Selection of Structure-Specific Inhibitors of the HIV Rev–Rev Response Element Complex

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Abstract: We devised a single-step selection method to identify short sequences within the folded Rev response element (RRE) of the human immunodeficiency virus (HIV) RNA genome that are proximal and able to bind short oligonucleotides. The method employed a library of partially randomized tethered oligonucleotide probes (TOPs) complementary to all regions of the RRE and an RNase H cleavage assay which identified those RRE regions preferred by the TOPs. Six short sequences were identified. Two TOPs synthesized on the basis of this selection, S1-5-S2 and S1-1-S2, were potent, concentration-dependent inhibitors of a RRE function *in vitro* and abolished interaction of the RRE with the HIV regulatory protein Rev at nanomolar concentration (25 °C, 0.8 nM RRE, 45 nM Rev). TOPs S1-5-S2 and S1-1-S2 exhibited greater potency and faster association kinetics than traditional oligonucleotides targeted to the same regions. The random TOP selection procedure described here allows rational design of oligonucleotide-based ligands for RNA that exploit (rather than avoid) the complex structure of the RNA target.

The manipulation of sequence-specific protein binding and gene expression using antigene or antisense technology requires oligonucleotides that bind DNA or RNA with high affinity and specificity.[1-4] Oligonucleotides capable of selective recognition of RNA must accommodate, in addition, both the kinetic[5,6] and the thermodynamic[6,7] consequences of a nonuniform RNA conformation. Although formally single-stranded, RNA molecules base-pair intramolecularly and the resultant secondary structures fold to generate tertiary architecture ("self-structures") of greater complexity than that found in DNA.[8-11] The structural complexity inherent in RNA complicates recognition because it limits the accessibility of single-stranded regions that must be differentiated for specific binding. It is estimated that 11–15 nucleotides are necessary to define a unique mRNA sequence in a eukaryotic cell,[12] yet few well-characterized RNA secondary structures contain contiguous single-stranded regions of this size. Secondary structures, moreover, do not reveal which single-stranded regions in a folded RNA are accessible to oligonucleotides.[13,14] Antisense oligonucleotides that must disrupt structure to pair with their complements, if they bind, often do so with slow association kinetics, or low affinities, or both.[5,6]

We have described a class of synthetic molecules that bind RNA on the basis of both sequence and structure, thereby accommodating the structural complexity of RNA.[15,16] Tethered oligonucleotide probes (TOPs) consist of two short oligonucleotides joined by a tether whose length and composition are varied using chemical synthesis (Figure 1B). In contrast with traditional antisense oligonucleotides that recognize a single, contiguous RNA sequence, TOPs recognize two short, noncontiguous sequences that are proximal in the folded RNA.[6,15,16] Because TOPs have the potential to bind simultaneously to two accessible sequences, rather than one long sequence that may not be fully accessible, we predicted that TOPs could exhibit very high affinities for structured RNA targets.[6] We also predicted that TOPs might equilibrate more rapidly with their targets than oligonucleotides that must disrupt a stable conformation in order to bind.

The human immunodeficiency virus (HIV) contains at least two structured RNAs that interact with viral proteins and mediate novel genetic regulatory pathways. One such RNA is a 234 nucleotide sequence located within the *env* coding region called the Rev response element (RRE). Interaction of the RRE with the viral protein Rev[17,18] regulates the appearance of unspliced or singly spliced viral mRNAs in the cytoplasm of infected cells.[19-27] Without these mRNAs, structural proteins do not accumulate and the virus cannot replicate.[28,29] The primary binding region for Rev is located between RRE nucleotides G39 and C104 (Figure 1A).[20,30,31] The secondary structure proposed for the RRE in the absence of Rev is based on free energy

