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ARTICLE *in* BIOORGANIC & MEDICINAL CHEMISTRY · JULY 1997

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Bioorganic & Medicinal Chemistry, Vol. 5, No. 6, pp 1123–1129, 1997

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Inhibition of Rev·RRE Complexation by Triplex Tethered Oligonucleotide Probes

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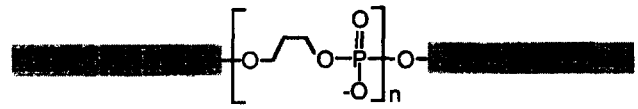
Abstract—We have described a class of molecules, called tethered oligonucleotide probes (TOPs), that bind RNA on the basis of both sequence and structure. TOPs consist of two short oligonucleotides joined by a tether whose length and composition may be varied using chemical synthesis. In a triplex TOP, one oligonucleotide recognizes a short single-stranded region in a target RNA through the formation of Watson–Crick base pairs; the other oligonucleotide recognizes a short double-stranded region through the formation of Hoogsteen base pairs. Binding of triplex TOPs to an HIV-1 Rev Response Element RNA variant (RRE^{AU}) was measured by competition electrophoretic mobility shift analysis. Triplex TOP·RRE^{AU} stabilities ranged between −9.6 and −6.1 kcal mol^{-1} under physiological conditions of pH, salt, and temperature. Although the most stable triplex TOP·RRE^{AU} complex contained 12 contiguous U·AU triple helical base pairs, complexes containing only six or nine triple helical base pairs also formed. Triplex TOPs inhibited formation of the RRE·Rev complex with IC_{50} values that paralleled the dissociation constants of the analogous triplex TOP·RRE^{AU} complexes. In contrast to results obtained with TOPs that target two single-stranded RRE regions, inhibition of Rev·RRE^{AU} complexation by triplex TOPs did not require pre-incubation of RRE^{AU} and a TOP: triplex TOPs competed efficiently with Rev for RRE^{AU} and inhibited RRE^{AU}·Rev complexation at equilibrium. © 1997 Elsevier Science Ltd.

Introduction

There is considerable current interest in the design of molecules able to bind large RNA molecules with high affinity and specificity.[1–7] Molecules with these properties facilitate the study of macromolecular recognition and design and have utility in the targeted inactivation or destruction of messenger RNAs.[8] However, the task of recognizing an RNA molecule—especially a large RNA molecule—is complicated. Most large RNAs are believed to be comprised of irregular arrangements of several short single- and double-stranded regions joined by structures such as loops, bulges and pseudoknots.[9,10] Biochemical studies[11,12] and a growing number of high-resolution structures[13,14] reveal that large RNAs, like large proteins, contain globular cores that are largely inaccessible to solvent and, hence, to solvent-borne molecules. This structural complexity severely complicates efforts to design oligonucleotide-based RNA-binding molecules, as most single-stranded regions are either inaccessible or too short to form high-affinity complexes.[15]

We have described a family of molecules, called tethered oligonucleotide probes (TOPs), that recognize RNA by accommodating their varied complex structures.[15–19] A TOP is composed of two short oligonucleotides joined by a flexible synthetic tether (Fig. 1). Each oligonucleotide recognizes a short, accessible region within the RNA and the tether traverses the distance between the two regions. Initially, we designed and tested TOPs capable of recognizing two noncontiguous single-stranded regions within the spliced leader RNA from *L. collosoma* (SL RNA)[16–18] or the HIV-I Rev Response Element RNA (RRE)[15] (Fig. 2A). In both cases, TOPs with high affinity and specificity for their targets were prepared. In the case of the RRE, the most potent TOPs recognized nucleotides 45–51 and 69–76 within stem II; these two single-stranded regions were termed sites 1 and 2, respectively (Fig. 2B). Site 1 spanned a portion of the binding site for the RRE ligand Rev. Not only did these TOPs bind the RRE efficiently, they prevented formation of the Rev·RRE complex with inhibitory constants (IC_{50} values) that paralleled their RRE affinities.

More recently, we described a set of triplex TOPs capable of recognizing single- and double-stranded



5' oligonucleotide	n	3' oligonucleotide	Label
dCTGTACCG	O		S1
	O	UUUUUUUUUUUU	S4
dCTGTACCG	1	UUUUUUUUUUUU	S1-1-S4
dCTGTACCG	1	UUUUUUUUU	S1-1-$S4^9$
dCTGTACCG	1	UUUUUU	S1-1-$S4^6$
dCTGTACCG	1	UUCUUUCUUUUC	S1-1-$S4^{UC}$
dCTGTACCG	1	CCCCCCCCCCCC	S1-1-$S4^C$
dCTGTACCG	5	dGCGCCCA	S1-5-S2

Figure 1. Sequences of TOPs and oligonucleotides used in this study. With the exception of **S1-5-S2**, which is composed solely of deoxyribonucleotides, all 5′-oligonucleotides are composed of deoxyribonucleotides and all 3′-oligonucleotides are composed of ribonucleotides.

