Nuclease-Resistant Composite 2′,5′-Oligoadenylate−3′,5′-Oligonucleotides for the Targeted Destruction of RNA: 2−5A-Iso-antisense

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A new modification of 2−5A-antisense, 2−5A-iso-antisense, has been developed based on a reversal of the direction of the polarity of the antisense domain of a 2−5A-antisense composite nucleic acid. This modification was able to anneal with its target RNA as well as the parental 2−5A-antisense chimera. The 2−5A-iso-antisense oligonucleotide displayed enhanced resistance to degradation by 3′-exonuclease enzyme activity such as that represented by snake venom phosphodiesterase and by that found in human serum. 2−5A-Iso-antisense was able to effect the degradation of a synthetic nontargeted substrate, [5′-^{32}P]p$C_{11}U_2C_7$, and two targeted RNAs, PKR and BCR mRNAs, in a cell-free system containing purified recombinant human 2−5A-dependent RNase L. These results demonstrated that the novel structural modification represented by 2−5A-iso-antisense provided a stabilized biologically active formulation of the 2−5A-antisense strategy.

Introduction

The small oligonucleotide known as 2−5A, (pp)pA2′p5′A2′p5′A, plays a vital role in mediating the antipicornavirus effects of interferon, and it has been speculated that 2−5A may also assume some fundamental role in cell differentiation and growth.[1−4] There are three essential components of the 2−5A system: 2−5A synthetases, a group of enzymes which, upon activation by double-stranded RNA, synthesize 2−5A from ATP; a latent 2−5A-dependent ribonuclease (RNase L) which, after activation by 2−5A, can degrade mRNA; and a 2′,5′-phosphodiesterase which degrades 2−5A to AMP and ATP and may limit the cellular toxicity of 2−5A. These enzymes act to defeat cell infection by encephalomyocarditis virus through the following mechanism. Treatment of a cell with interferon induces enhanced levels of 2−5A synthetase. Then dsRNA, formed as an intermediate in viral replication, activates the synthetase to generate 2−5A from ATP. The 2−5A activates the latent RNase L which then degrades mRNA, thereby inhibiting translation.

We have reported[5−7] a targeted mRNA destruction method which derives from the covalent linkage of a 3′,5′-antisense oligodeoxyribonucleotide and a 2′,5′-oligoadenylate activator of the 2−5A-dependent RNase. This composite nucleic acid, through the antisense domain, targets the chimera to a particular mRNA sequence which is then degraded after a localized activation of the latent RNase L by the 2−5A component of the chimera. The antisense component of the chimera provides a high degree of specificity normally missing from RNase L cleavages. The antisense region of the composite molecule also might facilitate uptake of 2−5A since antisense oligonucleotides are taken up by intact cells.[8] Finally, this approach brings into play the potent catalytic action of the latent RNase L,[9−11] perhaps thereby substantially increasing the potency of the antisense approach.[12] Recently, 2−5A-antisense has provided an effective inhibitor of the replication of respiratory syncytial virus.[13]

In this paper, we report a novel modification of 2−5A-antisense, called 2−5A-iso-antisense, based on a reversal of the direction of the polarity of the antisense domain of the 2−5A-antisense composite nucleic acid. When compared to the original 2−5A-antisense structure,[5,6,14] this new formulation shows significantly enhanced resistance to degradation by 3′-exonuclease-like enzyme activity as exemplified by snake venom phosphodiesterase and by phosphodiesterase activity found in human serum.

Materials and Methods

Synthesis of 2−5A-Antisense Chimeric Oligonucleotides. Reagents and Chemicals. The following were used for initiation of synthesis on solid support: **dG3′-lcaa-CPG** [5′-*O*-dimethoxytrityl-*N*^{2}-isobutyryl-2′-deoxyguanosine-3′-lcaa-CPG]; **dT3′-lcaa-CPG** [5′-*O*-dimethoxytritylthymidine-3′-lcaa-CPG]. **CPG** represents controlled pore glass, and **lcaa** is an abbreviation for long chain alkylamine.