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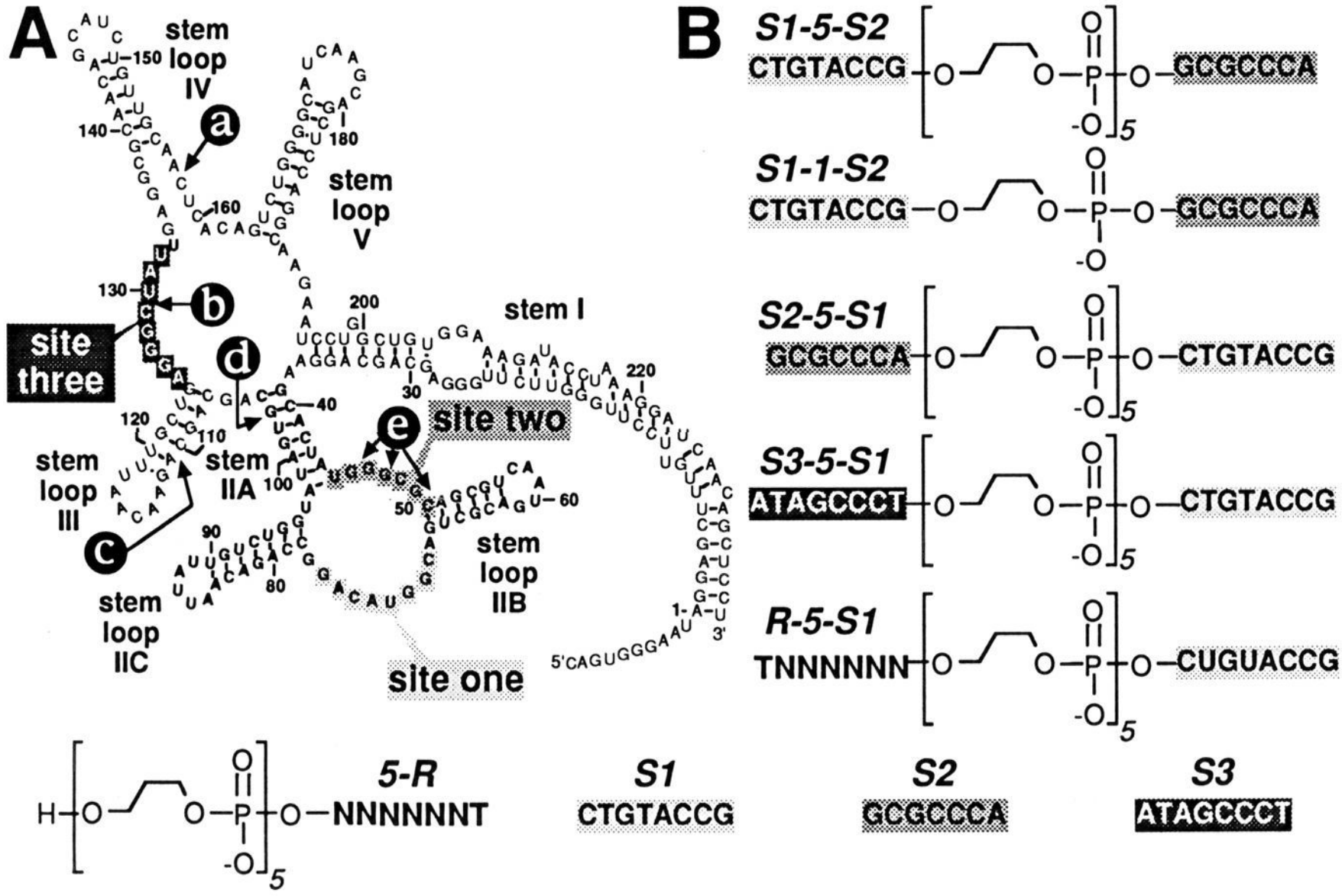

Figure 1. (A) Proposed secondary structure of the RRE in the absence of Rev.[20,32] Additional Watson–Crick or non-Watson–Crick interactions within the primary Rev binding region (G39–C104) may exist in the free RRE or be induced by the binding of Rev.[32–35] RNase H cleavage sites described in the text are indicated by arrows. (B) TOPs and single-site probes used in this study.

calculations[20] and chemical probing experiments.[32] Additional Watson–Crick or non-Watson–Crick interactions within the primary Rev binding region may exist in the free RRE or may be induced by the binding of Rev.[32–35]

Although the molecular mechanism of the Rev response is complex, molecules capable of blocking the Rev:RRE interaction have the potential to block viral replication.[36,37] Examination of the proposed RRE secondary structure revealed several short, single-stranded regions that might be appropriate targets for a TOP. In order to identify which regions would be most suitable for TOP binding, we designed a single-step selection experiment[38–43] using a random-TOP (R-TOP) library to identify single-stranded regions within the RRE that are proximal to one another and able to bind short oligonucleotides. Herein we describe this selection experiment and report that two TOPs synthesized on the basis of our results, S1-5-S2 and S1-1-S2 (Figure 1B), are potent, concentration-dependent inhibitors of Rev:RRE complex formation. Moreover, TOPs S1-5-S2 and S1-1-S2 exhibit greater potency and faster association kinetics than traditional oligonucleotides of similar length that must disrupt native secondary structure to bind fully.

Experimental Section

All buffer pH values refer to measurements at 25 °C.

