Combinatorial Screening and Rational Optimization for Hybridization to Folded Hepatitis C Virus RNA of Oligonucleotides with Biological Antisense Activity*

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We describe our initial application of a biochemical strategy, comprising combinatorial screening and rational optimization, which directly identifies oligonucleotides with maximum affinity (per unit length), specificity, and rates of hybridization to structurally preferred sites on folded RNA, to the problem of design of antisense oligonucleotides active against the hepatitis C virus (HCV). A fully randomized sequence DNA oligonucleotide (10-mer) library was equilibrated with each of two folded RNA fragments (200 and 370 nucleotides (nt)), together spanning the 5′-440 nt of an HCV transcript (by overlapping 130 nt), which were varied over a range of concentrations. The equilibrations were performed in solution under conditions determined to preserve RNA structure and to limit all RNA-DNA library oligonucleotide interactions to 1:1 stoichiometry. Subsequent Escherichia coli RNase H (endo Ribonuclease H: EC 3.1.26.4) cleavage analysis identified two preferred sites of highest affinity heteroduplex hybridization. The lengths and sequences of different substitute chemistry oligonucleotides complementary to these sites were rationally optimized using an iterative and quantitative analysis of binding affinity and specificity. Thus, DNA oligonucleotides that hybridized with the same affinity to the preferred sites in the folded RNA fragments found by screening as to short (25 nt) RNA complements were identified but were found to vary in length (10–18 nt) from site to site. Phosphorothioate (P=S) and 2′-fluoro (2′-F) uniformly substituted oligonucleotides also were found, which hybridized optimally to these sites, supporting the design of short (10–15 nt) and maximally specific oligonucleotides that are more nuclease-resistant (via P=S) and have higher affinity (via 2′-F) than DNA. Finally, the affinities of DNA and uniform 2′-F, P=S-substituted 10–20-mer oligonucleotide complements for the best hybridization site, from HCV nt 355 to nt 364–374, closely corresponded to antisense mechanism inhibition activities in an in vitro translation assay and in a human cell-based HCV core protein expression assay, respectively. These results validate our strategy for the selection of hybridization-optimized and biologically active antisense oligonucleotides targeting HCV RNA and support the potential for utility in further applications.

In order to ensure sequence-unique recognition of antisense and ribozyme oligonucleotide drug candidates with a chosen site on any given transcript or transcript precursor RNA, a necessary and sufficient requirement for a minimum of 15–17 complementary base pairs has been proposed (1, 2), and typically 20 are sought (3, 4). However, both the kinetics and the thermodynamics of antisense oligonucleotide hybridization may be profoundly attenuated when an energetic cost is incurred to disrupt secondary and tertiary structures of folded RNA that block the hybridization (5–8). Further, the complexity of folded RNA suggests that there should be great variability of RNA structure-dependent hybridization parameters for oligonucleotides complementary to different positions along the primary sequence. In addition, examination of known RNA secondary structures reveals that identification of structurally ideal RNA hybridization sites for oligonucleotides as long as 20-mers1 is improbable. Therefore, it should be expected that the usual piecemeal sampling (“walks”) at arbitrary intervals along linear RNA transcript sequences with a manageable set (typically 10–50) of complementary 20-mer oligonucleotides (3, 4) generally will identify a few, at best, that give sufficient net binding affinity, after the payment for RNA structure disruption, to support adequate biological inhibitory activity in cell-based screens. At effective concentrations, oligonucleotides chosen in this manner may then bind with near to, equivalent, or even higher affinity at alternative RNA sites on the same or other transcript(s) that are closely sequence-related, but more favorably structured, resulting in diminished site and transcript specificity (9–13).

In efforts to advance antisense oligonucleotide identification beyond the “linear thinking” of sequence complementarity, computer RNA folding algorithms as well as enzymatic and chemical reagent mapping approaches have been used to determine RNA secondary structure. These procedures do not predict tertiary interactions and may be unreliable (14–16), and the latter require use of multiple reagents or enzymes and can be tedious to perform. Even when single-stranded regions of RNA are accurately located, subsequent indirect predictions for the kinetics and thermodynamics of complementary oligonucleotide hybridization may be in error by many orders of magnitude (5) due to steric, topological, and tertiary structural constraints not evident in secondary structure determinations. Attempts to enhance the hybridization of antisense oligonucleotides to transcripts by rational design, including the use of

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1 The abbreviations used are: -mer(s), -oligomer(s); HCV, hepatitis C virus; nt, nucleotide(s); NCR, noncoding region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; tr-ICAM-1, truncated intercellular adhesion molecule 1; PAGE, polyacrylamide gel electrophoresis; IVT, in vitro translation; P=S, phosphorothioate; 2′-F, 2′-deoxy-2′-fluoro; RP-HPLC, reverse phase high pressure liquid chromatography; CPG, controlled pore glass.

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tethered oligonucleotide probes (17–19), pseudo-half-knots (20, 21), symmetric secondary structures (22, 23), and stem bridging (24), while all enjoying some early stage success, require identification of particular RNA structural elements and are limited in scope when viewed against the potential structural diversity of available RNA targets in cells. Most recently, potentially powerful combinatorial or semicombinatorial strategies have been applied to the problem of oligonucleotide recognition of RNA structure. Although (partial) randomization of the RNA substrate recognition nucleotides of trans-cleaving ribozymes (25, 26) is promising, the methodology is not directly applicable to small antisense oligonucleotides. Probing hybridization to structured RNA with multiple length fragments of a given antisense nucleic acid generated by semicombinatorial solid phase synthesis (27) or by solution-based alkaline fragmentation of end-labeled RNA (28) represent innovative screening approaches. However, solid phase screening may bias the former; conserved end fragments of the latter do not mimic therapeutic antisense oligonucleotides; and both methods require unique synthesis of (the equivalent of) full-length antisense nucleic acid for a complete search of a given RNA metabolite. All methods currently are subject to restrictions on the length of target RNA that can be accommodated, which may or may not be easily surmounted to eventually provide more efficient searches of long transcript RNAs.