These foregoing solid supports were used to synthesize oligonucleotides with the normal 3′ → 5′ phosphodi-

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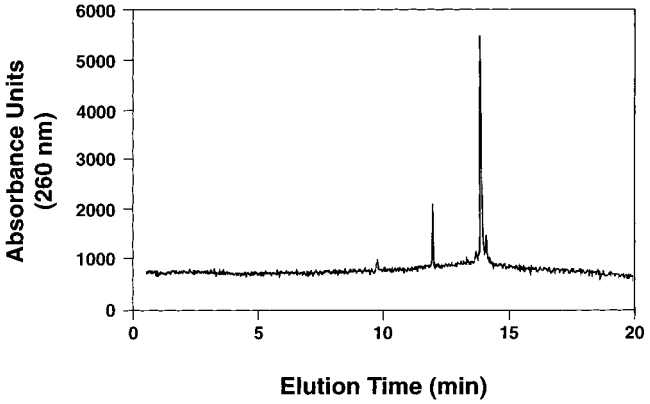

Figure 1. Capillary gel electrophoresis (CGE) of **2−5 A-iso-anti PKR** chimera. The CGE was performed on an Applied Biosystems 270A-HT capillary instrument using MICRO-GEL_{100}-filled capillaries (Applied Biosystems, Inc, Foster City, CA). Capillary dimensions were 50 mm i.d., with an effective length of 27 cm. The running buffer was 75 mM Tris phosphate (pH 7.6) with 10% methanol. UV detection was at 260 nm. The peak at 12 min was the standard reference 12-mer, $d(AGTC)_3$.

ester bonds. All were 1 μmol size and were commercially available products of Applied Biosystems (Foster City, CA).

The following solid supports were obtained from Glen Research (Sterling, VA) and were used to synthesize oligonucleotides with the reversed polarity 5′ → 3′ phosphodiester bonds ("*tail-to-tail*"). These dimethoxytrityl-protected nucleosides are attached to controlled pore glass (**CPG**) through a succinyl group and a long chain alkylamine (**lcaa**) linker. All were 1 μmol size and included **dC-5′ lcaa-CPG** [3′-*O*-dimethoxytrityl-N^4-benzoyl-2′-deoxycytidine-5′-lcaa-CPG] and **dG-5′ lcaa-CPG** [3′-*O*-dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine-5′-lcaa-CPG]. Otherwise, the procedures and reagents for synthesis of 2′,5′-oligoadenylate−antisense chimeras followed previously published procedures.[6,14,15]

Purification of the Oligonucleotides. Chimeras were purified by polystyrene reverse-phase ion-pair chromatography (PRP-IPC).[16] The oligonucleotide was converted to the sodium salt and dialyzed as described.[16]

Oligonucleotide Purity Confirmation. The purities of (2′,5′)-oligoadenylate−antisense chimeras were determined by HPLC and capillary gel electrophoresis (CGE).[14] The purity was obtained by the integration of peak area detected at 260 nm. Figure 1 shows a CGE of 2−5A-iso-antiPKR.

Mass spectra were determined as described previously[14] using matrix-assisted desorption ionization (MALDI-MS).

Characterization of (2′,5′)-Oligoadenylate−Antisense Chimeras. The nucleotide compositions of the standard "tail-to-head" chimeric oligonucleotides were determined by enzymatic digestion with snake venom phosphodiesterase[14] (*Crotallus durissus*) (Pharmacia).

