Incorporation of a Non-Nucleotide Bridge into Hairpin Oligonucleotides Capable of High-Affinity Binding to the Rev Protein of HIV-1†

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ABSTRACT: A bridge containing a rigid *trans*-stilbene group, $-P(O)(O^-)O(CH_2)_3NHC(O)-C_6H_4-CH{=}CHC_6H_4C(O)NH(CH_2)_3OP(O)(O^-)-$, has been incorporated into several oligonucleotide sequences based on the minimal Rev Binding Element (RBE) of HIV-1. This bridge was found to be as effective as a UUCG tetraloop in stabilizing short RNA duplex structures containing mismatched bases and bulged out nucleotide residues and to be more effective than either a TTTT loop or a triethyleneglycol linker in stabilizing similar DNA structures. Evaluation of stilbene-containing RNA RBE sequences of varying length for their ability to bind the Rev protein of HIV-1 showed that a 22-nucleotide stilbenedicarboxamide conjugate bound Rev almost as well as a 94-base fragment of the Rev Responsive Element (RRE). A DNA hairpin mimetic with the same sequence was incapable of Rev binding. Taken together, these experiments serve as an example for how *in vitro* selection and chemical modification can be combined to generate high-affinity mimetics of nucleic acid sequence and structure.

HIV-1 gene expression is regulated in part by a viral protein, Rev (Felber et al., 1989; Sodroski et al., 1986). Rev is an RNA binding protein that interacts with a highly structured portion of the viral genome known as the Rev Responsive Element (RRE; Figure 1a)[1] and facilitates the transport of unspliced and singly-spliced viral RNAs from the cell nucleus to the cytoplasm (Malim et al., 1989; Hammarskjold et al., 1989; Cochrane et al., 1990). In the absence of Rev, these RNAs, which code for HIV-1 structural proteins, are not exported to the cytoplasm. Molecules capable of binding to Rev might therefore exhibit anti-HIV-1 activity by sequestering the protein away from the viral genome and concomitantly inhibiting the synthesis of new viral particles. In fact, when RNA decoys of Rev are expressed in tissue culture cells they show potent antiviral activity (Lee et al., 1993, 1995).

Recent work has focused on improving such RNA decoys by selecting Rev-binding RNA molecules (aptamers) from random sequence nucleic acid pools (Bartel et al., 1991; Giver et al., 1993; Jensen et al., 1994). The primary Rev-binding element (RBE; Figure 1b) is localized within stem loop IIB of the RRE and was used as a starting point for constructing the randomized pools in several of these selections. Comparison of wild-type and selected RNA molecules allowed sequence and structural features that contributed to high-affinity Rev binding to be identified. The sequence of the wild-type RBE is shown in Figure 1b; nucleotides found to be highly conserved or invariant by *in vitro* genetic selections are indicated. The RBE can be seen to consist of an asymmetric internal loop surrounded by traditional Watson−Crick base pairs. The internal loop of the RBE features non-Watson−Crick base pairs [G_{47}:A_{73} and G_{48}:G_{71}, numbering according to Malim et al. (1990)] and a bulged U (U_{72}) residue. NMR data have confirmed several of these structural features (Battiste et al., 1994; Peterson et al., 1994). Molecular models of the wild-type RBE and several aptamer sequences show that the non-Watson−Crick pairings promote widening of the major groove and access to the α-helical RNA binding domain of Rev (Leclerc et al., 1994; Scanlon et al., 1995). The non-canonical pairs may also play a more direct role in binding: substitution of the G:G or G:A pairings with N^7-deaza-dG disrupts interactions with Rev (Iwai et al., 1992). Moreover, when a peptide corresponding to the RNA binding domain of Rev docks in the major groove of the RBE, the RNA undergoes a conformational change and the G:G pairing is subsequently stabilized (Battiste et al., 1994; Peterson et al., 1994).

While RNA decoys of Rev are effective when expressed in cells, it is still difficult to deliver such nucleic acids exogenously. Since data from *in vitro* selection studies and structural determinations have largely defined the RBE sequences and structures that are important for Rev recognition, it should prove possible to convert the RBE to a more accessible lead compound by removing extraneous residues and engineering the RNA for increased stability. An ideal method to achieve both of these goals is to introduce a chemical link between the strands of a helix. A number of modified nucleotide and non-nucleotide cross-linkers have already been incorporated across the strands of synthetic DNA and RNA helices in efforts to increase their stability

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[1] Abbreviations: RRE, Rev responsive element; RBE, rev binding element; T_m, temperature at the maximum point in a first derivative plot of an oligonucleotide melting curve; Σ, **X**, **Y**, and **Z** are linkers between oligonucleotide strands, as defined in Tables 1 and 2.