For these reasons, there clearly is a need for additional and better general methods to directly identify oligonucleotides that hybridize to folded RNA of undetermined structure with minimal energetic cost (29) of RNA structure disruption. Such oligonucleotides would maximize rates of hybridization (or “kinetic accessibility”) and, hence, as previously shown, also would maximize affinity per unit length (5) and the effectiveness of in vivo antisense inhibition (28). Enhanced specificity also should be realized when the probability is greatly reduced of finding close sequence-related RNA sites with comparably favorable structure for oligonucleotide hybridization. Oligonucleotides with these properties most likely would be restricted in length to 10–15 nt, but they would have sufficient biological potency because of the fullest possible realization of hybridization affinity, which could be enhanced further with alternative chemical compositions. The potential problem of formation of internal oligonucleotide structure that would be further stabilized by high affinity chemical compositions, and which would lessen the net hybridization affinity with RNA (8), would also be minimized with shorter oligonucleotides. Other potential advantages of shorter oligonucleotides would be enhancement of primary sequence mismatch specificity (30–32) and product release after RNase-, ribozyme-, or artificial agent-mediated RNA cleavage (30), improved cellular uptake (33), reduced nonspecific protein binding (11), and less expensive synthesis and purification.

To address this existing need for alternative methods to identify oligonucleotides optimized for hybridization to transcript RNA, we have developed a strategy schematically depicted in Fig. 1. In an initial solution-based combinatorial approach, we sequentially use short, fully randomized (i.e. equimolar) sequence DNA oligonucleotide library hybridization affinity screening and Escherichia coli RNase H (endoribonuclease H; EC 3.1.26.4) cleavage analysis (Fig. 1A) to identify energetically preferred hybridization sites on folded RNA. We then follow up by quantitative rational optimization of affinity and specificity of hybridization of individual oligonucleotides to these sites (Fig. 1B). Here we report the application of this approach to the problem of antisense recognition of the 5′-noncoding region (NCR) and initial coding region of a hepatitis C virus (HCV) transcript. HCV is the major cause of post-transfusion acute hepatitis, which may often progress to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (34), and therapeutic options for treatment of patients remain limited and largely ineffective (35). The 5′-NCR is attractive for antisense targeting because it is relatively long (~340 nt) and has highly conserved primary sequence (36, 37) and secondary structure (38) among HCV isolates worldwide.

EXPERIMENTAL PROCEDURES

Preparation of 5′ HCV Transcript Fragments—Plasmid pGEM42-NCE 12 contains an HCV genomic insert (nt 1–1357) obtained from HCV type II isolated from sera of a chronically infected Japanese patient. Sense oligonucleotide primer for the 370-nt fragment contained the 17-mer T7 RNA polymerase promoter followed by nt 1–20 of the HCV insert, and antisense oligonucleotide primer was complementary to nt 333–370. Sense oligonucleotide primer for the 200 nt fragment contained the T7 promoter followed by nt 240–260 of HCV, and antisense oligonucleotide primer was complementary to nt 403–440. Transcript fragments were prepared from polymerase chain reaction-synthesized duplex DNA fragments of pGEM42-NCE 1 using the Ambion MegaScript kit according to instructions and were extracted with phenol-chloroform-isooctyl alcohol, precipitated with ethanol, and then purified using Boehringer Mannheim G-50 Quick Spin columns as instructed. RNA transcripts were 5′-end-labeled with [32P]using calf-intestinal alkaline phosphatase (Boehringer Mannheim), using [γ-32P]ATP (ICN Biochemicals) and T4 polynucleotide kinase (Promega) and were 3′-end-labeled with [32P]phosphorytosine phosphate (ICN Biochemicals) and T4 RNA ligase (Boehringer Mannheim) (5) and were purified by 8% denaturing PAGE and were extracted with phenol-CHCl3, and precipitated with ethanol; specific activities were ~5000 cpm/fmol.

Enzymatic Structure Mapping and Footprinting—RNases ONE (Promega), T1 and CL3 (Boehringer Mannheim), A (Life Technologies, Inc.), and V1 (Pharmacia Biotech Inc.) were variously used in 10 μl containing, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 3 mg of tRNA, and 100 cpm 32P-labeled RNA transcript fragment in the presence (for footprinting) or absence (for mapping) of prehybridized individual oligonucleotides for 5 min at 37 °C. Reactions were quenched with 5 μl of 9 μl urea, and products were resolved on 10% denaturing PAGE. Enzymatic activities were adjusted to limit the extent of digestion to 10%. Gels were quantitatively imaged using a Molecular Dynamics PhosphorImager, and digitized values corresponding to all sequence positions where enzymatic cleavage was observed were used in all analyses.

Synthesis of Oligonucleotides and Randomized Libraries—DNA and 2′-deoxy, P=O oligonucleotides were synthesized by standard phosphoramidite chemistry on an ABI synthesizer (model 380B) and purified as described (3, 4), and 2′-F, P=O oligonucleotides were synthesized and purified similarly, as described previously (35). Oligoribonucleotides were synthesized using an ABI synthesizer (380B) and were purified as described previously (5). Briefly, 5′-dimethoxytrityl 2′-2′′-tert-butyldimethylsilyl nucleotide 3′-O-phosphoramidites with phenoxyacetyl protecting groups on the exocyclic amines of A, C, and G were used. The wait step after pulse delivery of tetrazole was 900 s. Base deprotection was achieved by overnight incubation at room temperature in methanolic ammonia, and the 2′-silyl group was removed at room temperature in 1M tetrabutylammonium fluoride in tetrahydrofuran. RNA oligonucleotides were purified using a C18 Sep-Pak cartridge followed by ethanol precipitation.