To carry out digestion of "tail-to-tail" 2−5A-antisense chimeras, 0.2 *A* unit of oligonucleotide was dissolved in 100 μL of 50 mM ammonium acetate buffer (pH 6.0), then 0.15 unit of spleen phosphodiesterase was added (5 μL of 5 units/0.17 mL of ammonium sulfate), and the mixture was incubated at 37 °C for 4 h. The resulting solution was treated with Microcon-10 and then subjected to HPLC analysis. Specific conditions required

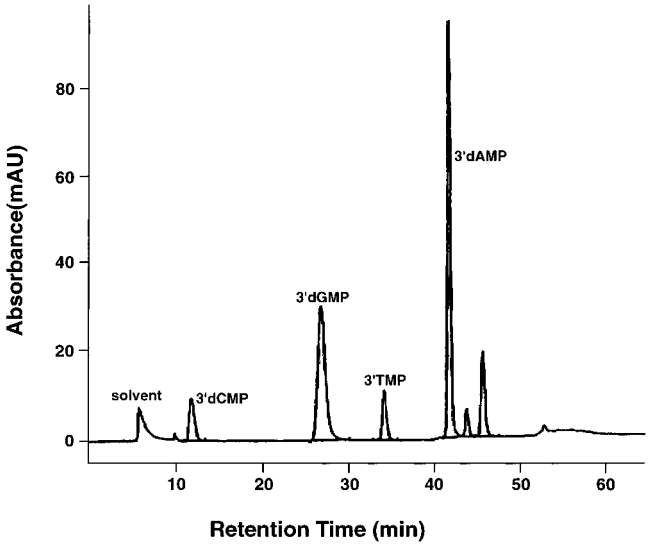

Figure 2. Reverse-phase chromatogram of spleen phosphodiesterase digested products of **2−5A-iso-sensePKR** on a Beckman Ultrasphere ODS (4.6 × 250 mm) column. The analysis was performed on a HP 1050 HPLC system. The elution program was the same as given in the legend to Figure 4.

for the HPLC analyses of such spleen phosphodiesterase digests were developed (data not shown). A typical digestion result is given in Figure 2.

Resistance of 2′,5′-Oligoadenylate−Antisense Chimeras to Degradation. The following procedures were used to study degradation of the oligonucleotides by phosphodiesterase activities.

1. Preparation of Snake Venom Phosphodiesterase Enzyme Stock. Snake venom phosphodiesterase (Pharmacia, 10 μL, containing 5 units of enzyme) was dissolved in 40 μL of Tris chloride buffer (50 mM, pH 8.0) and kept at 4 °C until use. Fresh stock of enzyme was prepared daily for these experiments. Immediately before a degradation experiment, 15 μL of the above stock solution was withdrawn and diluted with water to prepare a stock of 1.5 units of enzyme/mL. This dilution was used only once and then discarded.

2. Preparation of Degradation Reaction Mixture. A typical reaction mixture contained 0.2 A_{260} unit of oligonucleotide to be evaluated, 50 μL of 2 M NaCl, 50 μL of 1 M Tris buffer (pH 8.0), 100 μL of 100 mM $MgCl_2$, and 785 μL nuclease-free water. The final concentrations were as follows: 0.1 M NaCl, 50 mM Tris (pH 8), 10 mM $MgCl_2$, 0.2 A_{260} unit/mL of oligonucleotide, and 0.02 unit/mL of snake venom phosphodiesterase. To initiate the reaction, 15 μL of the above 1.5 unit/mL enzyme stock was added at 37 °C, and the ultraviolet absorption of the reaction mixture in a thermostated cuvette held at 37 °C was monitored by a Varian DMS 220 UV−visible spectrophotometer. As the oligonucleotides were degraded, the formation of smaller oligomers and individual mononucleotides gave a hyperchromic absorbance effect. This assay has certain limitations which have been outlined previously.[15] Results are given in Figure 3.

