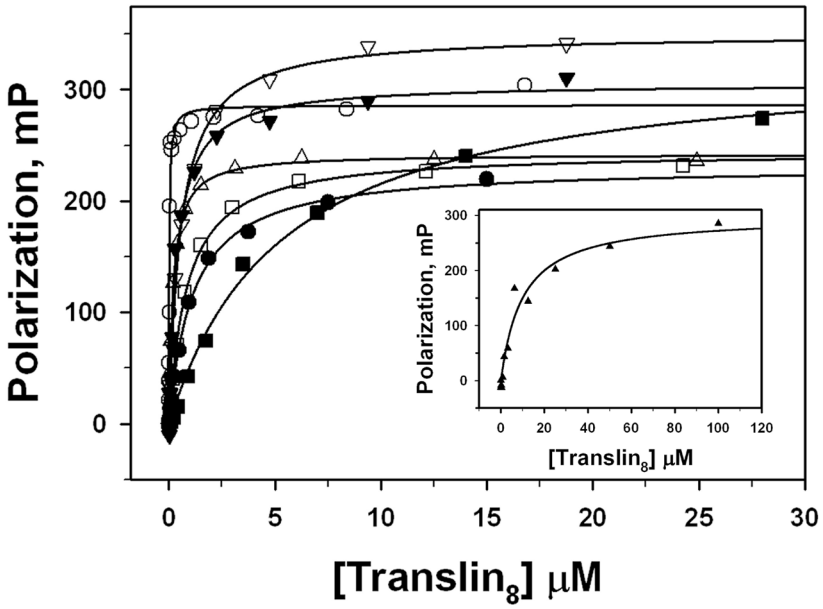

Figure 2.

A plot of translin binding to rGA, rCA, dGA, dCA, rGAG, dGAG and PRM-2. GA sequences are represented as circles, CA sequences are represented as squares, GAG sequences are represented as inverted triangles, and PRM-2 is represented as triangles. RNA sequences are shown as open symbols and DNA sequences are shown as filled symbols. Inset: a plot of translin binding to d(PRM-2). Both plots were constructed using the translin octamer as the binding protein.

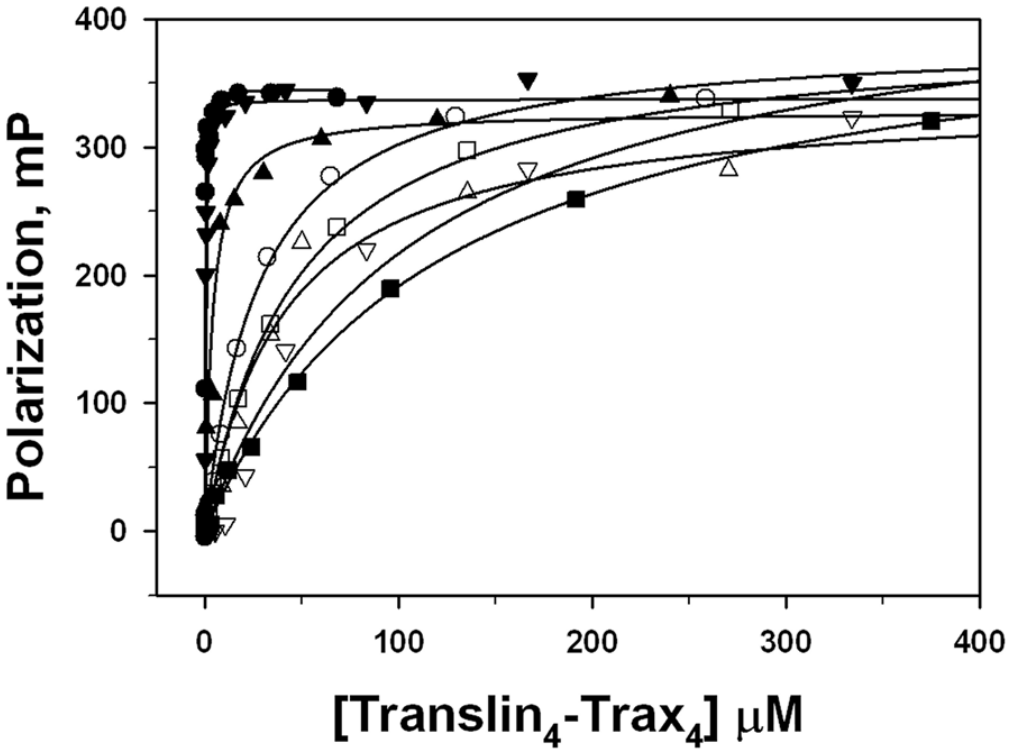

Figure 3.