Randomized DNA oligonucleotide libraries were made (40) on an ABI synthesizer (model 394) using experimentally determined adjusted proportions of phosphoramidites of each of the four nucleotide bases (as-sayed by ratio of incorporation into all possible dimers) such that, when mixed into a single vial, equimolar incorporation of all four bases at each sequence position was reproducibly obtained, thus ensuring equimolar representation of all possible sequence oligonucleotides (calculated as 41/2 = 1,048,576 sequences). Briefly, phosphoramidites were mixed in a single vial on the fifth port of the ABI-394 synthesizer, and the coupling wait step was increased to 5 min. The ratio of phosphoramidites in the mixture was tested by making a single coupling to dT-CPG, cleaving and deprotecting the product, and analyzing the crude dinucleotide material on RP-HPLC. Portions of the individual phosphoramidites were adjusted accordingly, and the procedure was

2 R. Hanecak, unpublished observations.
reported until equal amounts of the four dimers were obtained. The 3’-position was sequence-randomized by mixing the four base CPGs, removing the 5’-dimethoxytrityl on the synthesizer, cleaving and deprotecting the product, and analyzing by RP-HPLC. The proportion of each CPG was adjusted until equal amounts of each base were obtained. Each 1 μmol of the four base CPGs was analyzed by RP-HPLC. After cleavage, deprotection with ammonium hydroxide at 55 °C for 16 h the dimethoxytrityl-off oligonucleotide libraries were purified by RP-HPLC. The libraries were analyzed by denaturing PAGE and diluted to a final library concentration of 10 μM. Limit hydrolyses by snake venom phosphodiesterase I (U.S. Biochemical Corp.) and examination of products by absorption on RP-HPLC confirmed the equimolar representation of bases. A few sequences may be underrepresented, without affecting this result, due to relatively poor aqueous solubility (i.e. of long strings of G nucleotides).

**Combinatorial Hybridization Affinity Screening and RNase H Cleavage Analysis**—Prior to mixing for hybridization, the diluted transcript fragment and DNA library were each heated independently to 90 °C for 1 min and cooled slowly to 37 °C. Hybridizations were done over 20 h at 37 °C in 30 μl containing hybridization buffer, 1000 cpm of 32P end-labeled transcript (1 nM total RNA), and 1 mM DNA library (calculated concentration, 100 μM individual sequences). Some hybridizations proceeded for up to 40 h with no change in the results (not shown), except for enhanced nonspecific background degradation of transcript in some cases. Dithiothreitol was added to 1 mM and then added, and mixes were resolved at 10°C on native 20% PAGE using 44 ng of tr-ICAM-1 RNA (30 nM final concentration), 5 μM antisense oligonucleotide ranging from 10 μM to 10 μM. Mixes were heated at 90 °C for 5 min, cooled slowly to 37 °C, and incubated for 20 h at 37 °C. 10 μl of loading buffer (15% Ficoll, 0.25% bromphenol blue, 0.25% xylene cyanol) was then added, and mixes were resolved at 10°C on 20% PAGE using 44 ng Tris-borate and 1 mM MgCl2 running buffer for ~4 h at 122 W. Gels were quantitatively imaged using a Molecular Dynamics Phosphor-Imager. The log linear range of the assay was determined (not shown) to cover from 50 μM to 10 μM in apparent Kd values for antisense oligonucleotides.

**In Vitro Translation Assay**—HCV transcript was prepared as above except that it encompassed the first 5’ 1.4-kilobase pairs. Heterologous control truncated intercellular adhesion molecule 1 (tr-ICAM)-1 transcript was synthesized similarly (3). In vitro translation (IVT) reactions contained (in 15 μl) 300 ng of HCV RNA (44 mM final concentration), 100 ng of tr-ICAM-1 RNA (30 mM final concentration), 5 μl rabbit reticulocyte lysate (Promega), 8.6 μg of [35S]methionine (1175 Ci/mmol), 15 μl IVT amino acid mix minus methionine (Promega), 8 units of RNasin, and oligonucleotides (30 μl to 1 μM). Before mixing, RNAs were heated at 65 °C for 5 min and then at 37 °C for 15 min. Reaction mixes were incubated at 37 °C for 1 h and then quenched with an equal volume of Laemmli gel loading buffer, boiled for 8 min, and placed on ice. After microcentrifuging for 8 min, samples were electrophoresed on a 14% polyacrylamide gel (Novex) for 2 h at 125 V. Gels were fixed (10% trichloroacetic acid, 3% glycerol, 20 min), vacuum-dried, and subjected to PhosphorImager analysis.

**Cell-based HCV Core Protein Expression Assay**—Simian virus 40 large tumor antigen-immortalized human hepatocytes HSad-17e (41) were selected for expression of HCV sequences after calcium phosphate-mediated transfection with a neomycin resistance expression vector containing HCV type II sequences (nt 1–1357, including the 5’-NCR, core protein, and the majority of the envelope protein E1 gene) fused to the human cytomegalovirus immediate early promoter (35). HCV sequence-expressing HSad-17e cells were seeded into six-well dishes at a density of 5 × 105 cells/well, rinsed once with Optimem (Life Technologies). Cells were treated with oligonucleotides for 4 h in the presence of 5 μg/ml N-[2-(6-azauridine)-2,3-dideoxy-N-[2-(5-fluoropropyl)-n-trimethylammonium] chloride, and then rinsed once and refed with growth medium. Cells were lysed in 1 × Laemmli sample buffer 18–20 h after treatment with oligonucleotide and then boiled, and cell debris was removed by centrifugation. Proteins were separated on 16% SDS-PAGE and transferred to polyvinylidene fluoride membrane. Western blots were blocked in phosphate-buffered saline containing 2% normal goat serum and 0.3% Tween 20 were probed with a polyclonal antibody derived from serum taken from an HCV type II-positive patient and a monoclonal antibody to glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) (Advanced Immunochemical), rinsed, and incubated with [125I]iodolabeled goat anti-human antibody, and immunoreactive proteins were subjected to Phosphorimage analysis. HCV RNA levels were not measured, because they previously have been shown not to be reduced by antisense oligonucleotides uniformly modified with 2′-substitutions that do not support RNase H activity (35).

**RESULTS AND DISCUSSION**

**Strategy for Combinatorial Screening for Preferred Hybridization Sites**

**Library Design**—We used short (10-mer) DNA libraries in order to restrict the library complexity (calculated as 410 = 1,048,576 sequences) so that all possible sequences would be synthesized with equimolar base representation at each sequence position and in equivalent and sufficient amounts (40). The use of short oligonucleotide libraries also avoids negatively biasing selections for suboptimal recognition with long oligonucleotides, as discussed earlier (9–13), keeps the heteroduplex affinity at the selected preferred sites within the operational range of measurement of readily accessible assays in order to facilitate subsequent quantitative optimization, and attenuates the stability of intramolecular interactions (i.e. stem-loops) of library sequences. Short oligonucleotides with very stable internal structure will diminish the affinity of hybridization with RNA (7, 8) and will not be selected by affinity-based screening. Unlike libraries of other nucleic acid screening strategies, the libraries used in this strategy have no internal fixed sequence positions (42) or external conserved flanking sequences (43–45).