3. Degradation by Human Serum Components. Fresh human blood was obtained, allowed to clot at 4 °C, and then centrifuged at 4 °C. The supernatant serum was removed and used for the stability experi-

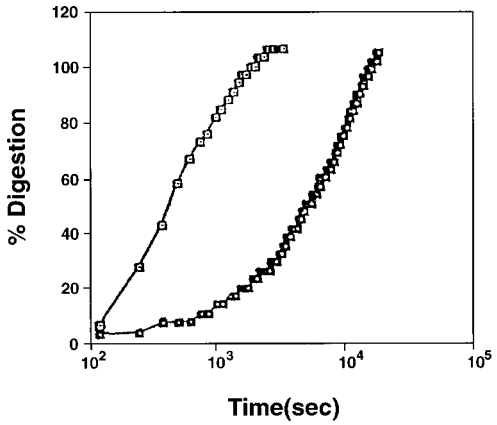

Figure 3. Digestion of phosphodiester-backbone oligonucleotides, **2−5A-antiPKR** (open squares) and **2−5A-iso-antiPKR** (filled squares), with snake venom phosphodiesterase (SVPD). 0.2 A_{260} of oligonucleotide was dissolved in 50 μL 2 M NaCl, 50 μL 1 M Tris/HCl (pH 8.0), 100 μL of 100 μM $MgCl_2$ and 785 μL H_2O. After 15 μL of 1.5 units/mL SVPD was added, the reaction mixture was incubated at 37 °C in a thermally regulated cell of a UV spectrophotometer, and the A_{260} was recorded against time.

ments. For analysis, HPLC was employed since the UV-absorbing chromophores in serum precluded the use of UV spectroscopy to follow the degradation of the oligonucleotides using hyperchromicity. The HPLC analysis system used was a Dionex PA 100 column (4 mm × 250 mm) using the following elution program: solvent A, 25 mM Tris, pH 7.0, 0.5% acetonitrile; solvent B, 25 mM Tris, pH 7.0, 0.5% acetonitrile, in 1 M ammonium chloride. Degradation experiments were carried out in the following buffer: 50 mM Tris, pH 7.8, 5 mM $MgCl_2$ The concentration of human serum present in the reaction mixtures was 10 vol %, the starting chimeric oligonucleotide concentration was 0.4 A_{260} unit/mL, and the temperature of incubation was 37 °C. Reactions were stopped by freezing aliquots in dry ice and storing at −80 °C until analysis. All HPLCs were run by co-injecting a standard with the sample to be analyzed. The results obtained are presented in Figure 4.

In Vitro Cleavage of PKR RNA by 2−5A-Antisense Chimeras. The PKR mRNA was synthesized, 5′-labeled, and purified exactly as described previously.[7] Briefly, human PKR cDNA,[17] a gift of Dr. Bryan Williams, Cleveland, was transcribed in vitro with T7 RNA polymerase. PKR mRNA was labeled at its 5′-terminus with [γ-^{32}P]ATP to specific activities of 10000−20000 cpm per mg of RNA and was then purified by electrophoresis in 6% polyacrylamide−8 M urea gels followed by elution as described.[7] Ribonuclease assays were performed as described[7] with modifications. PKR mRNA (50 nM), 2−5A-iso-antiPKR (where present), and recombinant RNase L (75 ng) were mixed in buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, 8 mM β-mercaptoethanol, and 100 mM KCl to a final volume of 20 μL on ice. Incubations were at 37 °C for 30 min. Reactions were terminated with the addition of gel sample buffer (U.S. Biochemicals). Degradation of [^{32}P]-labeled PKR mRNA was monitored according to published procedures[7] by electrophoresis in 6% polyacrylamide−8 M urea gels, autoradiography, and analysis in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