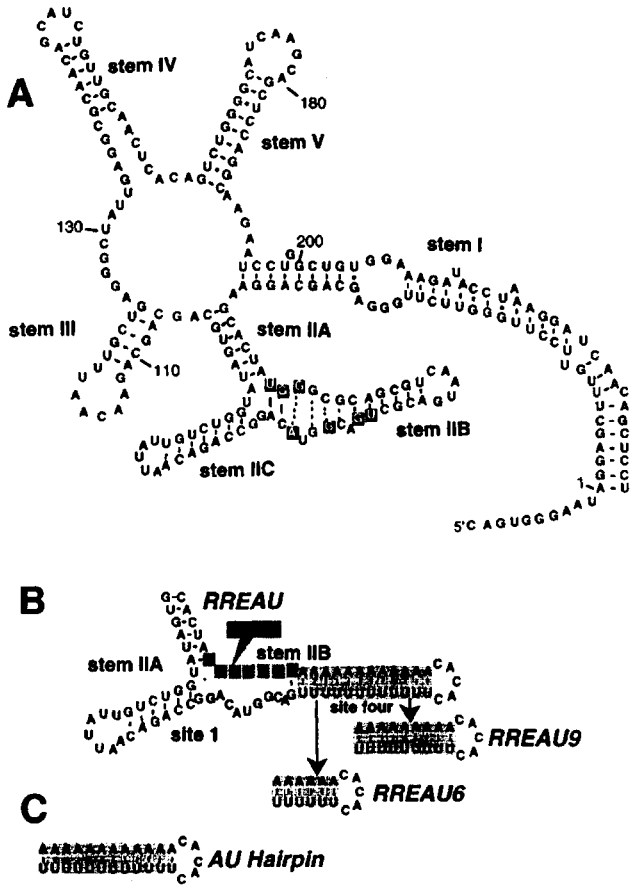


Figure 2. Sequences of (A) wild-type RRE; residues shown by NMR[30] to contact Rev are shaded. (B) RRE^{AU}, RRE^{AU9}, and RRE^{AU6} and (C) the AU hairpin.

regions within a large, structured RNA.[19] Triplex TOPs contain one oligonucleotide designed to recognize a short single-stranded region through the formation of Watson–Crick base pairs[20] and a second oligonucleotide designed recognize a short double-stranded region through the formation of Hoogsteen base pairs.[21] The RNA target for our triplex TOPs, RRE^{AU} (Fig. 2B), contained 12 contiguous A–U base pairs that functioned as a third strand recognition site. This site was termed site 4. Triplex TOPs designed to recognize sites 1 and 4 formed RRE^{AU} complexes with nanomolar dissociation constants. Moreover, triplex TOP·RRE^{AU} stabilities were less sensitive to changes in salt concentration and temperature than were model triple helices of comparable length. To further explore the limitations of RNA recognition by triplex TOPs, here we investigate the effect of triple helix length on RRE^{AU}·TOP stability and Rev·RRE^{AU} inhibition.

Results and Discussion

Design of RRE^{AU} and triplex TOPs

Because the wtRRE (Fig. 2A) lacked an extended polypurine–polypyrimidine site suitable for triple helix formation, we designed a series of variant RREs containing 6, 9, or 12 contigous A·U base pairs. These RREs were termed RRE^{AU6}, RRE^{AU9}, and RRE^{AU}, respectively (Fig. 2B). The natural RRE ligand Rev[22–24] binds to discrete residues in stem IIB of wtRRE.[25–30] We reasoned that lengthening stem IIB while retaining these residues would maintain the overall fold and function of the RRE and allow us to assess both the RRE affinities of triplex TOPs as well as their potencies as Rev·RRE inhibitors. RRE^{AU} and wtRRE bound Rev with similar affinities, with half-maximal binding at 20 nM Rev (Fig. 3). Triplex TOPs designed to recognize RRE^{AU} and its analogues were comprised of a 5′-oligodeoxyribonucleotide complementary in a Watson–Crick sense to the eight bases comprising site 1 and a 3′-oligoribonucleotide complementary in a Hoogsteen sense to the extended region of stem IIB (site 4) (Figs 1 and 2B). Molecules lacking the 5′-oligodeoxyribonucleotide (**S4**) or the 3′-oligodeoxyribonucleotide (**S1**) were prepared as controls, as were TOPs that were not perfectly complementary to the RRE target (**S1-1-S4**^{UC}, **S1-1-S4**^{C}). TOP **S1-5-S2** recognized sites 1 and 2.