Synthesis of Tethered Oligonucleotide Probes. Oligonucleotides were synthesized following the solid-phase phosphoramidite method[44] on an Applied Biosystems 380B Synthesizer. TOPs were synthesized using methods described previously.[15] Deoxyribonucleoside 2-cyanoethyl phosphoramidites were purchased from ABI. Ribonucleoside 2-cyanoethyl phosphoramidites were purchased from Peninsula or ABI. TOPs containing only deoxyribonucleotides were cleaved from the solid support (NH_4OH, 1 h, 25 °C), deprotected (NH_4OH, 8 h, 55 °C), and purified by use of denaturing 20% (20:1 cross-linked) polyacrylamide gels. The randomized sequences in TOPs R-5-S1 and 5-R were generated by reacting an equimolar mixture of the four nucleoside phosphoramidites during the appropriate synthesis cycles. TOPs containing ribonucleotides (or both ribonucleotides and deoxyribonucleotides) were cleaved from the solid support (3:1 EtOH:NH_4OH, 1 h, 25 °C), deprotected (3:1 EtOH:NH_4-OH, 8 h, 55 °C), desilylated (1 M $(n\text{-Bu})_4NF$, THF, 12 h, 25 °C), and purified by use of denaturing 20% (20:1 cross-linked) polyacrylamide gels.[45] Oligonucleotide homogeneity was assessed by high-resolution gel electrophoresis of 5′-end labeled ([γ-^{32}P]ATP, T4 polynucleotide kinase) material.[46] All TOPs were >95% homogeneous by this criterion.

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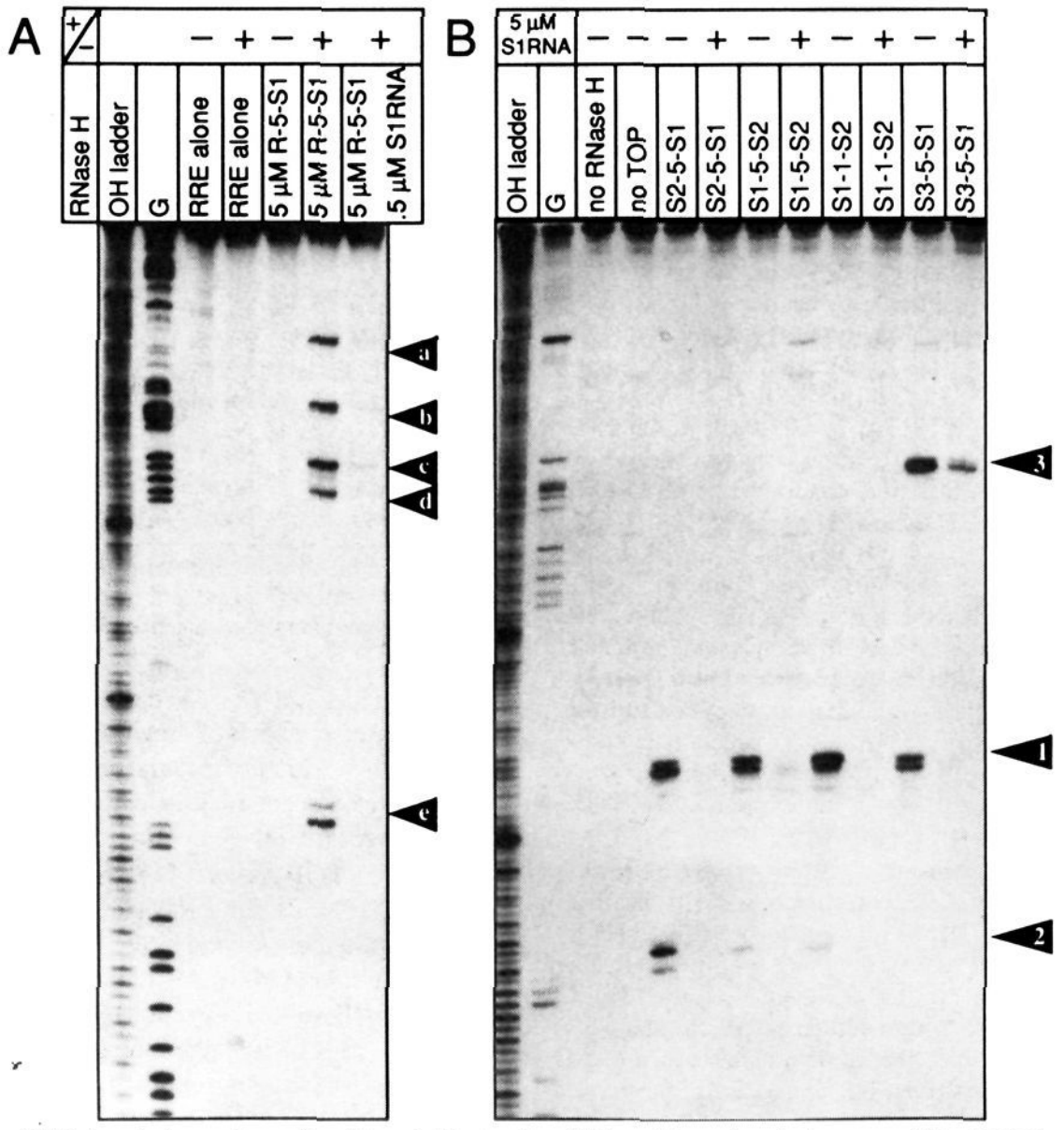