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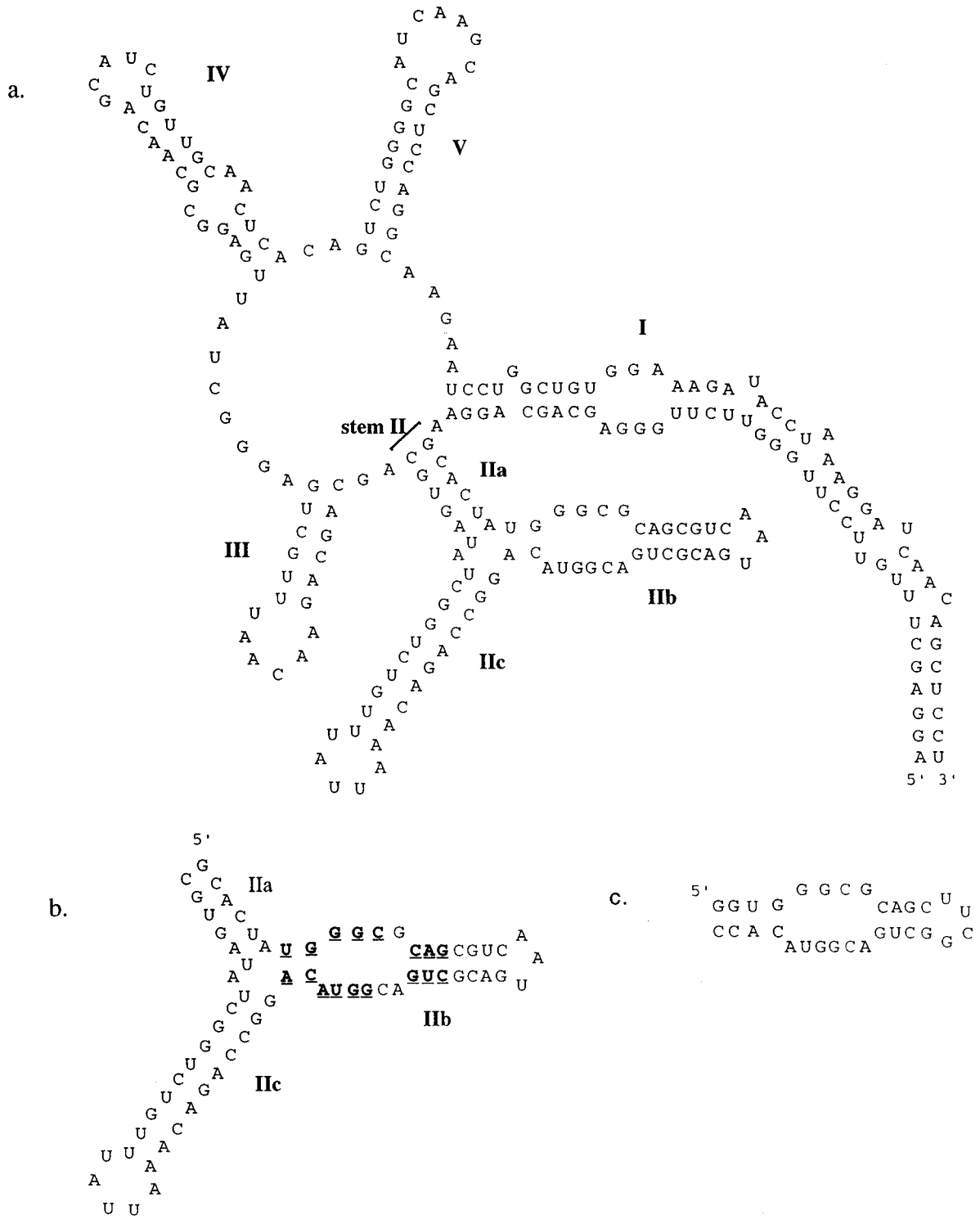