Equilibrium dissociation constants of TOP·RRE^{AU} complexes

Dissociation constants of TOP·RRE^{AU} complexes were determined by use of a competition[31] electrophoretic mobility shift assay.[32] This assay allowed us to monitor the fraction of 5′-^{32}P **S1-5-S2** bound to RRE^{AU} as a function of the concentration of added competitor TOP and determine the dissociation constants of TOP·RRE^{AU} complexes that were not stable during gel electrophoresis.[15] Two factors guided our choice of **S1-5-S2** as the labeled probe in the competition assay. First, **S1-5-S2** bound RRE sites 1 and 2 with high affinity to form complexes that were stable during gel electrophoresis.[15] Second, the stability of the **S1-5-S2**·RRE complex depended on simultaneous interaction between **S1-5-S2** and both sites 1 and 2; **S1-5-S2** did not form stable RRE^{AU} complexes if either site 1 or site 2 were inaccessible.[15] All of the triplex TOPs described in this report interacted with site 1 of the RRE, thus their interaction with sites 1 and 4 precluded interaction of **S1-5-S2** at sites 1 and 2.

Triplex TOP·RRE^{AU} complexes

Previously we reported equilibrium dissociation constants of TOP·RRE^{AU} complexes under conditions where the Rev·RRE^{AU} complex was not stable.[19] In an attempt to correlate the affinity of a triplex TOP for RRE^{AU} with its potency as an inhibitor of Rev·RRE^{AU} complexation under a standard set of conditions, we measured TOP·RRE^{AU} dissociation constants under conditions where the Rev·RRE^{AU} complex was stable (Fig. 4, Table 1). These conditions differed in several respects from the conditions reported previously.[19] The Rev binding buffer contained potassium ions rather than sodium ions as the primary monovalent cation; it also contained a divalent cation (1 mM $MgCl_2$) and two nonspecific competitors (BSA and $tRNA^{Phe}$).

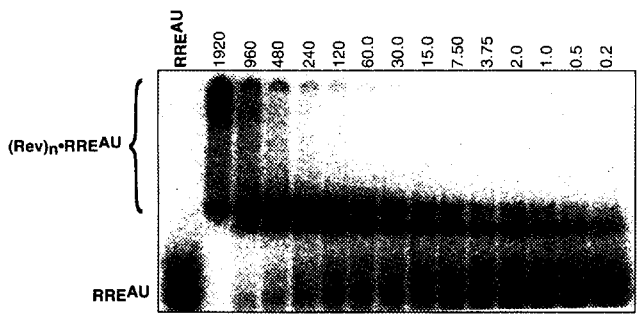


Figure 3. Autoradiogram of a 6% native polyacrylamide gel illustrating the concentration-dependent binding of Rev to ^{32}P-RRE^{AU}. The concentration of Rev, in nanomolar units is shown above each lane.

Under these new conditions, TOP·RRE^{AU} stabilities ranged between −9.6 to −6.1 kcal mol^{-1} and paralleled those determined previously.[19] Triplex TOP **S1-1-S4,** whose RRE^{AU} complex was stabilized by eight Watson–Crick and 12 Hoogsteen base pairs, displayed the highest RRE^{AU} affinity (ΔG_{obs} = −9.6 kcal mol^{-1}). Comparison of the stabilities of the **S1**·RRE^{AU} (ΔG_{obs} = −6.5 kcal mol^{-1}) and **S1-1-S4**·RRE^{AU} complexes revealed that formation of the site 4 triple helix contributed a net 3.1 kcal mol^{-1} of binding energy to the complex. This value represented the sum of terms: a favorable contribution provided by triple helix formation within the context of RRE^{AU} and other contributions that most likely resulted from structural reorganization of the RNA or TOP. Comparison of the stabilities of the **S1-1-S4**^{UC} (ΔG_{obs} = −6.1 kcal mol^{-1}) and **S1-1-S4**^{C} (ΔG_{obs} = 7.8 kcal mol^{-1}) complexes with the **S1-1-S4**·RRE^{AU} complex revealed that mismatches within the triple helix had a significant effect on stability. Taken with RNase H assays that provide evidence for site-specific interaction of **S1** with RRE site 1,[15] these data support a model in which the 3′- and 5′-oligonucleotides within **S1-1-S4** recognize sites 1 and 4 through Watson–Crick and Hoogsteen interactions, respectively.