Figure 2. Autoradiograms of 8% denaturing polyacrylamide gels illustrating RNase H catalyzed cleavage of the RRE in the presence of TOPs. All lanes contain 6 nM [5′-^{32}P]RRE, 0.2 mM HEPES (pH 7.5) (0.5 mM in Figure 2B), 15.25 mM Tris (pH 7.5) (14.75 mM Tris in Figure 2B), 26.25 mM NaCl, 185 mM KCl, 2 mM $MgCl_2$, 2 mM $Mg(OAc)_2$, 0.1 mM EGTA, 0.7 mM EDTA, 27.5 μg/μL BSA, 4 ng/μL tRNA, 5.5 mM DTT, 5.4% glycerol (6.25% glycerol in Figure 2B), and 10–30 units of RNasin (Promega). (A) R-TOP R-5-S1. Oligonucleotide concentrations are indicated above each lane. Arrowheads and letters correspond to cleavage sites described in the text and mapped in Figure 1A. (B) TOPs S2-5-S1, S1-5-S2, S1-1-S2, and S3-5-S1. TOPs are present at a concentration of 20 nM. Arrowheads correspond to the locations of target sites one, two, and three.

Synthesis of RRE RNA. RNA comprising RRE nucleotides 7336–7579 of HIV-1 HXB-3[47] (renumbered 1–234 in Figure 1A) was prepared by T7 RNA polymerase (New England Biolabs) catalyzed runoff transcription[48,49] of a DNA template prepared by mutagenic PCR[50] amplification of pRRE provided by J. Kjems.[32] Transcribed RRE was purified by preparative gel electrophoresis and ethanol precipitation. Nucleotides 1–203 of the RRE transcript were verified using primer extension sequencing.[51,52] The purified RRE transcript was dephosphorylated using CIAP (Boehringer Mannheim) and 5′-phosphorylated with [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear) and T4 polynucleotide kinase (New England Biolabs) using protocols recommended by the suppliers.

RNase H Assays. 5′-^{32}P end-labeled RRE was renatured at a concentration of 60 nM in renaturation buffer (10 mM Tris (pH 7.5), 50 mM NaCl) by heating at 80 °C for 5 min and cooling at 25 °C for 15 min. Renatured RRE was added to the appropriate TOP or oligonucleotide dissolved in water and incubated for 1 h at 25 °C. RNase H (Pharmacia) (0.9 units) was added, and the reactions were incubated for 3 h at 25 °C. Final concentrations were as follows: 6 nM RRE, 0.2 mM HEPES (pH 7.5) (0.5 mM in Figure 2B), 15.25 mM Tris (pH 7.5) (14.75 mM Tris in Figure 2B), 26.25 mM NaCl, 185 mM KCl, 2 mM $MgCl_2$, 2 mM $Mg(OAc)_2$, 0.1 mM EGTA, 0.7 mM EDTA, 27.5 μg/μL BSA, 4 ng/μL tRNA, 5.5 mM DTT, 5.4% glycerol (6.25% glycerol in Figure 2B), 10–30 units of RNasin (Promega). RNase H reactions were quenched by the addition of 10 μg of tRNA, NaCl to a final concentration of 360 mM, and 3 volumes of ethanol. The reaction products were precipitated at −70 °C, diluted to constant cpm/μL with 5 M urea, and fractionated on denaturing (7 M urea) 8% (20:1 cross-linked) polyacrylamide gels alongside hydroxide and G-specific cleavage lanes. Sites of RNase H cleavage were determined to ±1 base resolution.[6] Less than 10% of the RRE was cleaved under these conditions, suggesting that each cleavage band resulted from RNase H attack on complexes with full-length RRE.

Determination of Dissociation Constants. Equilibrium dissociation constants of the TOP:RRE complexes were measured using direct and competition electrophoretic mobility shift assays[53] as described previously.[6] For competition assays (used for all TOPs except S3-5-S1), the fractional saturation of 0.5 nM 5′-^{32}P end-labeled S1-1-S2 bound to 120 nM unlabeled RRE was monitored as a function of the concentration of unlabeled competitor TOP. Final buffer conditions were as follows: 11.25 mM Tris (pH 7.5), 26.25 mM NaCl, 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 7.5 μg/μL BSA, 4 ng/μL tRNA, 1.5 mM DTT, 10% glycerol. A 1-h incubation time was used to ensure that equilibrium had been established. For direct mobility shift assays (used for S3-5-S1), the fractional saturation of 0.5 nM 5′-^{32}P end-labeled S3-5-S1 was measured as a function of the concentration of unlabeled RRE. Final buffer conditions were as follows: 11.25 mM Tris (pH 7.5), 26.25 mM NaCl, 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 7.5 μg/μL BSA, 4 ng/μL tRNA, 1.5 mM DTT, 10% glycerol. Resulting mixtures were incubated 3 h at 25 °C before electrophoresis. Data were analyzed as previously described.[6]