FIGURE 1: Sequence and structural features of the Rev binding element. (a) Rev Responsive Element. The sequence and predicted secondary structure of the RRE are shown. Stems are labeled according to Malim et al. (1990). (b) Rev Binding Element. The sequence and predicted secondary structure of the stem II of the RRE are shown. Stem IIB contains the primary Rev binding element. Residues that have been revealed by *in vitro* selection or mutagenesis experiments to be important for function are bolded and underlined. Numbering of residues is according to Malim et al. (1990). (c) Minimal RBE. This sequence has been shown by Bartel et al. (1991) to bind Rev and was used as a starting point for the synthesis of RNA mimetics.

and resistance to nucleases. Nonspecific cross-links can be generated using compounds such as psoralens (Tomic et al., 1987), epoxides (Millard & White, 1993), mustards (Millard et al., 1990), and nitrous acid (Kirchner et al., 1992). More specific cross-links can be introduced in a variety of ways. Ethylene glycol linkers have been shown to greatly enhance the stability of small, chemically accessible RNAs such as the TAR element of HIV-1 (Ma et al., 1993a,b). Similarly, the introduction of disulfide links into nucleic acids has been shown to enhance the stability of short DNA (Cain et al., 1995) and RNA (Goodwin & Glick, 1994) hairpins, as well as DNA triplexes (Goodwin et al., 1994). Finally, other nucleotide linkers, such as 6edDTP (Cowart & Benkovic, 1990) and psoralen-modifed deoxyadenosine (Pieles et al., 1989), have been used to form specific post-synthesis cross-links.

In the present study, we have synthesized oligonucleotide mimetics related to the RBE. In order to incorporate cross-linkers into the mimetics, the sequence and structural features essential for Rev-binding were first incorporated into hairpin structures (Figure 1c). Structural models suggested that the distance between the 5′-OH of G_{47} and the 3′-OH of A_{73} is 18.1 Å, while the distance between the 3′-OH of G_{48} and the 5′-OH of G_{71} is 19 Å. Therefore, covalent bridges capable of spanning these distances should be particularly useful. Ethylene glycol oligomer linkers of varying lengths have been shown to span from 13 to 31 Å (Rumney & Kool, 1995). Another promising linker for this purpose is a stilbenedicarboxamide derivative previously developed in one of our laboratories (Letsinger & Wu, 1994) which permits base stacking in adjacent residues and has been shown to lead to extraordinary stabilization in matched DNA duplexes (Letsinger & Wu, 1995). Our approach was to investigate whether this arene bridge can similarly stabilize hybridization of short RNA strands and whether small RNA mimetics containing this structural element can bind Rev effectively. If these molecules are capable of binding to Rev, then their enhanced stability may make them more effective as therapeutics than unmodified nucleic acids.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Oligonucleotides were synthesized on long-chain alkylamino controlled pore glass supports (80−100 mesh, 500 Å) on a Millipore Expedite DNA synthesizer (Bedford, MA). Columns containing 1 μmol of loaded nucleoside were used to initiate syntheses, and Milligen-Biosearch protocols for phosphoramidite reagents were employed (Caruthers et al., 1987). The synthesis of the stilbene-derived phosphoramidite is described elsewhere (Letsinger & Wu, 1995). This reagent was used in place of a nucleoside phosphoramidite reagent to introduce the stilbene bridging unit at the proper position during synthesis of the oligonucleotide chain on the solid support. The triethylene glycol-derived phosphoramidite (Spacer-9) (Durand et al., 1990) was purchased from Glen Research (Sterling, VA). 2′-*O*-*tert*-Butyldimethylsilyl-protected ribonucleoside β-cyanoethyl phosphoramidites with *p*-*tert*-butylphenoxyacetyl N-protecting groups were used for synthesis of oligoribonucleotides.

The DNA products were synthesized with a 5′-terminal DMT group and purified by reversed-phase high-performance liquid chromatography (RP HPLC). Samples were applied to a Hewlett Packard Hypersil ODS 5m 4.6 × 200 mm column on a Dionex chromatograph (Sunnyvale, CA) and developed with a 30 mM aqueous triethylammonium acetate (TEAA) buffer at pH 7, 1 mL/min flow rate, and a 1%/min gradient of 95:5 acetonitrile:30 mM TEAA. After concentration of the eluant and detritylation with 80% aqueous acetic acid (30 min), the fully deprotected oligonucleotides were analyzed by RP HPLC and, if necessary, rechromatographed.