A plot of translin•trax binding to rGA, rCA, dGA, dCA, rGAG, dGAG, PRM-2 and d(PRM-2). GA sequences are represented as circles, CA sequences are represented as squares, GAG sequences are represented as inverted triangles, and PRM-2 is represented as triangles. RNA sequences are shown as open symbols and DNA sequences are shown as filled symbols. This plot was constructed using the translin•trax hetero-octamer as the binding protein.

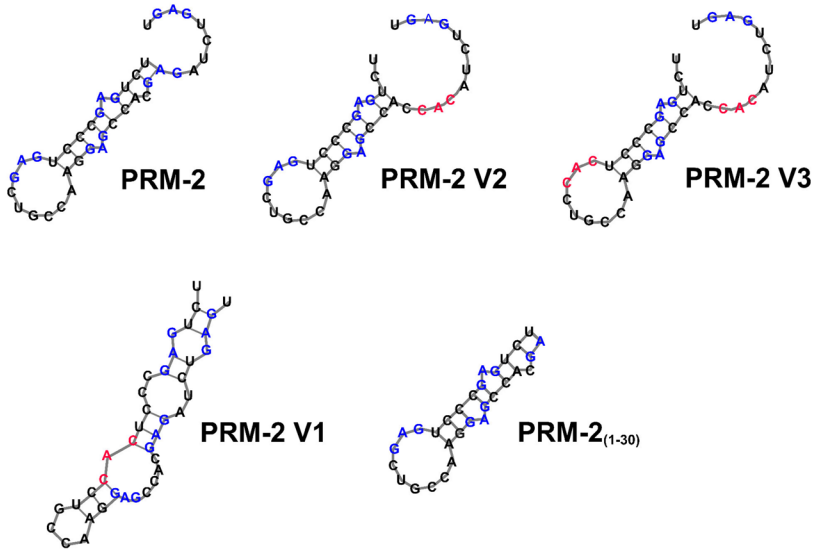

Figure 4.
The predicted secondary structures for PRM-2 and the 4 PRM-2 variant sequences as determined using the Vienna RNA web server [17].

Table 1

Dissociation constants for translin$_8$ and translin$_4$•trax$_4$ binding to RNA and DNA.

Name	Sequence	translin$_8$ K_d (μM)	translin$_4$•trax$_4$ K_d (μM)
PRM-2	UCU**GAG**CCCU**GAG**CUGCCAAGGAGCCAC**GAG**AUCU**GAG**U	0.2	40
rGA	AGAGAGAGAGAGAGAGAGAGAGAGA	.02	28
rCA	ACACACACACACACACACACACACA	0.9	48
rGAG	AGAGGAGGAGGAGGAGGAGGAGGAG	0.5	104
dPRM-2	d(TCT**GAG**CCCT**GAG**CTGCCAAGGAGCCAC**GAG**ATCT**GAG**T)	10	4
dGA	d(AGAGAGAGAGAGAGAGAGAGAGAGA)	1	0.10
dCA	d(ACACACACACACACACACACACACA)	5	12
dGAG	d(AGAGGAGGAGGAGGAGGAGGAGGAG)	0.4	0.18

Table 2

Dissociation constants for translin$_8$ binding to PRM-2 and variant PRM-2 sequences.

Name	Sequence	translin$_8$ K_d (μM)
PRM-2	UCU**GAG**CCCU**GAG**CUGCCAAGGAGCCAC**GAG**AUCU**GAG**U	0.2
PRM-2 Variant 1	UCUGAGCCCUCACCUGCCAAGGAGCCACGAGAUCUGAGU	1
PRM-2 Variant 2	UCUGAGCCCUGAGCUGCCAAGGAGCCACCACAUCUGAGU	0.2
PRM-2 Variant 3	UCUGAGCCCUCACCUGCCAAGGAGCCACCACAUCUGAGU	0.3
PRM-2$_{(1–30)}$	UCUGAGCCCUGAGCUGCCAAGGAGCCACGA	0.2