Rev. Rev protein was obtained from Henrik Steen-Olsen (Hoffmann-LaRoche, Nutley, NJ) and was used without further purification. Rev

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was stored at −70 °C at a concentration of 4.5 μM in storage buffer (10 mM Tris (pH 7.5), 20 mM NaCl, 204 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 7.5 μg/μL BSA, 4 ng/μL tRNA, 1.5 mM DTT, 10% glycerol). Immediately prior to a binding reaction, a 20-μL aliquot of the Rev stock was thawed and diluted to the appropriate concentration with binding buffer (10 mM Tris (pH 7.5), 20 mM NaCl, 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 7.5 μg/μL BSA, 4 ng/μL tRNA, 1.5 mM DTT, 10% glycerol).

IC_{50} Determination. 5′-^{32}P end-labeled RRE was renatured at a concentration of 6 nM in renaturation buffer by heating at 80 °C for 5 min and cooling at 25 °C for 15 min. Renatured RRE was added to freshly diluted (water) oligonucleotide or TOP and incubated for 1 h at 25 °C in a buffer containing 11.7 mM Tris (pH 7.5), 28.3 mM NaCl, 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 7.5 μg/μL BSA, 4 ng/μL tRNA, 1.5 mM DTT, and 10% glycerol. To this solution was added Rev (freshly diluted in binding buffer) to a final concentration of 45 nM. Final concentrations: 0.8 nM RRE, 11.25 mM Tris (pH 7.5), 26.25 mM NaCl, 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 7.5 μg/μL BSA, 4 ng/μL tRNA, 1.5 mM DTT, 10% glycerol. After 15 min at 25 °C, the samples were loaded onto nondenaturing (50 mM Tris (pH 8.8), 50 mM glycine) 10% (79:1 cross-linked) polyacrylamide gels running at 300 V. Gels were maintained at 4–6 °C during electrophoresis by immersion in running buffer (50 mM Tris (pH 8.8), 50 mM glycine) cooled by a circulating, temperature-controlled water bath. The amounts of complexed and free RRE were quantified using a Betagen 605 Blot Analyzer. The IC_{50} was defined as the concentration of inhibitor required to reduce the maximum observed fraction RRE complexed by 50%.

Pre-equilibration, Inhibition Experiment. 5′-^{32}P end-labeled RRE was renatured in renaturation buffer at a concentration of 6 nM by heating at 80 °C for 5 min, then cooling at 25 °C for 15 min. Renatured RRE was then added to tubes containing 2X binding buffer. At 60, 40, 30, 20, 10, or 0 min before addition of Rev, TOP or oligonucleotide (390 nM freshly diluted in water) was added. After addition of Rev, the reactions were incubated 15 min at 25 °C, then loaded onto nondenaturing (50 mM Tris (pH 8.8), 50 mM glycine) 10% (79:1 cross-linked) polyacrylamide gels running at 300 V. Final concentrations were as follows: 45 nM Rev, 0.8 nM RRE, 11.25 mM Tris (pH 7.5), 26.25 mM NaCl, 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 7.5 μg/μL BSA, 4 ng/μL tRNA, 1.5 mM DTT, 10% glycerol. Gels were maintained at 4–6 °C during electrophoresis by immersion in running buffer cooled by a circulating, temperature-controlled water bath. The amounts of complexed and free RRE were quantified using a Betagen Blot Analyzer.

Results and Discussion

Random-TOP Selection Protocol. In order to identify which regions of the RRE would be most suitable for TOP binding, we designed a single-step selection experiment[38-43] that used a random-TOP (R-TOP) library to identify single-stranded regions within the RRE that were proximal to one another and able to bind short oligonucleotides. The R-TOP library consisted of 4096 (4^6) different molecules, each containing the oligoribonucleotide complementary to an eight nucleotide region within the primary Rev binding site (C69–G76, site one) joined with a tether to a different heptadeoxyribonucleotide of random sequence. The heptadeoxyribonucleotide pool was generated by randomizing six bases within the sequence d(N_6T), where N represents an equimolar mixture of all four possible bases (Figure 1B). In our selection experiment, the oligonucleotide of defined sequence (the "anchor") positions the R-TOP at site one, allowing the oligonucleotide of variable sequence (the "selector") to search for accessible regions of appropriate sequence. All members of the R-5-S1 library should be able to bind the RRE at site one, but only some should be able to bind at a second site simultaneously and exhibit a corresponding increase in RRE affinity.[6,15,16] Under equilibrium conditions of limiting RRE, the predominant RRE:R-TOP complexes should include only those members of the R-TOP pool that bind most avidly to the RRE. To determine the locations of the second binding sites, we used an RNase H cleavage assay and end-labeled RRE. Since RNase H selectively hydrolyzes RNA:DNA hybrid duplexes, we could identify those RRE regions hybridized to the "selector" oligonucleotide by subjecting the RNase H reaction products to high-resolution gel electrophoresis.[6,15,16,54] We chose to anchor the R-5-S1 library to site one on the basis of previous results showing that nucleotides within this site are protected from chemical probes in the presence of Rev[32] and react readily with RNase H in the presence of a complementary DNA octamer (not shown).