**Combinatorial Hybridization Affinity Screening**—Combinatorial affinity screening (Fig. 1A) was performed at hybridization equilibrium, in solution, under quasiphysiologic conditions (i.e. pH, temperature, salt species, and concentration). Screening on solid supports or employing physical separation was avoided because of the potential for the introduction of bias and the difficulties inherent in satisfying the following requisite conditions.

The concentration of DNA library must be held (typically at 1 μM) such that the calculated concentration (100 μM for a 1 μM library) of individual library sequences (IX10 in Fig. 1) is limiting and significantly less (by >100-fold) than the Kd for heteroduplex formation, intermolecular interactions among complementary library sequences are strongly disfavored. These conditions also allow using a [RNA] that is still low enough (even up to 10 μM) that physical aggre- gation is mitigated (at least for RNA < 400 nt). Most importantly, the only statistically significant population of bound RNA-DNA complexes has 1:1 stoichiometry, so all RNA-DNA interactions behave as if measured independently (i.e. are “unlinked”) and there is no “melting out” of individual RNA mol-
ecules. By these means, unbiased affinity selection in a single round by massive parallel co-processing of all unlinked possibilities for heteroduplex hybridization is achieved.

Hybridization Affinity Screening Assay—We currently use an RNase H cleavage assay to enable positional identification of affinity-selected heteroduplex regions on the end-labeled RNA via RNA cleavage product size separation on high resolution denaturing PAGE; from the sequences of the RNA at cleavage sites, the complementary sequences of bound oligonucleotides are inferred. The cleavage assays are performed sequentially to the combinatorial affinity hybridizations in the same solutions. It should be noted that, for reasons not well understood, RNase H cleavage at all nt positions of heteroduplexed DNA oligonucleotides with folded RNA is not (often) observed. This has two implications. First, cleavage of even one nt position is a secondary event dependent on the primary event of preferred hybridization of at least one DNA library oligonucleotide at sequences encompassing the cleavage site(s). Second, it is not possible to predict the exact length and sequence of preferentially hybridizing DNA oligonucleotides from affinity cleavage patterns. This requires further optimization (see below).

The RNase H cleavage method of analysis of DNA oligonucleotide hybridization with folded RNA has high sensitivity (≤10 pM) for heteroduplex in the presence of much larger amounts of unbound RNA and library sequences. It also detects multiple hybridization sites simultaneously, and it can identify preferred hybridization sites that are longer than the (10-mer) library oligonucleotides, since affinity cleavage patterns on gels are the sum of all patterns for individual 1:1 complexes, including those that are sequence-overlapping. This summation can contribute to, at most, a severalfold enhanced cleavage product yield at sites allowing hybridization of oligonucleotides longer than those of the library. However, at the best sites we observe (i.e. Fig. 2C and results not shown) considerable amplification (≥100-fold at sites with high affinity and rates of hybridization) of cleavage products over the maximum yield calculated from the limiting concentration of individual library sequences that can be present in 1:1 complexes at equilibrium without amplification. Thus, we posit that there is, in addition, multiple turnover of oligonucleotides during RNase H digestion at favorable sites. This speculation remains unconfirmed and is a subject of further study, but the end result is considerably improved assay sensitivity over the calculated limit values.

Dependence on (E. coli) RNase H cleavage creates the possibility that some RNA sites acceptable for oligonucleotide hybridization may be overlooked because they are sterically inac-

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4 T. W. Bruice and W. F. Lima, unpublished results.
FIG. 2. Strategy of Fig. 1 as applied to HCV. A, HCV genomic landmarks, transcript fragments, combinatorial hybridization affinity-screening RNase H cleavage (as per Fig. 1A), enzyme structure mapping results, and first and second round oligonucleotide sequences of optimization (as per Fig. 1B). 370- and 200-nt RNA fragments were chosen as described under “Results and Discussion.” Positions of the two most preferred hybridization sites identified by affinity cleavage patterns encompassing nt 29–38 and nt 343 to 368 are indicated with telescoped enlargement of the sites shown below. 1, affinity cleavage sites; open boxes, consensus of single-stranded enzymatic mapping. Oligonucleotide nomenclature is as follows: first number denotes 5’ sequence hybridization start site on the RNA fragment, and second number denotes the oligonucleotide length going 5’ → 3’ along the RNA. B, affinity cleavage and enzymatic structure mapping for the 130-nt overlap region of the 370- and 200-nt transcript.
cessible to RNase H or are not contiguous in the primary sequence (i.e. are stem-bridging). However, one advantage may be that those sites that are identified may be more predictive for recruitment of intracellular RNase H (albeit mammalian in therapeutic applications), which often is required for robust antisense biological inhibitory activity (3, 4, 46). Accurate assessment of the ability of this biochemical strategy to predict biological efficacy will require thorough testing as it may be adversely affected by any number of elements of the cellular milieu, including interference by RNA binding proteins (see below), transcript coding sequence scanning by ribosomes, and unfavorable differential subcellular localization of target RNA metabolite, antisense oligonucleotide, and RNase H. The favorable results for the initial investigation targeting HCV RNA are reported below and suggest that additional studies are warranted.

Application of Combinatorial Hybridization Affinity Screening to 5’ HCV RNA

We started with a 5’ 370-nt HCV RNA fragment (Fig. 2A), because it encompasses the entire 5’-NCR, translation start codon, and 30 nt of the coding region and the secondary structure previously has been determined by mapping with multiple single strand- and double-strand-specific endoribonucleases (38). It is large enough to expect a folded structure, at least upstream of the artificial 3’-end (see below), closely approximating that in the full-length transcript under the same conditions. Application of combinatorial hybridization affinity screening analysis gave results summarized in Fig. 2, A–C. Acceptable quality single nt resolution of RNA cleavage products on standard denaturing PAGE typically is limited to ~100 nt from the end label, with lower resolution useful to at least another 50 nt. Within these limits, using the 5’-end-labeled transcript fragment, we detected several lesser quality affinity cleavage sites, characterized by only a few low intensity bands on PAGE, and one modestly preferred site starting at about nt 29 (the “29-site”) with a larger number of higher intensity cleavage products on PAGE. The 29-site aligns well with results of our own (Fig. 2A) and the previously reported independent enzymatic structure-mapping results (38) showing a large internal loop structure as the only substantially less structured domain within the first 5’ 125 nt. This site is also where the greatest spontaneous background hydrolytic cleavage in mock experimental controls is observed (Fig. 2C), indicating single-stranded local structure, as expected. The 370-nt fragment also was 3’-end-labeled, and the combinatorial screening and analysis was repeated. Again, only several lesser preferred affinity cleavage sites were found (at nt 267–269, 302–304, and 351–352), having a few low intensity cleavage products on PAGE, consistent with the high degree of enzyme-mapped secondary structure (38) and recently determined higher order structure (47) in this region (Fig. 2A). The cleavage sites from nt 267–269 are consistent with a binding site(s) for a 10-mer oligonucleotide(s) at overlapping a site from nt 264–282 for which a phosphorothioate antisense oligonucleotide gave demonstrable inhibition in both in vitro translation and cell culture experiments (48).