The data presented here suggest that triplex TOP·RRE^{AU} stability is relatively insensitive to changes in counterion identity. This observation further supports our earlier conclusion that triplex TOPs are less sensitive to changes in the concentrations and identities of cations than are model triple helices. The stability of the **S4**·AU triple helix (Fig. 2C), like those of other model triple helices,[33–37] was highly sensitive to the presence of divalent cations: the T_m of the S4·AU hairpin complex was 12 °C in a buffer containing 300 mM sodium ion (2 μM total strand concentration) and increased to 21 °C when the buffer was supplemented with 5 mM Mg^{2+} (data not shown). The insensitivity of triplex TOP·RRE stability to the presence of divalent cations presumably reflects the comparatively small contribution of the site 4 triple helix to the stability of the **S1-1-S4**·RRE^{AU} complex. Notably, even the modest 3.1 kcal mol^{-1} contribution of triple helix formation converts a micromolar ligand (**S1**) into a nanomolar ligand (**S1-1-S4**) and permits the selective recognition

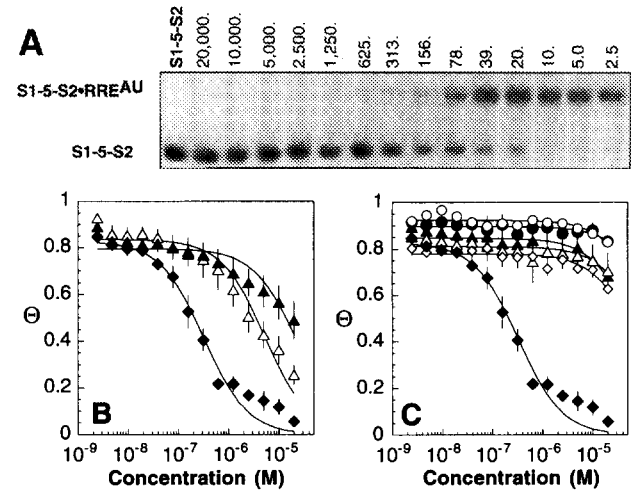


Figure 4. Determination of equilibrium dissociation constants of TOP and oligonucleotide·RRE^{AU} complexes. (A) Representative autoradiogram of a 6% native polyacrylamide gel illustrating the effect of **S1-1-S4** on the fraction of 5′-^{32}P **S1-5-S2** bound to RRE^{AU}. The **S1-1-S4** concentration, in nanomolar units, is shown above each lane. (B) and (C) Plots illustrating concentration-dependent inhibition of **S1-5-S2**·RRE^{AU} complexation by various TOPs and oligonucleotides. ◆, **S1-1-S4**; △, **S1-1-S4**^{9}; ▲, **S1-1-S4**^{6} and ◇, **S1**. Also illustrated in graph (C): ○, inhibition of **S1-5-S2**·RRE^{AU9} by **S1**; and ●, **S1** inhibition of **S1-5-S2**·RRE^{AU6} by **S1**. The *y* axis represents θ, the fraction of 5′-^{32}P **S1-5-S2** bound to RRE^{AU}. The *x* axis represents the concentration of TOP or oligonucleotide. The line represents the best fit of the data to equation 1 (see Experimental).

of two sites that individually are not recognizable above the level of a nonspecific interaction.

Effect of shortening the triple helix

A series of TOPs were synthesized to determine the smallest number of Hoogsteen base pairs required for high-affinity RRE^{AU} recognition. TOPs **S1-1-S4**^{6} and **S1-1-S4**^{9} contained the 3′-oligodeoxyribonucleotide complementary to RRE^{AU} site 1 joined through a short phosphodiester tether to six or nine consecutive uridine residues (Fig. 1). These TOPs were identical to **S1-1-S4** except that they formed triple helices containing only six or nine U·AU base pairs, respectively. Dissociation constants of the complexes formed between these TOPs and RRE^{AU} are listed in Table 1. **S1-1-S4**^{6} and **S1-1-S4**^{9} formed complexes with RRE^{AU} that were 1.3 and 2.5 kcal mol^{-1} less stable, respectively, than the **S1-1-S4**·RRE^{AU} complex. Despite these losses in binding free energy, both complexes were still more stable than an analogous complex lacking a triple helix: under identical conditions the **S1**·RRE^{AU} complex exhibited a free energy of −6.5 kcal mol^{-1}. Thus, when presented with an extended (12 bp) third strand recognition site, both truncated TOPs formed high-affinity RRE^{AU} complexes. Complex stability was, however, dependent on the presence of the extended third strand recognition site. Dissociation constants were also determined for complexes between **S1-1-S4**^{6} and **S1-1-S4**^{9} and RRE^{AU} analogues (Fig. 2B) containing only 6 (RRE^{AU6}) or 9 (RRE^{AU9}) AU base pairs within stem IIB. The **S1-1-S4**^{9}·RRE^{AU9} and **S1-1-S4**^{6}·RRE^{AU6} complexes displayed binding free energies of −6.5 and