Table 1. Dissociation Constants of TOP:RRE Complexes and IC_{50} Values for Inhibition of the Binding of Rev

probe	K_d (nM)	$\Delta G°_{obs}$[b] (kcal mol^{-1})	IC_{50} (nM)
S1	4361. ± 294	−7.3	>17 000.
S2	17664. ± 451	−6.5	>25 000.
S3	ND	ND	>25 000.
S1-5-S2	20. ± 4	−10.5	14.
S2-5-S1	103. ± 6	−9.5	216.
S1-1-S2	34. ± 6	−10.2	14.
S3-5-S1	463 ± 109.[a]	−8.6	3 900.
S1 + S2[c]	ND	ND	3 180.
S1 + S3[c]	ND	ND	2 900.
S1EXT	ND	ND	393.[d]
S2EXT	ND	ND	198.[d]
GRE-20A	ND	ND	>25 000.
$tRNA^{phe}$	ND	ND	8 880.

[a] Determined by direct band shift titration. [b] $\Delta G°_{obs} = RT \ln(1/K_d)$ where $R = 0.001\ 987\ 2$ kcal mol^{-1} deg^{-1}; $T = 298$ K. [c] Equimolar amounts of each oligonucleotide were used. [d] 14 h of incubation.

Random-TOP Assay. Treatment of 6 nM 5′-^{32}P labeled RRE with 5 μM R-5-S1 and RNase H led to cleavage of the RRE at five discrete loci (second sites) (Figures 1A and 2A). Cleavage was observed at G46, G49, and C51 in the loop separating stems IIA and IIB (e in Figure 2A), G103 in stem IIA (d), C110 in stem III (c), C129 in the loop separating stems III and IV (b), and C155 in the loop separating stems IV and V (a). In order to demonstrate that the RNase H cleavage bands observed in the presence of R-5-S1 resulted from simultaneous binding of an R-TOP to site one and each of the second sites, we performed a competition experiment using the oligoribonucleotide CUGUACCG (S1RNA) complementary to the sequence at site one (Figure 1B).[15,16] RNase H cleavage at each of the five second sites was inhibited by 0.5 μM S1RNA (Figure 2A). In addition, no RNase H cleavage was observed when the RRE was incubated with 5 μM of an oligonucleotide library (5-R, Figure 1B) that was not anchored to site one (data not shown). Taken together, these results demonstrate that the RNase H cleavage observed at each second site resulted from simultaneous hybridization of an R-TOP at site one and a second site. In summary, the R-TOP library identified five regions of RRE sequence space—second sites—that were available to a TOP anchored to site one (Figure 1A).

Two of the five selected regions (sites two and three) are protected from chemical probes in the presence of Rev.[32] Therefore, we asked whether TOPs targeted to sites one and two or one and three might bind the RRE with high affinity and block binding by Rev. Although the R-5-S1 pool contained one of four possible tether orientations, we synthesized TOPs containing tethers in two orientations as well as tethers of different length (Figure 1 (bottom)). Single-site probes complementary to sites one, two, or three (S1, S2, or S3, respectively) were prepared for comparison. Control experiments demonstrated that each TOP (20 nM) elicited RNase H cleavage of the RRE only at its complementary sites and that cleavage at both TOP binding sites was inhibited by S1RNA (Figure 2B).

Dissociation Constants of RRE:TOP Complexes. TOPs were characterized first in terms of their affinity for the RRE (Table 1). Equilibrium dissociation constants of most TOP:RRE complexes were measured using a competition assay in which the

(54) Donis-Keller, H. *Nucleic Acids Res.* **1979**, *7*, 179. This assay identifies those RRE regions that form duplexes which are good RNase H substrates. There may be regions within the RRE that bind an R-TOP well and are not detected in this assay.