For all RNA purification procedures, distilled water was treated with 0.03% diethyl pyrocarbonate and autoclaved prior to use, and buffers were prepared and used under sterile conditions. After synthesis, the CPG-bound oligomers were detritylated and the oligomers were cleaved from the support and partially deprotected by treatment with 2 mL of 3:1 ammonium hydroxide/ethanol for 12 h at 55 °C (Stawinski et al., 1993). The crude 2′-*O*-silylated RNA recovered from a given run was then divided into two equal portions. To each portion was added 0.4 mL of a 1 M solution of tetrabutylammonium fluoride (TBAF) in THF (Hogrefe et al., 1993). After 24 h at ambient temperature, 0.6 mL of 2 M TEAA was added to quench the reaction. Each sample was then concentrated on a speed-vac to ∼0.5 mL and desalted on a Pharmacia (Piscataway, NJ) NAP-5 column. In a protocol similar to that used by Whoriskey et al. (1995), crude, fully deprotected RNA mimetics were analyzed and purified (10 A_{260} units/injection) by ion exchange (IE) HPLC on a Dionex NucleoPac PA-100 (4 × 250 mm) column equipped with a Dionex NucleoPac PA-100 guard column (4 × 50 mm), at pH 12 (10 mM NaOH) with a 2%/min gradient of 1.0 M NaCl in 10 mM NaOH. Aqueous acetic acid (8%, ∼10 μL) was immediately added to each 1.0 mL of the collected RNA-containing fractions to bring the pH to 7. Following desalting on a Pharmacia NAP-10 column, the purified RNA mimetics were lyophilized and then reanalyzed under the same chromatographic conditions. No detectable degradation was observed (for a representative example see Figure 2). The recovery of RNA mimetics varied depending on the length of the oligonucleotide but was generally ∼2 A_{260} units/10 A_{260} units of crude injected product. The oligoribonucleotides (see Table 2) and their respective elution times (min) are as follows: **7Σ**, 25.6; **7Z**, 28.0; **8**, 30.9; **9**, 30.6; **10**, 35.5; **11**, 35.9; **12Σ**, 33.1; **12Z**, 37.1; **13**, 33.0; **14**, 35.2; and **15**, 35.1.

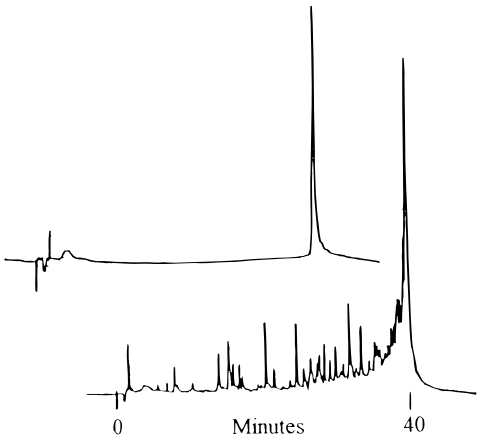


FIGURE 2: Ion exchange HPLC, pH 12 (see Experimental Section for conditions), for crude oligomer **13**. Inset, oligomer **13** after purification.

Determination of Melting Temperatures. Melting curves were recorded on a Perkin-Elmer (Foster City, CA) λ-2 UV/VIS spectrometer using solutions that were either 0.1 or 1.0 M in NaCl, 0.01 M in phosphate buffer (pH 7), and 5 μM in oligonucleotide. To ensure complete annealing of the intramolecular hybrids, all samples were warmed to 80 °C and slowly cooled to 0 °C prior to recording thermal denaturation profiles. Changes in absorbance at 260 nm were monitored while the temperature was decreased at a rate of 1 °C/min. T_m values were obtained from first-derivative plots.

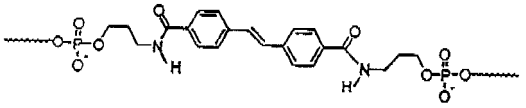
Gel-Shift Analysis. Competition experiments were carried out between labeled RRE and cold RNA mimetics, and the results were monitored by gel-shift analysis. Wild-type RRE stem II RNA (Figure 1a) was prepared as previously described (Giver et al., 1993). Briefly, a PCR product derived from plasmid pD2 was transcribed *in vitro* using an

Table 1: Oligodeoxyribonucleotide Conjugates[a]

	compounds	T_m (°C) 0.1 M NaCl	T_m (°C) 1.0 M NaCl
1	5' TGG-GCG 3' ACATGGC	<5	<5
2	5' TGG-GCG-Σ, X, or Y 3' ACATGGC-Σ		
	Σ	~58	~60
	X	40	48
	Y	36	40
3	Σ-TGG-GCG 3' ACATGGC 5'	~62	~65
4	5' GTGG-GCG-CAG 3' CACATGGCAGTC	<10	20
5	5' GTGG-GCG-CAG-Σ 3' CACATGGCAGTC	60	68
6	Σ-GTGG-GCG-CAG 3' CACATGGCAGTC 5'	61	71

[a] Watson−Crick base pairs are indicated by a dot between the bases. The dash represents a phosphodiester bond opposite an extra base in a bulge.