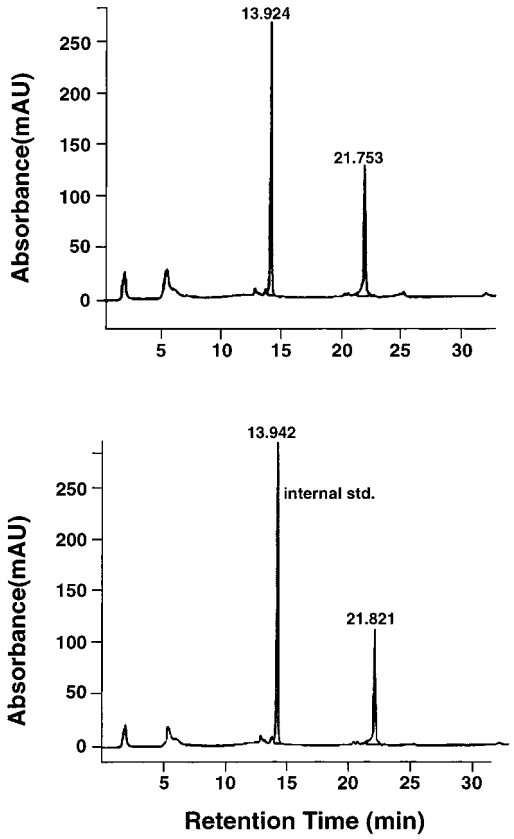

Figure 4. Dionex PA-100 anion exchange chromatograms of **2−5A-iso-antiPKR** in human serum for 0 h (top) or 30 h (bottom). This was co-injected with a reference standard dodecamer.

Application to bcr/abl RNA. The chimeric tail-to-tail oligonucleotide, **2−5A-iso-antiBCR**, was designed to be directed against the AUG start site and 3′ flanking sequence of bcr/abl mRNA and had the following sequence:

p5′A2′[p5′A2′p]$_3$O(CH$_2$)$_4$OpO(CH$_2$)$_4$Opd-
(3′TAC CAC CTG GGC CAC CCG5′)

To determine the ability of **2−5A-iso-antiBCR** to direct RNase L to the 5′-region of bcr/abl mRNA, two regions of the bcr/abl mRNA were produced in vitro. One RNA segment of 659 bases, referred to as 5′ bcr RNA, is the 5′-region of the bcr/abl mRNA including the target sequence. The other, control, RNA segment of 473 bases, called bcr/abl RNA, is from the bcr/abl fusion region and does not contain the complementary sequence to **2−5A-iso-antiBCR**. A complete coding sequence cDNA for the p210 protein version of bcr/abl in vector pGEM 4Z (plasmid [0]p210) was a generous gift from Dr. Owen Witte. Restriction fragments from this plasmid were subcloned to produce two derivative plasmids encoding a 5′-bcr RNA segment and a bcr/abl fusion-site RNA. The former plasmid, pGEM 4Z bcr 5′ was constructed by digesting plasmid [0]p210 first with *Eco*RI followed by a double digestion with *Sal*I and *Xho*I. An *Eco*RI−*Xho*I fragment encoding the 5′-bcr segment was subcloned into pGEM 4Z (predigested *Eco*RI and *Sal*I). To produce 5′-bcr RNA, plasmid pGEM 4Z bcr 5′ was linearized with *Xho*I and then transcribed with SP6 RNA polymerase. The second

plasmid was constructed by digesting [0]p210 with *Hin*dIII and Asp718, releasing a fragment encoding the bcr/abl fusion segment. The *Hin*dIII−Asp718 fragment was subcloned into the plasmid pBluescript II KS+ (predigested with *Hin*dIII and *Kpn*I) to produce plasmid pBluescript bcr/ablI. To produce the bcr/abl RNA segment, plasmid pBluescript bcr/ablI was digested with Asp718 and transcribed with T7 RNA polymerase.