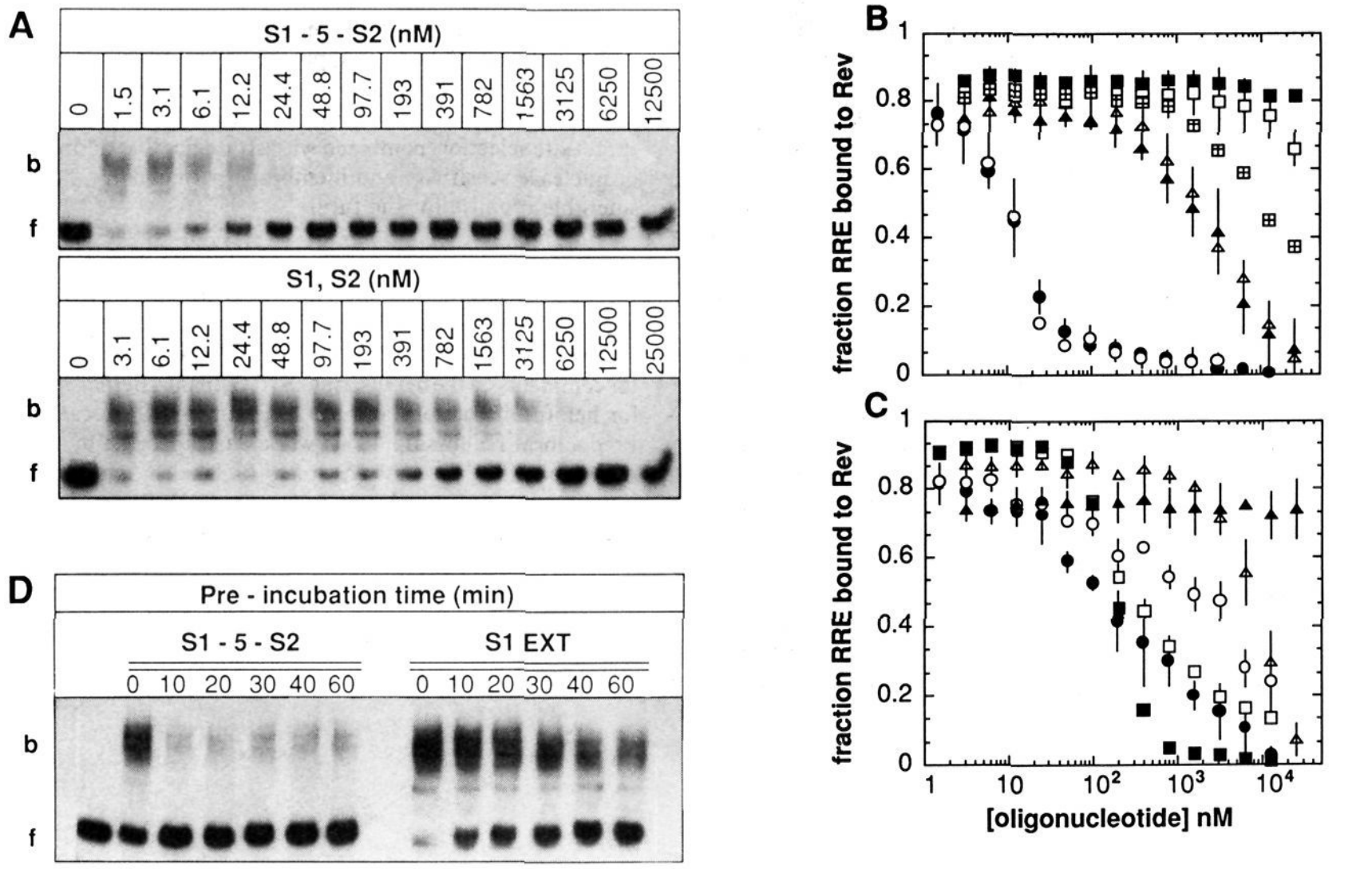

Figure 3. Inhibition of Rev:RRE copmlex formation by TOPs. (A) Autoradiograms illustrating electrophoretic mobility shift assays used to compare S1-5-S2 to a mixture of S1 and S2 as inhibitors of Rev:RRE complexation. Oligonucleotide concentrations are indicated above each lane. Free (f) and bound (b) RREs are indicated. (B,C) Semilogarithmic plots illustrating the fraction of RRE bound to Rev as a function of inhibitor concentration. B: ●, S1-5-S2; ○, S1-1-S2; ▲, S1 + S2; △, S1 + S3; ⊞, S1; ■, S2; □, S3. C: ●, S2-5-S1; ○, S3-5-S1; ▲, GRE-20A; △, tRNAPhe; □, S1EXT; ■, S2EXT. Points represent the average of three trials (two for S1EXT and S2EXT). Error bars represent one standard deviation. (D) Autoradiograms illustrating electrophoretic mobility shift assays demonstrating the effect of preincubation time on Rev inhibition by S1-5-S2 and S1EXT.

fractional saturation of 5′-^{32}P labeled S1-1-S2 bound to unlabeled RRE was monitored as a function of an unlabeled competitor probe.[6] Direct mobility shift assays used for the S3-5-S1:RRE complex monitored the fractional saturation of 5′-^{32}P end-labeled S3-5-S1 as a function of the concentration of unlabeled RRE. Examination of the K_ds reveals that TOPs targeted to sites one and two bound the RRE with dissociation constants in the nanomolar range and displayed higher RRE affinities than those targeted to sites one and three. All TOPs showed significantly higher RRE affinities than single-site probes S1 or S2, which bound with dissociation constants in the micromolar range. Tether orientation had a measurable effect on RRE affinity: S1-5-S2 bound the RRE with a 20 nM dissociation constant, whereas S2-5-S1 bound with a 103 nM dissociation constant. This difference in affinity was not due to the inherent stabilities of the two different duplexes, as S1-5-S2 and S2-5-S1 bound their respective complementary RNA sequences with similar affinities of ~100 pM (data not shown). That tether orientation affected RRE affinity suggested either that S2-5-S1 was incompletely hybridized to its target or that hybridization required disruption of secondary or tertiary interactions within the RRE itself, or both. The apparent stability of the RRE complexes with S1-5-S2 and S1-1-S2 suggested that these TOPs might provide a stable block to RRE recognition by Rev.