Σ =



X = −TTTT−; **Y** = −OP(O)(O⁻)O(CH$_2$CH$_2$O)$_3$P(O)(O⁻)O−.

Ampliscribe kit (Epicenter Technologies, Madison, WI). The RRE was radiolabeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Rev protein was the generous gift of Maria Zapp and was overexpressed in *Escherichia coli* and purified as described in Zapp et al. (1991). Labeled RNA (1 pmol) was heated in 8 μL of 1× Rev binding buffer (50 mM KCl, 50 mM Tris-HCl, pH 8) at 65 °C for 3 min and then cooled to ambient temperature. Each hairpin RNA mimetic or cold RRE competitor was similarly heat equilibrated in 10 μL of buffer. Labeled RRE and cold mimetic were then mixed together, Rev protein (~1 pmol) and RNA (2 μg, ~80 pmol) were added to the mix, and the reaction was incubated for 1 h on ice. At the conclusion of the reaction a 6× nondenaturing dye solution (4 μL) was added, the entire reaction was loaded on an 8% native gel [30:1, 0.5× TBE (0.045 M Tris-borate, 0.001 M EDTA)], and the gel was run at 5 W, 4 °C for about 2 h. The gels were dried and exposed overnight to a Molecular Dynamics (Sunnyvale, CA) Phosphorimager screen, and the number of counts in each band was quantitated.

RESULTS AND DISCUSSION

Preliminary experiments were carried out with oligodeoxyribonucleotides to determine to what extent the stilbenedicarboxamide bridge would stabilize the hybridization of short DNA sequences containing non-Watson−Crick base pairs (dGA and dGG) and bulged residues. The T_m data in Table 1 show that this bridge is in fact a very effective structural stabilizer. Both in the case of duplex **1**, a system containing thirteen nucleotides with one bulge and four canonical base pairs, and duplex **4**, a system containing twenty-two nucleotides with two bulges and eight canonical base pairs, covalent linkage of the strand termini by the stilbenedicarboxamide derivative led to an increase in T_m of more than 50 °C in 0.1 M NaCl (compare conjugate **2Σ** with control duplex **1** and conjugate **5** with control duplex **4**). For these systems the effect was comparable whether the bridge was joined at the right or left end of the duplex (compare **3** with **2Σ** and **6** with **5**). As demonstrated by the data for **2Σ**, **2X**, and **2Y**, the stilbenedicarboxamide bridge is considerably more effective in stabilizing these complexes than either a tetrathymidylate loop or a triethyleneglycol bridge.

Table 2: Oligoribonucleotide Conjugates[a]

	compounds	T_m (°C) 0.1 M NaCl	T_m (°C) 1.0 M NaCl
7	5' UUUUUU-Σ, or **Z** 3' AAAAAA-Σ	44 40	49 50
	Σ	~40	~46
	Z		
8	5' UGG-GCG-Σ 3' ACAUGGC-Σ	~42	46
9	Σ-UGG-GCG 3' ACAUGGC 5'	52	56
10	5' UGG-GCG-CA-Σ 3' ACAUGGCAGU-Σ	50	55
11	Σ-UGG-GCG-CA 3' ACAUGGCAGU 5'		
12	5' GUGG-GCG-CAG-Σ, or **Z** 3' CACAUGGCAGUC-Σ	60 60	68 66
	Σ		
	Z	62	68
13	Σ-GUGG-GCG-CAG 3' CACAUGGCAGUC 5'	57	64
14	5' CUGG-ACUC--CG-Σ 3' GACAACGAGUUGC-Σ	58	65
15	Σ-CUGG-ACUC--CG 3' GACAACGAGUUGC 5'		

[a] Σ represents the stilbenedicarboxamide bridge, as in Table 1. **Z** represents the oligonucleotide bridge, −UUCG−. Other abbreviations are the same as in Table 1.