The RNA segments were dephosphorylated with alkaline phosphatase (Boehringer) and then incubated with proteinase K and phenol extracted prior to labeling at the 5′-termini with 2 units of T4 polynucleotide kinase (USB) and 50 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The RNAs were purified from 6% polyacrylamide−8 M urea gels for use in the cleavage reactions. Reactions were performed in the absence or presence of **2−5A-iso-antiBCR** (25 nM) with 50 nM each of 5′-radiolabeled 5′-bcr RNA and 5′-radiolabeled bcr/ablI RNA in buffer containing 25 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 8 mM β-mercaptoethanol, and 100 mM KCl. RNase L (20 ng) was added to a final volume of 20 μL, and incubations were at 37 °C for 30 min. This RNase L preparation was made in SF21 insect cells from a human cDNA (Zhou et al., 1993) subcloned in a baculovirus vector (Clontech) and was purified with the use of fast protein liquid chromatography (Pharmacia).[9] Reactions were terminated with 10 μL of formamide stop buffer. RNA was analyzed in 6% polyacrylamide−8 M urea gels (30 × 40 × 0.04 cm). Degradation of [^{32}P]-labeled RNA was monitored by analysis in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Evaluation of 2−5a-Antisense Chimeras by RNase L-Catalyzed Cleavage of the Synthetic Substrate [5′-^{32}P]-p$C_{11}U_2C_7$. This assay was initially described by Carroll et al.[18] Synthetic oligoribonucleotide r$C_{11}U_2C_7$ was prepared by Midland Certified Reagent Co. (Midland, TX), labeled with polynucleotide kinase and [γ-^{32}P]ATP (DuPont-NEN, Wilmington, DE), and purified on a Chromaspin-10 column (Clontech, Palo Alto, CA). For cleavage assays, the cleavage buffer was 25 mM Tris HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 100 μM ATP, and 10 mM DTT. For each assay, components were added in the following order: 14−18 μL cleavage buffer, 2 μL 2−5A-antisense chimera at 10× the desired final concentration, and 2 μL of a RNase L solution to give a final concentration of 160 nM RNase L. Cleavage reaction mixtures were held on 10 min on ice after addition of RNase L. The substrate [5′-^{32}P]-p$C_{11}U_2C_7$ (2 μL of a solution to give a final substrate concentration of 10 nM) was added last and the mixture incubated at 37 °C for 15 min. Then 20 μL of RNase-free loading buffer was added, and the samples were applied to a 1 mm 20% PAGE−8 M urea gel which was electrophoresed at 350 V for 6 h at 1 °C. Gels were exposed to film, developed, and scanned.

Results

Composite Oligonucleotides Synthesized. The following 2−5A-iso-antisense oligonucleotides were prepared for this study:

p5′A2′[p5′A2′]$_3$O$(CH_2)_4$OpO$(CH_2)_4$Opd-
(5′GTA CTA CTC CCT GCT TCT G3′)

or **2−5A-antiPKR**, the first-generation "tail-to-head" chimera;[5,6,14]

p5′A2′[p5′A2′]$_3$O$(CH_2)_4$OpO$(CH_2)_4$Opd-
(3′ GTC TTC GTC CCT CAT CAT G5′)

or **2−5A-iso-antiPKR**, a "tail-to-tail" chimera;

p5′A2′[p5′A2′]$_3$O$(CH_2)_4$OpO$(CH_2)_4$Opd-
(3′ CAT GAT GAG GGA CGA AGA C5′)

or **2−5A-iso-sensePKR**, a "tail-to-tail" control chimera for **2−5A-iso-antiPKR**;

p5′A2′[p5′A2′]$_3$O$(CH_2)_4$OpO$(CH_2)_4$Opd-
(3′TAC CAC CTG GGC CAC CCG5′)

or **2−5A-iso-antiBCR**, a "tail-to-tail" chimera;

p5′A2′[p5′A2′]$_3$O$(CH_2)_4$OpO$(CH_2)_4$Opd-
(5′GCC CAC CGG GTC CAC CAT3′)

or **2−5A-antiBCR**, a first-generation "tail-to-head" chimera;[5,6,14]

p5′A2′[p5′A2′]$_3$O$(CH_2)_4$OpO$(CH_2)_4$Opd-
(5′GCC CAC CGG GTC CAC CAT3′3′C)

or **2−5A-antiBCR3′**-tail, a second-generation chimera with a single terminal inverted phosphodiester bond.[15]

The synthetic approach was similar in coupling and protecting group chemistry as described previously.[6,14] Figure 1 shows a capillary electropherogram of one HPLC purified product, **2−5A-iso-antiPKR**, demonstrating it to be at least 95% pure. The peak at approximately 12 min in the electrophoregram is a standard oligonucleotide 12-mer, d$(AGTC)_3$, which was co-injected as a reference.