Table 1. Equilibrium dissociation constants of triplex TOP·RRE complexes and inhibition constants for Rev inhibition

TOP or oligo	RRE analogue	K_d[a] (nM)	ΔG_{obs}[a] (kcal mol^{-1})	ΔG_{obs}[b] (kcal mol^{-1})	IC_{50} (nM)
S1-1-S4	RRE^{AU}	93 ± 15	−9.6 ± 0.3	−8.7 ± 0.1[c]	53 ± 10
S1-1-S4^{9}	RRE^{AU}	814 ± 200	−8.3 ± 0.3	N.D.	470 ± 120
S1-1-S4^{6}	RRE^{AU}	5900 ± 1080	−7.1 ± 0.4	N.D.	3500 ± 520
S1-1-S4^{9}	RRE^{AU9}	16,800 ± 8320	−6.4 ± 0.3	N.D.	N.D.
S1-1-S4^{6}	RRE^{AU6}	11,800 ± 2490	−6.7 ± 0.2	N.D.	N.D.
S1	RRE^{AU}	17,700 ± 3050	−6.5 ± 0.1	−6.1 ± 0.7	17,400 ± 1900
S1	RRE^{AU9}	12,300 ± 2950	−6.7 ± 0.2	N.D.	N.D.
S1	RRE^{AU6}	27,900 ± 8040	−6.2 ± 0.3	N.D.	N.D.
S4	RRE^{AU}	N.D.	N.D.	N.D.	2800 ± 860
S1-1-S4^{UC}	RRE^{AU}	30,900 ± 3670	−6.1 ± 0.3	−6.6 ± 0.3	90,300 ± 33,000
S1-1-S4^{C}	RRE^{AU}	1850 ± 380	−7.8 ± 0.1	−5.9 ± 0.3	7900 ± 1030
tRNA	RRE^{AU}	19,800 ± 3630	−6.4 ± 0.2	N.D.	21,000 ± 2410

Values for ΔG°_{obs} were calculated from the relationship $\Delta G^{\circ}_{obs} = -RT \ln (1/K_d)$ where $R = 0.0019872$ kcal mol^{-1} K^{-1} and $T = 298$ or 277 K.
[a]In Rev binding buffer at 25 °C.
[b]In triple helix binding buffer at 4 °C.[19]
[c]ΔG°_{obs} for **S1-1-S4**·RRE^{AU} at 25 °C is −9.4 kcal mol^{-1}.

−6.7 kcal mol^{-1}, respectively; these values were approximately equal to that of the **S1**·RRE complex.

Inhibition of Rev·RRE^{AU} complexation

A series of experiments was performed to determine if triplex TOP **S1-1-S4** could inhibit Rev·RRE^{AU} complexation at equilibrium. In each experiment, we monitored the fraction of 5′-^{32}P-RRE^{AU} bound to Rev as a function of added triplex TOP. Results are expressed in terms of an IC_{50} value, which is equal to the concentration of TOP required to reduce by one half the fraction of 5′-^{32}P-RRE^{AU} bound to Rev. In the first experiment (method 1), fixed concentrations of Rev and 5′-^{32}P-RRE^{AU} were incubated for 70 min with varying concentrations of **S1-1-S4** or **S1** before the fraction of 5′-^{32}P-RRE^{AU} bound to Rev was determined by gel electrophoresis. In the second experiment (method 2), RRE^{AU} was preincubated with Rev for 10 min followed by addition of **S1-1-S4** or **S1** and an additional 1 h incubation. In the third experiment (method 3), RRE^{AU} was preincubated with **S1-1-S4** or **S1** for 1 h followed by the addition of Rev and an additional 10 min incubation.

Table 2 lists the IC_{50} values determined for **S1-1-S4** and **S1** in each experiment. The data show that the potencies of **S1-1-S4** and **S1** as inhibitors of Rev·RRE^{AU} complexation were largely independent of the order in which the reaction components were mixed. The IC_{50} value observed for **S1-1-S4** when Rev, RRE^{AU} and **S1-1-S4** were added simultaneously (44 nM) was within experimental error of the IC_{50} value observed when Rev and RRE^{AU} were preincubated (55 nM) and slightly higher than the IC_{50} value observed when **S1-1-S4** and RRE^{AU} were preincubated (19 nM). The observation that the IC_{50} value was relatively independent of the order in which the components were mixed indicated that the system had reached equilibrium. IC_{50} values listed in Table 1 were determined using method 2.