The short affinity cleavage pattern (covering ~10 nt) at the 29-site suggested the possibility of tight restrictions on the length of optimally binding complementary oligonucleotides. This site also is very near the 5’-end of the transcript, and inhibition of viral translation by antisense complementarity to it would be expected to be incomplete due to the initiation of translation at downstream sequences comprising the internal ribosome entry site (IRES) (36, 49). Although a cap structure may be present at the 5’-end of HCV RNA expressed in H8Ad17c cells, extensive secondary structure within the 5’-NCR and the presence of multiple AUG codons upstream of the normal initiator AUG should interfere with ribosome scanning and result in retention of an internal ribosome entry (35). Therefore, we wanted to see if there was a larger preferential hybridization site in the initial coding region downstream of the AUG translation start codon at nt 342. The 370-nt fragment is not long enough to confidently analyze the initial coding region for hybridization sites, because of potential “end effects” at the artificial 3’-end (see below). Therefore, we made a 200-nt fragment centered on this AUG. Radioisotopic labeling of each end, in turn, allowed for complete combinatorial hybridization affinity screening with RNase H cleavage analysis to single nt resolution. The affinity cleavage results for the central portion of this fragment (Fig. 2A), removed from artificial 5’- and 3’-ends (as below), indicated a good candidate hybridization site at ~340–370 nt. It was characterized by a long sequence stretch (covering ~28 nt) of high density and intensity of affinity cleavage products seen on denaturing polyacrylamide sequencing gels.

Overlap of RNA Fragments: End Effects, Structure Mapping, and Affinity Cleavage

RNA fragments are shorter than full-length transcripts and have 5’, 3’, or both termini that correspond to sequence positions internal to the transcripts; thus, such termini are artificial ends. Since they are lacking contiguous 5’ and/or 3’ sequences, the local structure in the vicinity of such unnaturally positioned ends may be different than for the corresponding sequences in full-length transcripts. These differences, as detected by enzymatic mapping and/or oligonucleotide hybridization affinity, may be termed end effects. In this study, the 370-nt fragment has a 3’ artificial end, and the 200-nt fragment has both 5’ and 3’ artificial ends; but we have designed substantial sequence overlap (130 nt) of the 3’-end of the 370-nt fragment with the 200-nt fragment and of the 5’-end of the 200-nt fragment with the 370-nt fragment. For both RNase H affinity cleavage of hybridized oligonucleotides and consensus enzymatic structure mapping, there was good correspondence between the 370- and 200-nt fragments for much of the 130-nt overlap region (Fig. 2B). Differences that may best be ascribed to local end effects were evident for the 200-nt fragment from nt 240 to ~251 and for the 370-nt fragment from nt 350 to 370. The 315–325-nt region was a clear outlier, which probably is most consistent with a large steric effect and/or long range structure interaction occluding access to mapping enzymes and RNase H for the 370-nt fragment but not for the 200-nt fragment. This interpretation is consistent with the recent identification of a large RNA pseudoknot structure, involving upstream (nt 126–134) and downstream (nt 305–311, 315–323, and 325–331) sequences of the 5’-NCR, both present only in the 370-nt fragment (47). Finally, although most affinity cleavage sites aligned roughly with single-stranded domains determined by enzymatic structure mapping for either fragment, the latter fragments (notation as in A). C, representative RNase H cleavage analysis at the 29-site for combinatorial hybridization affinity screening with a 1 M randomized 10-mer DNA library and the 5’-32P 370-nt RNA fragment at 40 M = 4K for the highest affinity heteroduplex. The far right Transcript only lane is end-labeled transcript at the beginning of the procedure. The far left no library lane is end-labeled transcript after being subjected to the entire mock (no DNA library) protocol. D, representative enzymatic footprinting analysis (using 0.02 and 0.002 units of RNase CL3) of binding at the 355-site on the 5’-32P 200-nt RNA fragment of the 355-20 DNA oligonucleotide titrated over the range of concentrations indicated. All results were obtained from experiments performed as described under “Experimental Procedures.”
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was not always usefully predictive of the former. For example, at the most interesting candidate hybridization site at nt 340–370 on the 200-nt fragment, the majority of nt positions of affinity cleavage align poorly, if at all, with consensus single-stranded sequences. A similar situation holds for the less dramatic affinity cleavage site at nt 295–301, and there is no affinity cleavage seen at (predominantly) single-stranded nt 302–315. These results are consistent with a previous study of an RNA stem-loop fragment from mutant Ha-ras mRNA (5) for which consensus enzymatically mapped single-stranded positions of the loop were as unfavorable for complementary oligonucleotide hybridization as was the double-stranded stem. Together, these observations suggest that (i) some single-stranded regions that are favorable for oligonucleotide hybridization are missed by (larger) mapping enzymes, presumably due to steric occlusion and, more importantly, (ii) only a subset of RNA single-stranded stretches long enough for favorable antisense oligonucleotide hybridization will actually support it. Therefore, the more efficient and accurate approach for identification of this subset is combinatorial hybridization affinity cleavage using only RNase H instead of the far more tedious and less informative determination of secondary structure by mapping using multiple enzymes.