We then turned to RNA mimetics, first looking at UUUUUU-Σ-AAAAAA (**7Σ**) to see if a stilbenedicarboxamide bridge would also favor the hybridization of RNA fragments. A markedly stabilized structure (T_m 44 °C, 0.1 M NaCl) was indeed obtained. The T_m values for this compound are comparable to those for UUUUUU-(UUCG)-AAAAAA (**7Z**, Table 2), which contains a tetranucleotide sequence found to be unusually effective in stabilizing hairpin conformations of short oligoribonucleotide duplex segments (Cheong et al., 1990; Varani et al., 1991). In line with the observation that poly(U)/poly(A) dissociates at a lower temperature than poly(dT)/poly(dA) (Felsenfeld, 1967), the T_m for **7Σ** was 14 °C lower than that for the corresponding deoxyribonucleotide conjugate, dTTTTTT-Σ-dAAAAAA (T_m 58 °C; Letsinger & Wu, 1995). The fluorescence spectrum of **7Σ** exhibits a distinctive band, λ_{max} 330 nm, that is characteristic of a non-associated stilbenedicarboxamide group (Letsinger & Wu, 1995). This spectrum is therefore consistent with a cross-linked duplex conformation for **7Σ** and is inconsistent with formation of a bimolecular duplex that aligns the stilbene groups in proximity.

Thermal dissociation data for the oligoribonucleotide conjugates are listed in Table 2 and representative melting curves are shown in Figure 3. In all cases the stilbenedi-

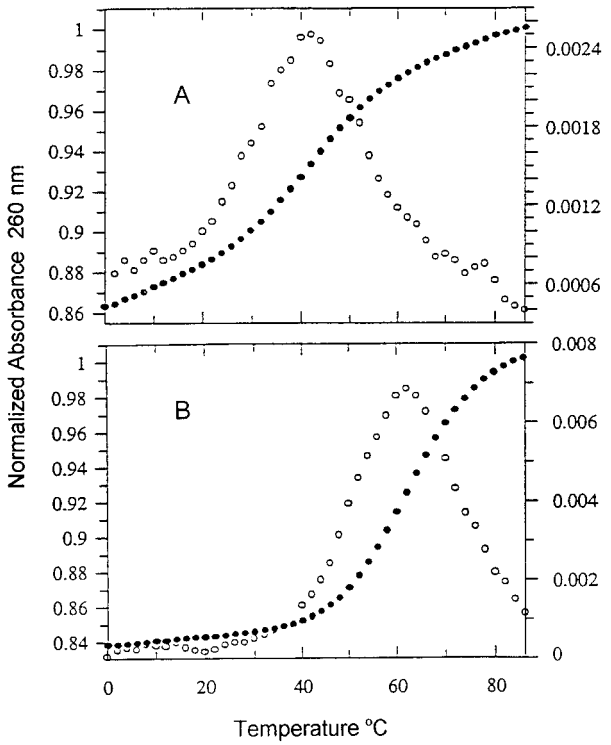


FIGURE 3: Thermal denaturation (●) and derivative curves (○) for (A) oligomer **9** and (B) oligomer **13**, in 0.1 M NaCl, 10 mM Tris-HCl, pH 7.0.

carboxamide bridge afforded unusually stable hybridized structures. The associative complexes of the short RNA mimetics, **8** and **9**, are less stable than the corresponding DNA analogues and exhibit broad transitions. The T_m values for these compounds are independent of oligonucleotide concentration over a 10-fold range, consistent with a monomolecular denaturation that may involve a hairpin conformation (data not shown). The longer RNA mimetics (**10**−**15**) exhibit sharper transitions (see Figure 3 for a representive profile) and considerably higher T_m values. As in the case of **7**Σ and **7Z,** the thermal stability of the low-temperature conformation of the stilbenedicarboxamide conjugate (**12**Σ) is comparable to that for an oligonucleotide (**12Z**) containing a tetraloop, UUCG, in place of a stilbenedicarboxamide bridge.