Curves illustrating concentration-dependent inhibition of Rev binding by triplex TOPs are shown in Figure 5. In general, TOPs and oligonucleotides exhibited IC_{50} values that were within a factor of three of the dissociation constants of their RRE^{AU} complexes. TOP **S1-1-S4** was the most potent inhibitor by at least 100-fold with an average IC_{50} value of 53 nM. Neither of the oligonucleotides from which **S1-1-S4** was composed was an effective inhibitor of Rev·RRE^{AU} complexation as judged by this assay: the **S1** and **S4** oligonucleotides displayed IC_{50} values of 17 and 2.8 μM, respectively. Moreover, a mixture of **S1** and **S4** did not inhibit Rev·RRE^{AU} complex formation more successfully than did each oligonucleotide alone, indicating little or no communication between sites 1 and 4.[15] As expected, **S1-1-S4**^{9} and **S1-1-S4**^{6} were less potent inhibitors than **S1-1-S4**, displaying IC_{50} values of 470 nM and 3.5 μM, respectively. TOPs **S1-1-S4**^{UC} and **S1-1-S4**^{C} were poor inhibitors, with IC_{50} values of 90 and 7.9 μM, respectively.

Conclusions

Here we demonstrate that triplex TOPs recognize several Rev Response Element RNA (RRE) variants in solution, and inhibit binding of the RRE to its protein ligand Rev. Triplex TOPs inhibit Rev·RRE complexation at nanomolar concentrations, and are as potent as TOPs that recognize two single-stranded regions of RRE^{AU}. Notably, inhibition of Rev·RRE^{AU} complexation by triplex TOPs does not require preincubation: triplex TOPs compete efficiently with Rev for RRE^{AU} at equilibrium. Thermodynamic studies indicate that although the most stable triplex TOP·RRE^{AU} complex studied contains 12 contiguous U·AU triple helical base pairs, stable complexes containing only six or nine triple helical base pairs also form under certain conditions. Moreover, triplex TOP·RRE complexes are less sensitive than model triple helices to salt and temperature. These properties suggest that triplex TOPs may prove more useful than simple oligonucleo-

Table 2. Effect of addition order on IC_{50} for Rev inhibition

TOP or oligonucleotide	IC_{50} (nM)		
	Method 1	Method 2	Method 3
S1-1-S4	44 ± 15	55 ± 10	19 ± 6
S1	20,500 ± 6000	ND	17,500 ± 2000

tides for the recognition and inactivation of structured RNAs in vitro and in vivo. Moreover, the observation that short RNA triple helices can form readily under physiological conditions of pH, salt, and temperature suggests that these structures may contribute to the stabilities of large folded RNAs.[38,39]

Experimental

Preparation of TOPs, RRE^{AU}, RRE^{AU6} and RRE^{AU9}

TOPs were prepared as described previously.[16,18] RREs were prepared by in vitro transcription using T7 RNA polymerase and chemically synthesized DNA templates.[40] Transcribed products were purified by preparative denaturing gel electrophoresis (20% acrylamide, 19:1 acrylamide:bis-acrylamide, 7 M urea), and desalted by passage over a 5 mL column of G-25 Sephadex (Pharmacia) or by dialysis using a Microcon-10 column (Amicon, Beverly, MA). Aliquoted TOP and RNA stocks were stored in diethyl pyrocarbonate-treated (DEPC) water at −20 °C and thawed prior to use in Rev inhibition and competition electrophoretic mobility shift experiments. The sequence of RRE^{AU} between nucleotides 42 and 69 and RRE^{AU6} between nucleotides 44 and 77 was confirmed by enzymatic methods (Pharmacia) using protocols recommended by the supplier.

Determination of equilibrium dissociation constants

Equilibrium dissociation constants of TOP·RRE^{AU} complexes were determined by use of a competition electrophoretic mobility shift assay in which the fraction of 5′-^{32}P **S1-5-S2** bound to RRE was monitored as a function of the concentration of unlabelled competitor TOP (20 μM–2.44 nM).[15] Briefly, RRE^{AU} (2.5 μM in 5 mM Hepes (pH 7.5), 25 mM NaCl) was renatured by heating at 85 °C for 2 min and cooling at 25 °C for 10 min prior to each competition assay. Binding reactions were incubated in Rev binding buffer (10 mM Tris (pH 7.5), 20 mM NaCl, 150 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 20 ng/μL $tRNA^{Phe}$, 10 ng/μL BSA, and 10% glycerol) for 1.5 h at 25 °C and applied immediately to 1.5 × 140 × 150 mm 6% polyacrylamide gels (79:1 acrylamide:bis-acrylamide) prepared with running buffer (45 mM Tris-borate (pH 7.0), 1 mM EDTA). Gels were maintained between 3 and 6 °C during electrophoresis by immersion in buffer cooled by a circulating, temperature-controlled bath. Radioactivity was quantified on a Betagen 605 Blot Analyzer (Betagen Inc., Waltham, MA) or a Storm 840 Phos-