Characterization of Combinatorial Screening-identified Hybridization Sites and Oligonucleotide Optimization

Strategy—We followed the quantitatively rigorous approach in Fig. 1B in order to characterize the hybridization and to rationally optimize the sequence, length, and chemical composition of oligonucleotides complementary for identified sites. An iterative testing cycle was used to minimize the total number of oligonucleotides synthesized and analyzed. The rational optimization goal was design of oligonucleotides with simultaneous realization of two attributes. (i) The most obvious one is high affinity ($K_a$) for the preferred sites on folded RNA (but not so high as to lose sequence mismatch specificity) (31, 32), since affinity generally is thought to correlate with biological antisense potency (31, 32, 50, 51). (ii) Of equal importance for full realization of the antisense therapeutics promise is to achieve the highest possible transcript hybridization specificity. An accessible indicator of this is the site specificity, $K_{rel}$, which we define for any candidate oligonucleotide as the ratio of the value of $K_a$ to the value of the intrinsic potential affinity, $K_{a,irr}$, for hybridization to sequence complementary, short, and unstructured RNA external standards (Fig. 1B); ideally, $K_{rel} = K_a/K_{a,irr} ≥ 1.0$. In other words, any oligonucleotide that binds with sequence complementarity to an identified, structurally preferred transcript site as well as it can to the same excerpted RNA sequence without structure is unlikely to bind to any other sequence-related (or even identical sequence) transcript site nearby as well and so will be maximally specific.

In practice (Fig. 1B), $K_a$ is determined by titrating individual candidate oligonucleotides against folded RNA fragments and assaying by enzymatic footprinting. Values for $K_{a,irr}$ are similarly determined except that short sequences of RNA (≤25 nt) are excerpted from the preferred hybridization sites on the longer fragments, and a gel mobility shift assay is used. Although this cannot claim to give "ideal" intrinsic binding affinities, it is reasonable to expect that there cannot be a very large energetic cost of disruption of structure in either the preferred target RNA site or in the complementary DNA oligonucleotides, or else they would have been screened against (8). Since, in addition, the control RNA oligonucleotides used to determine $K_{a,irr}$ values by gel mobility shift are much shorter than the parent RNA fragments used in combinatorial screening, potential inhibition of hybridization by higher order structure involving sequences flanking, or some distance removed from, the hybridization sites should be minimized. Finally, in preliminary assay calibration experiments, affinity constants for hybridization of 10-mer DNA oligonucleotides complementary to a 47-mer mutant Ha-ras mRNA stem-loop determined by either the enzymatic footprinting or gel shift methods were in agreement within a 2–3-fold variance. Under our assay conditions, neither method was very sensitive to oligonucleotide sequence for mixed sequence oligonucleotides of a given length and backbone chemistry (Fig. 3).

DNA Oligonucleotides—The results of applying this rational approach for DNA oligonucleotides complementary to the combinatorial screen-identified HCV transcript fragment sites starting at nt 29 and 340 are shown in Fig. 2D and Fig. 3, A–D. The highest observed affinity binding ($K_a$) of 10-mers was restricted to one (or perhaps two) oligonucleotide(s) for the 29-site (Fig. 3A), consistent with expectations from the relatively short affinity cleavage pattern, and to at least two oligonucleotides (separated by 10 nt) for the site from nt 355–380 (Fig. 3C), also consistent with the longer affinity cleavage pattern. Importantly, experimentally apparent optimal binding of at least one 10-mer DNA oligonucleotide(s) was found for the 29-site; $K_a$ and $K_{a,irr} > 10$ nM limit value (under the conditions used) for a 10-mer mixed sequence heteroduplex, and therefore, $K_a/K_{a,irr} > 1.0$. Since the site from nt 340–380 is so much longer, $K_a$ values were not initially obtained for all 10-mers, but $K_{rel}$ values for some of these oligonucleotides were of similar magnitude to that for the best one for the 29-site. Further, there was no evidence by either single-stranded RNA footprinting or RNase H affinity cleavage analysis for binding of any of these individual 10-mer oligonucleotides outside of the combinatorial screening-identified preferred sites (data not shown). These results validate the combinatorial hybridization affinity-screening protocol on the binding level and also lend support to the existence of “ideal” hybridization sites in folded transcript RNA, at least for 10-mer oligonucleotides.

The binding of DNA 20-mers was compared with that of DNA 10-mers at the 29-site (Fig. 3B). Only a small increase in $K_a$ is maximally obtained for the best 20-mer at the expense of a significant reduction in $K_{rel}$. Other 20-mers actually bind with lower or no better affinity than the best 10-mer, although the best 10-mer sequence is embedded in all 20-mers tested. These results underscore the strong contribution of RNA structure to oligonucleotide hybridization and the importance of matching
oligonucleotide length to RNA target site structure. The conclusion that “more (oligonucleotide length) is not always better” has now been shown for both sequence mismatch specificity (31) and optimal structural recognition-dependent affinity and specificity (this study).

The most preferred subsite for hybridization within the HCV transcript nt 340–380 region identified by affinity cleavage (Fig. 2A) is from nt 355–380 (the “355-site”) (Fig. 3C), and hybridization appears to be more favorable than at the 29-site. All 10-mer DNA oligonucleotides starting from nt 355–370 hybridize with $K_a$ within 100-fold of the highest affinity 10-mer for the 29-site (Fig. 3C). Thus, this site is relatively long (~25 nt) and appears structurally favorable for hybridization of oligonucleotides somewhat longer than 10-mers in order to realize greater affinity without undue loss of specificity. This conjecture was confirmed with a series of DNA oligonucleotides increasing in length from 10 to 20 nt, all starting at the 355-nt sequence position (Fig. 3D). It was found that $K_a$ values are proportional to length up to the 18-mer. Surprisingly, $K_{rel}$ values actually improve with length, such that the $K_{rel}$ value for the 18-mer is nearly ideal. It is not clear why this happens. One possibility might be that the already favorably preorganized site in folded transcript RNA (the latter point inferred from the 10-mer DNA data) requires only a modestly more costly reorganization with increasing length of DNA oligonucleotides, resulting in minimal lost energy from the net biochemical energetic gain (−$\Delta G'$) upon hybridization. In contrast, it might be expected that the net energy gain (−$\Delta G'$) upon hybridization of longer DNA oligonucleotides to control sequences would be diminished by an increasingly more costly de novo organization into longer helices of more nucleotides of a short random coil RNA complement. This would then result in a progressively larger value for $-\Delta G'$ for DNA oligonucleotides of increasing length, where $\Delta G' = \Delta G_a - \Delta G_c$. For whatever reason, a similar increase in $K_{rel}$ with increasing oligonucleotide length is also seen for the uniform $P_5S$ substitution (Fig. 3E).