We assayed whether the RNA mimetics could compete with stem II of the RRE, which contains all sequences necessary for Rev responsiveness and is known to bind Rev with high affinity (Zapp & Green, 1989; Heaphy et al., 1990). Binding was monitored using both gel mobility shift (Figure 4) and filter-binding assays (data not shown). The short oligomer conjugates, **8** and **9**, serve as mimetics for a small domain "involved with initial high affinity Rev recognition" (Pritchard et al., 1994). However, these mimetics were incapable of Rev binding, even at high concentration levels. Results from filter-binding assays, though not quantitated, were similar to those obtained through the gel-shift analysis. The intermediate length mimetics, **10** and **11**, also appeared to have little or no capacity for Rev binding (under our assay conditions $K_i > 1\ \mu M$). These compounds mimic the "core element" described by Bartel et al. (1991) minus one C:G base pair. The longest mimetics, **12**Σ and **13,** were found to bind to Rev at a level comparable to that for the stem loop IIB RNA. For compound **13** (bridge to the left as written), the K_i was about twice that of RRE stem loop IIB ($K_d \approx$

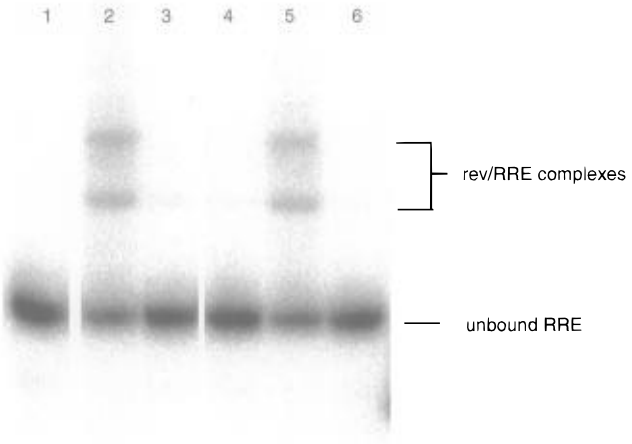


FIGURE 4: Gel-shift analysis. Oligomers were assayed for their ability to inhibit a Rev/RRE gel shift (see Experimental Section for conditions). Lane 1, 94 nt radiolabeled RRE RNA. Lane 2, complex of RRE RNA with Rev protein. Lane 3, complex with addition of oligomer **12**Σ. Lane 4, with addition of oligomer **13**. Lane 5, with addition of oligomer **5**. Lane 6,with addition of cold RRE RNA.

1−3 nM; Heaphy et al., 1990). The binding affinity of mimetic **12**Σ (bridge to the right) was slightly lower. On the basis of K_i values, our estimations of dissociation constants are ∼5 nM for mimetic **13** and ∼10 nM for mimetic **12**Σ. For further comparison, the binding affinity of **12Z**, which is stabilized by a UUCG tetraloop rather than a stilbenedicarboxamide group, was similarly examined. We estimated the K_d to be ∼20 nM for this compound. As expected, the control DNA mimetic **6** (with a sequence corresponding to the best RNA mimetic tested, oligomer **13**) was found incapable of Rev binding ($K_i > 1\ \mu M$).

Compounds **14** and **15** were designed to mimic aptamer sequences that bind Rev more effectively than the wild-type RRE (Giver et al., 1993). Although a quantitative assessment of K_d was not carried out with these mimetics, visualization of the titrations suggests that they bind Rev as well as mimetic **12**, but no better. There are several possible explanations for the failure to exhibit elevated affinity for Rev. The sequences incorporated into mimetics **14** and **15** were originally identified in longer sequences, and may not have the same effect in the shorter mimetics examined in this work. Alternatively, if the rigid stilbene bridge locks the strands of the aptamers in a nonideal conformation, this could explain why enhanced binding ability is not observed in the mimetics.

In summary, these examples demonstrate that a tailored, relatively rigid non-nucleotide bridge can be utilized to stabilize the hybridization of short DNA and RNA duplex segments containing mismatched and looped out nucleotides. A stilbenedicarboxamide bridge is found to be more effective than a TTTT tetraloop or a triethylene glycol linker in stabilizing DNA constructs and to be as effective as a UUCG tetraloop in stabilizing short RNA segments. Most importantly, RNA mimetics with a molecular weight of approximately 7800 daltons can still bind to Rev almost as well as the wild-type RRE. These mimetics are roughly 2100 Daltons smaller than the smallest known Rev-binding element, 18 300 daltons smaller than the best Rev-binding aptamer, and almost 70 000 daltons smaller than the RRE itself. These results suggest that such bridges used in conjunction with *in vitro* selections and chemical modifica-

tions to enhance resistance to nucleases, may prove useful in converting an array of oligonucleotides to small molecule mimetics for potential therapeutic applications.

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