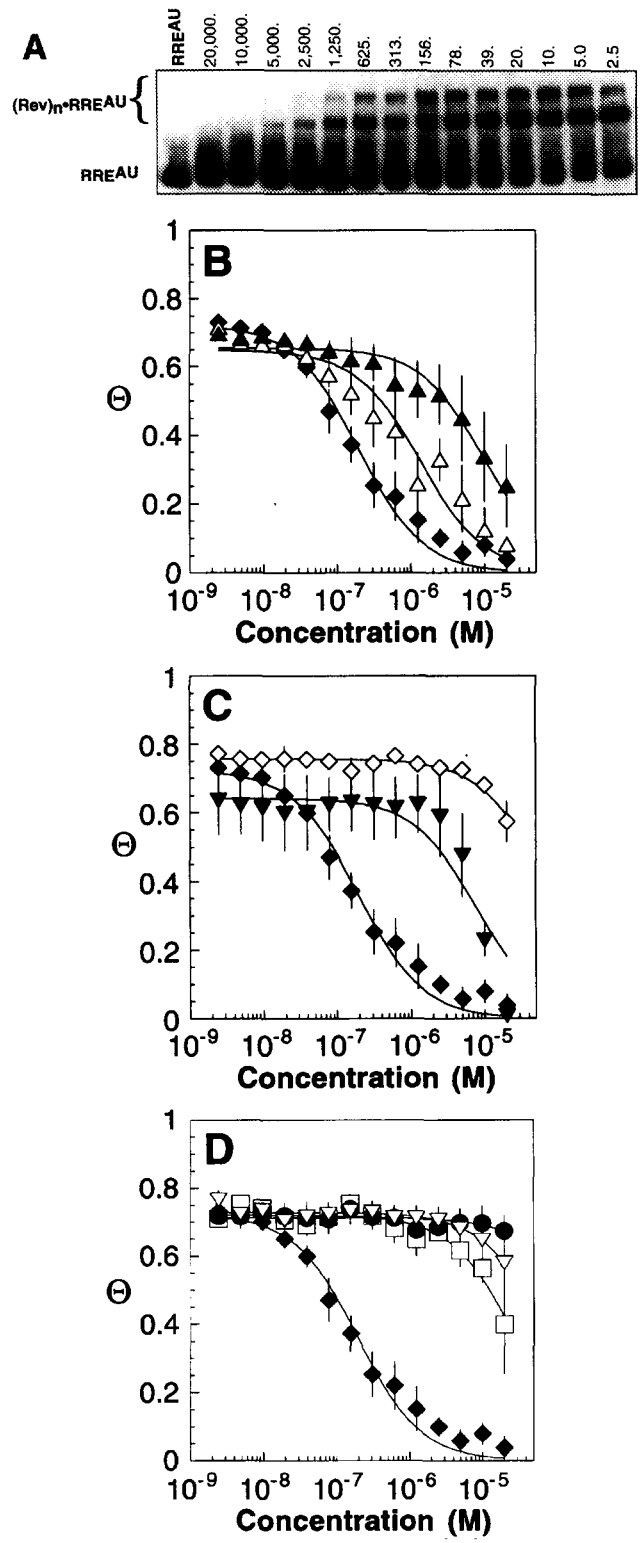


Figure 5. Determination of IC_{50} values for Rev inhibition. (A) Representative autoradiogram of a 6% native polyacrylamide gel illustrating the effect of **S1-1-S4** on the fraction of 5′-^{32}P RRE^{AU} bound to Rev. The concentrations of **S1-1-S4**, in nanomolar units, is shown above each lane. (B), (C), and (D). Plots illustrating concentration-dependent inhibition of Rev·RRE^{AU} complexation by ◆, **S1-1-S4**; △, **S1-1-S4**^{9}; ▲, **S1-1-S4**^{6}; ◇, **S1**; ▼, **S4**; ■, **S1-1-S4**^{UC}; □, **S1-1-S4**^{C}; and ▽, $tRNA^{Phe}$. The *y* axis represents θ, the fraction of 5′-^{32}P RRE^{AU} bound to Rev. The *x* axis represents the concentration of competitor TOP or oligonucleotide. The line represents the best fit of the data to equation 2 (see Experimental).