Backbone Chemistry-substituted Oligonucleotides—We expected that preferred DNA hybridization sites in folded RNA would also be preferred hybridization sites for other oligonucleotide chemistries, since all alternative chemistries discovered to date (39,52) that bind single-stranded RNA by Watson-Crick hybridization form right-handed heteroduplexes with helical conformations close to A or A' form, as does DNA. If true, then our combinatorial DNA library screening strategy would have significant predictive value for biological and therapeutic applications requiring enhanced nuclease resistance (i.e., most commonly using $P=S$) (3,4) and affinity per unit length (i.e., highly potentiated for 2'-F) (39). As shown in Fig. 3, the 355-site discovered using DNA oligonucleotides (Fig. 3D) also is favorable for hybridization of oligonucleotides substituted with alternative backbone chemical compositions (Fig. 3, E–F).

However, it also appears that more subtle variation of conformational preferences of heteroduplexes of different substitute oligonucleotide chemistries are manifested as deviations of
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maximum length limits for best possible binding at sterically and topologically constrained sites in folded RNA; evidence for variable topological-conformational constraints over the nt 355–380 site is seen in the uneven \( K_r \) profile (Fig. 3 C) for 10-mer DNA oligonucleotides. In fact, it follows that higher affinity, conformationally preorganized backbone chemistries (i.e. 2'-F) that form more rigidified heteroduplex helices (39) and are therefore less conformationally accommodating should exhibit decreased limit lengths for optimal binding at constrained sites, as observed: 20-mer \( P=S \) diester (Fig. 3E) and 18-mer phosphodiester (Fig. 3D) versus 14-mer 2'-F, \( P=S \) diester (Fig. 3F) limit lengths for the HCV transcript 355-site. Alternatively, or additionally, minimal internal structure in oligonucleotides longer than 14–16 nt could be stabilized by the 2'-F modification to the extent that the requisite cost of its disruption on net affinity of hybridization to the 355-site is significant. Although combinatorial screening for hybridization with 10-mer DNA oligonucleotides strongly biases against structure in these oligonucleotides, it cannot altogether eliminate the possibility of internal structure in longer oligonucleotides with selected 10-mer sequences embedded therein. However, our inability to generate plausible folded structures makes this explanation appear less probable. Also, the evidence points to little structure in the RNA target from nt 355–380, and so it is not likely there is much structure in the antisense complement spanning this region.

The generally reduced values of \( K_{rel} \) for oligonucleotides of both the lower affinity \( P=S \) and higher affinity 2'-F, \( P=S \) series compared with those of the DNA series may be attributed solely to the \( P=S \) modification. Chemical synthesis of the \( P=S \) substitution creates a random diastereomer distribution in oligonucleotides. A more restricted conformational subset of this total product distribution may hybridize well (hence a lower average \( K_r \) for the whole population) to more constrained sites in folded RNA fragments than the larger subset that may be expected to hybridize well (hence a higher \( K_{rel} \) for the whole population) to more conformationally accommodating, short, unfolded RNA oligonucleotides, thus accounting for these observed \( K_{rel} \) values. Other ways in which the \( P=S \) modification could negatively bias the observed \( K_{rel} \) values for this site compared with values for DNA may also be possible, but they remain to be identified. Regardless of the underlying cause, if this observation is found to apply to other preferred sites of hybridization on folded RNA, then this even less than expected affinity obtained with the \( P=S \) substitution would argue for minimizing its incorporation in future designs.

The possibility exists that some additional improvement in \( K_r \) and \( K_{rel} \) values for shorter length 2'-F, \( P=S \) oligonucleotides could be realized by a finer subsite mapping within the nt 350–380 site (i.e. 12–16-mers starting at nt 357 or 359 or centered at nt 365). Even so, results presented here with the nt 355 start site 2'-F, \( P=S \) oligonucleotide series (Fig. 3F) show that alternative chemistries to DNA (Fig. 3D) can be used successfully in the context of shorter (10–15-mer) oligonucleotides in order to simultaneously achieve enhanced nuclease resistance and affinity per unit length and the maximal specificity that is permitted by the structure of a given preferred hybridization site.

Correlations of in Vitro Optimization of Binding with Biological Antisense Activities

Prior to commencing this work, we felt that there was sufficient justification to support the premise that a biochemical strategy could predict biologically active antisense oligonucleotides often enough to prove useful. Briefly summarized, numerous examples are known of identity in structure and function of RNA elements either purified (and often truncated) in biochemical studies or (usually full-length) in cellular studies (6–8, 14–16, 22, 23, 53–57). High affinity RNA binding proteins often may not be effective competitors of antisense, since they usually recognize combinations of RNA sequence and structure elements that are not favorable for antisense hybridization (53–55). A major class of generally lower affinity and specificity RNA single-stranded binding proteins, RNA chaperones, alter folding kinetics, but not final structure, of RNA and, likewise, alter oligonucleotide hybridization kinetics but not specificity with folded RNA (58, 59). Finally, more directly relevant to the application described here, preliminary studies elsewhere\(^a\) have demonstrated that identical specificities are obtained for oligonucleotide hybridization to long, folded HCV 5’-NCR-coding region transcript fragments in the presence and absence of protein introduced with whole cell cytoplasmic or nuclear extracts.

Results from our in vitro oligonucleotide binding optimization strategy with purified RNA fragments (Fig. 3D) do show, in fact, a positive, predictive correlation with biological antisense inhibition activity in two assay formats. The first is a cell membrane-free IVT assay (Fig. 4). The IVT assay uses a much longer HCV 5’ 1.4-kilobase transcript fragment in the presence