TOPs Block RRE Binding by Rev. Experiments were performed to evaluate the ability of TOPs to block the binding of Rev to the RRE. Using an electrophoretic mobility shift assay we determined the concentration of TOP or oligonucleotide required to decrease the fraction RRE bound to Rev by 50% (Table 1, Figure 3). Curves illustrating concentration-dependent inhibition of Rev binding are shown in Figure 3. Single-site probes S1, S2, or S3 at a concentration of 1.5 μM had no effect on binding of Rev, and equimolar mixtures of S1 and S2 or S1 and S3 had little effect (IC_{50} = 3.2 μM and 2.9 μM, respectively) (Figure 3B). In contrast, S1-5-S2 and S1-1-S2 were potent, concentration-dependent inhibitors of Rev binding, exhibiting IC_{50} values of 14 nM (Figure 3A). Inhibition by the various TOPs depended on both tether orientation and target site location. S2-5-S1 was complementary to the same RRE nucleotides as S1-5-S2 but was 16-fold less effective. S3-5-S1 was complementary to sites one and three and showed a significant RRE affinity (as measured by K_d), yet was no more effective a Rev inhibitor than a mixture of S1 and S3. GRE-20A, a 19-mer noncomplementary oligonucleotide, showed no inhibition at 25 μM, and yeast tRNA^{Phe} exhibited an IC_{50} of 8.9 μM. Mobility shift assays detected no evidence for a stable complex between end-labeled S1-5-S2 or S2-5-S1 (0.5 nM) and the Rev protein at concentrations between 6 and 769 nM, suggesting that the observed inhibition was not the result of binding to Rev. The potency of S1-5-S2 and S1-1-S2 as Rev inhibitors demonstrates that TOPs designed to complement two RRE regions selected on the basis of accessibility bind with high affinity and thereby block the formation of an essential RNA–protein complex.

Comparison of S1-5-S2 and S1-1-S2 with Traditional Oligonucleotides of Similar Length. The previous results indicated that TOPs S1-5-S2 and S1-1-S2 are more potent Rev inhibitors than the short oligonucleotides from which they are composed. We also compared S1-5-S2 and S1-1-S2 with two traditional oligonucleotides of similar length that targeted sites one or two and the adjacent structured stem loops. S1EXT is a 18-mer (CTGTACCGTCAGCGTCAT) complementary to site one and the adjacent region of stem IIB (A59–G76), and the previously studied S2EXT[37] is an 18-mer (GACGCTGCGCCCATAGTG) complementary to site two and the adjacent regions of stems IIA and IIB (C40–C57). Both S1EXT and S2EXT were substantially weaker inhibitors than S1-5-S2 and S2-5-S1 (Table 1). Moreover,

whereas S1-5-S2 equilibrated quickly with the RRE, both S1EXT and S2EXT bound the RRE slowly. At a concentration of 390 nM, S1EXT required at least 40 min of preincubation with the RRE to reach maximum inhibitory activity. Similar results were obtained with S2EXT. In contrast, S1-5-S2 showed significant activity upon mixing with the RRE and Rev and reached maximal activity after a 10-min preincubation (Figure 3D). Although more extensive characterization of the binding kinetics remains to be completed, these results emphasize the advantages of a TOP over traditional oligonucleotides in the design of antisense agents for folded RNAs: by targeting two short sequences that are conformationally accessible, TOPs bind more rapidly and with higher affinity than oligonucleotides disruptive to secondary structure.

Although substantial efforts are being directed at targeting mRNA for therapeutic purposes,[1-4] target site selection remains an empirical process.[7,36] This is largely the result of our inability to identify which sequences in an RNA of complex structure are suitable for binding complementary oligonucleotides. Here we exploit the bifunctional binding mode of a TOP[6,15,16] to select sites within the HIV RRE that are proximal, accessible, and able to bind short oligonucleotides and, hence, function as target sites. TOPs targeted to these sites bind more rapidly and with higher affinity than traditional oligonucleotides targeted to the same regions. Although here we apply the R-TOP strategy to identify potential target sites in the RRE, analogous strategies could be used to map local structure in any large RNA. This strategy for target site selection, combined with strategies that address issues of nuclease sensitivity and membrane permeability, holds considerable promise for the future.

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