phorimager (Molecular Dynamics, Sunnyvale, CA). Dissociation constants were determined from best fits of the data to equation 1

$$\theta = K_d^{\text{S1-5-S2}} + \frac{K_d^{\text{S1-5-S2}}[\text{TOP}]_T}{K_d^{\text{TOP}}} + [\text{S1-5-S2}]_T + [\text{RRE}]_T - \sqrt{\left[\left(K_d^{\text{S1-5-S2}} + \frac{K_d^{\text{S1-5-S2}}[\text{TOP}]_T}{K_d^{\text{TOP}}} + [\text{S1-5-S2}]_T + [\text{RRE}]_T\right)^2 - 4[\text{RRE}]_T[\text{S1-5-S2}]_T\right]} \Big/ 2[\text{S1-5-S2}]_T \quad (1)$$

using a nonlinear least-squares analysis (Kaleidagraph v. 3.0.2) where $[\text{TOP}]_T$, $[\text{RRE}]_T$, and $[\textbf{S1-5-S2}]_T$ are the total concentrations of competitor TOP or oligonucleotide, RRE derivative, and 5′-^{32}P **S1-5-S2**, respectively. All of the values of K_d determined in this work are greater than 1 nM and should therefore be determined accurately by the competition method.[41]

Determination of Rev·RRE^{AU} complex stability

Direct electrophoretic mobility shift assays were used to determine the concentration of Rev required to result in 50% or 85% of a limiting concentration of 5′-^{32}P RRE^{AU} bound to Rev. Rev (Intracell, Cambridge, MA) was thawed partially at room temperature and diluted into Rev binding buffer (defined above) prior to the addition of renatured RRE^{AU}. Binding reactions were incubated at 25 °C for 12 min and applied directly to nondenaturing polyacrylamide gels as described above. The kinetic stability of the Rev·RRE^{AU} complex was determined as follows: Rev at a final concentration of 100 nM was added to a limiting amount of renatured 5′-^{32}P RRE^{AU} and aliquots of the binding reaction applied to a nondenaturing gel every 10 min for 90 min. The fraction of 5′-^{32}P RRE^{AU} bound to Rev was monitored as a function of time.

Determination of 50% inhibition constant (IC_{50}) values

The IC_{50} was defined as the concentration of TOP or oligonucleotide required to reduce by 50% the maximum observed fraction of RRE complexed to Rev. IC_{50} values were determined by use of a competition electrophoretic mobility shift assay in which the fraction of 5′-^{32}P RRE^{AU} bound to Rev was monitored as a function of the concentration of unlabeled competitor TOP.[15] Pre-aliquoted stocks of Rev protein (700 nM) in Rev binding buffer (10 mM Tris (pH 7.5), 20 mM NaCl, 150 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 20 ng/μL tRNA^{Phe}, 10 ng/μL BSA, and 10% glycerol) were thawed immediately before use. Binding reactions containing Rev and freshly renatured RRE^{AU} were incubated for 70 min at 25 °C and applied immediately to 1.5 × 140 × 150 mm 6% (79:1 acrylamide:bis-acrylamide) gels prepared with running buffer (45 mM Tris–borate (pH 7.0), 1 mM EDTA). Final concentrations were as follows: 0.01 nM ^{32}P-RRE^{AU}, 70 nM Rev, 20 μM–2.4 nM competitor TOP or oligonucleotide, 20 mM Tris (pH 7.5), 20 mM NaCl, 150 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 20 ng/μL tRNA^{Phe}, 10 ng/μL BSA, and 10% glycerol. Gels were maintained between 3 and 6 °C during electrophoresis by immersion in buffer cooled by a circulating, temperature-controlled bath. IC_{50} values were determined from best fits to equation 2

$$\theta = K_d^{\text{RRE}} + \frac{K_d^{\text{RRE}}[\text{TOP}]_T}{\text{IC}_{50\ \text{TOP}}} + [\text{Rev}]_T + [\text{RRE}]_T - \sqrt{\left[\left(K_d^{\text{RRE}} + \frac{K_d^{\text{RRE}}[\text{TOP}]_T}{\text{IC}_{50\ \text{TOP}}} + [\text{RRE}]_T + [\text{Rev}]_T\right)^2 - 4[\text{Rev}]_T[\text{RRE}]_T\right]} \Big/ 2[\text{RRE}]_T \quad (2)$$

using a nonlinear least-squares analysis (Kaleidagraph v. 3.0.2) where $[\text{TOP}]_T$, $[\text{Rev}]_T$, and $[\text{RRE}]_T$ are the total concentrations of competitor TOP or oligonucleotide, Rev, and RRE^{AU}, respectively. A Rev concentration of 70 nM resulted in 80–85% of the labeled RRE^{AU} bound to the Rev.

Acknowledgements

This work was supported by the NIH (GM 43501). A.C.M. is grateful to Hoffmann–LaRoche for a graduate fellowship. We are all grateful to Professor Thomas E. Shrader (Albert Einstein College of Medicine) for a gift of T7-911, a His_6-tagged version of T7 RNA polymerase.

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(*Received in U.S.A.* 16 *October* 1996; *accepted* 18 *February* 1997)