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\( \text{Fig. 5. Inhibition by 355-site 10–20-mer uniform 2'-F, } P=S \text{ antisense oligomers of cellular expression of HCV core protein.} \) 355-20R is the same sequence oligonucleotide as in Fig. 4. Assays were performed as described under “Experimental Procedures,” and expression of HCV core protein is shown as a percentage of expression of GAPDH internal control, where values are the mean ± S.D. of three or four independent determinations. The maximum reduction in HCV core protein levels observed in this preparation of cells treated with HCV antisense oligonucleotides at a dose of 200 nM (the highest concentration showing antisense inhibition specific for HCV core protein and without effect on GAPDH levels) is 50%. A similar inhibition limit was also observed for standard treatment with cycloheximide (not shown). This likely results from the stability of HCV core protein, which is constitutively expressed in the HCV sequence transfected H9Ad17-C cells and represents the basal level of stable, expressed core protein in these experiments.
of cellular RNA-binding proteins. The absence of any inhibition of translation of the HCV transcript by the 355-10 and 355-12, the randomized sequence 20-mer (355-20R, except at the highest concentration), or the sense sequence 20-mer (355-20S) DNA oligonucleotides or of the heterologous tr-ICAM internal control transcript translation by any of the oligonucleotides argues against a (length-dependent) nonspecific effect. It is apparent that a minimal threshold affinity (i.e. for a DNA 14-mer) is required to elicit an inhibitory biological response by 355-site antisense oligonucleotides. For the 355-14 to 355-20 length oligonucleotides the dose-response curves are reflective of binding isotherms (Fig. 4A), and higher affinity oligonucleotides have lower values (within experimental error) for the effective concentration giving 50% reduction (EC50) in HCV core protein production (Fig. 4B). These results suggest a direct correlation of inhibition by an antisense mechanism with hybridization affinity. Compression of the range of EC50 values with purified RNA (Fig. 4B) and sense activity (36, 48, 60).

The second biological assay measures HCV core protein expression in human cells. In this assay, the inhibitory activities of 10–20-mer 2'-F, P=S oligonucleotides targeting the 355-site are dose-responsive (Fig. 5) and closely correlate with their biochemical hybridization affinity to folded RNA transcript fragments (Fig. 3F). There is little or no activity of a randomized sequence 20-mer (355-20R), and none of the oligonucleotides tested inhibited expression of the internal control GAPDH. As with the affinities, there is a diminishing gain in inhibition obtained with the 2'-F rigidified backbone at longer than 14–16-mers. Nevertheless, the inhibitory activity of the 2'-F, P=S 355-20-mer oligonucleotide was unsurpassed (Fig. 6B) when compared with activities determined identically of the most active 20-mer 2'-F, P=S oligonucleotides of a linear sequence walk targeting the 355-site identified by combinatorial screening. Although the majority of these oligonucleotides showed no activity, a few gave only slight inhibition (Fig. 6A), and sequence-randomized controls (330-20R and 340-20R) gave little or no inhibition (Fig. 6B), our results are consistent with sequence-specific antisense inhibition and not with sequence-nonspecific, but length-dependent, P=S oligonucleotide inhibition (9, 11). Further, the most active antisense oligonucleotides identified by the walk are complementary to sites of preferred hybridization, as inferred from positions of RNase H cleavage after combinatorial oligonucleotide screening (i.e. 260-20 and especially 340-, 345-, 350-, and 355-20 oligonucleotides). Similar results using the HCV core protein expression assay recently were obtained from a walk with sequence-nonspecific, but length-dependent, P=S oligonucleotide sequences from the walk compared with the length-matched sequences (335-20) for the 355-site identified by combinatorial screening. 330-20R (330R) is a randomized sequence oligonucleotide (ACGGCGGTGTGATGCGTTCT) with the same base composition as 330-20, and 340-20R (340R) is a randomized sequence oligonucleotide (TACGTTCGATCGGG) with the same base composition as 340-20. Experiments of A and B (and of Fig. 5) were performed at different times with different cell preparations and thus show the typical greater interexperiment variability (i.e. for 260-20) than intraexperiment variability (as in B).

**Conclusions and Implications**

Combinatorial DNA oligonucleotide screening (Fig. 1A) and rational optimization (Fig. 1B) of oligonucleotide sequence, length, and chemistry for affinity and specificity of hybridization to structurally preferred sites on folded HCV RNA tran-
script fragments has been quantitatively established in vitro and biologically validated. This correlation lends further support to the growing value to biology of careful biochemical studies with purified RNA fragments and, in particular, shows that potential artifacts resulting from artificial ends of transcript fragments can be avoided. Further applications of this strategy will be required to better assess the general predictive value for efficacy of antisense oligonucleotides in living cells. In the present study, the identified preferred sites for DNA hybridization also hybridize well with oligonucleotides having unnatural chemistry-substituted backbones conferring enhanced nuclease resistance and hybridization affinity. The results of this study support the hypothesis that binding-optimized shorter oligonucleotides (i.e. 10–15-mers) may often have equivalent or higher affinity and significantly greater hybridization site specificity than longer ones (i.e. 20-mers). We recommend comprehensive biological testing focused on oligonucleotides optimized for hybridization to the various candidate sites identified by our biochemical strategy in order to determine those sites where antisense generates the most potent biological inhibition. In those cases where the correlation of in vitro binding and biological activity is not confounded by the cellular environment, the global RNA binding specificity, that is, the discrimination against binding to all intracellular RNA sites other than the one targeted, should be improved using oligonucleotides optimized in vitro for binding affinity and specificity for a particular preferred site.

As for the alternative semicombinatorial antisense fragment probing strategies (25, 28) described earlier, practical selections against RNAs much longer than is possible with the technology reported here is greatly desired. Primer extension copying of the RNase H-generated RNA fragments into DNA should facilitate improved resolution on denaturing PAGE. However, the preferred eventual assay to use with our strategy would be realized if combinatorial hybridization analysis could allow direct screening of libraries of oligonucleotides (preferably enzymatically) of unbound library oligonucleotides (i.e. using single-strand-specific DNases similarly to, and within the time period of, successful posthybridization RNase H treatment of this study), (ii) melting of the RNA-preferentially hybridized oligonucleotide heteroduplexes, and then (iii) some format of oligonucleotide matrix array (61) capture and imaging of released oligonucleotides. If successful, such an alternative method to RNase H cleavage for hybridization analysis could allow direct screening of libraries synthesized incorporating novel chemistries. In selecting these analog chemistries, our results suggest that less rigid backbones may better accommodate the variable topological and conformational constraints on available hybridization sites on folded RNA and thereby will more fully realize the potential affinity enhancement of the chemistry used.

Addendum—Subsequent to the initial submission of this manuscript it was reported (62) that high affinity chemistry-substituted antisense oligonucleotides as short as 7-mers can be potent and selective inhibitors of gene expression in cells, consistent with some of the assertions and results of this study.

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