Characterization of the Tail-to-Tail Chimeras Using Enzymatic Digestion. The tail-to-tail 2−5A-antisense chimeras were very resistant to the standard snake venom phosphodiesterase digestion conditions which we have previously employed to characterize the first generation "*tail-to-head*" chimeras. For instance, a 24 h digestion with the snake venom enzyme produced complete disappearance of the starting "tail-to-tail" chimera, **2−5A-iso-antiPKR**, but the digestion products could not be well quantitated. This resistance to degradation would be expected based on the "*tail-to-tail*" construction of the chimera and the resulting lack of accessible 3′-termini needed for efficient degradation by the snake venom enzyme. As an alternate approach to qualitative analysis of the 2−5A-iso-antisense chimeras, digestion by DNase I and snake venom phosphodiesterase produced from **2−5A-iso-sensePKR** the following products: 5′-dAMP, 5′-dGMP, 5′-dCMP, 5′-dAMP, 5′-AMP, and the residual undigested 2−5A domain containing AMP still conjugated to the two butanediol moieties (p5′A2′pBupBu). This latter product was the same as that which forms in the digestion of first-generation 2−5A-antisense chimeras with the snake venom enzyme[6,14] (data not shown). Nonetheless, this DNase I/venom PDE digestion also produced dephosphorylated products, and this prevented quantitative determination of base ratios. Inclusion of alkaline phosphatase with the DNase I and venom PDE failed to solve the quantitation problem, although it verifed

qualitatively the chimera nucleotide composition identities, namely, rA, dA, dC, dG, dT, and the nonphosphorylated ApBupBu (data not shown).

To achieve reliable deoxynucleotide composition analysis of the tail-to-tail chimeras, spleen phosphodiesterase was employed since it is well established that this enzyme can degrade DNA or RNA with an unblocked 5′-terminus to yield nucleotide 3′-monophosphates as products.[19] Application of spleen phosphodiesterase degradation resulted in a satisfactory analysis and characterization of the 3′,5′- deoxyribonucleotide (antisense) domain of the chimera; for example, Figure 2 shows the spleen phosphodiesterase digestion result for **2−5A-iso-antiPKR**. Under the applied conditions, degradation of the 2−5A-linker domain was not observed: instead, it appeaerd as a peak at 46 min in the HPLC (Figure 2). This is an expected[2] result since the structure obtained after enzymatic removal of the deoxyribonucleotides is a 2′-modified 2′,5′-oligoadenylate. Such 2′,5′-phosphodiester structures are inherently resistant to phosphodiesterases in general.[2] Moreover, such a fragment would be resistant to the spleen enzyme in particular since this oligoadenylate linker has no available free 5′-hydroxyl terminus necessary for the spleen enzyme attack.[19] In accord with this, no 5′-rAMP, which would have arisen from the 2′,5′-oligoadenylate region, was generated in the spleen phosphodiesterase digestion. However, as can be seen in Figure 2, the digested products included the deoxynucleotide 3′-monophosphates, identified by retention time and by determination of their UV spectra by the diode array of the HPLC system. The 3′-monophosphates were present in a ratio which confirmed the composition of the antisense domain of the chimera.

Another approach also corroborated the structure of the chimera. Previously, nuclease P1 has been shown to degrade 2−5A-antisense to a mixture of 5′-monodeoxyribonucleotides from the antisense domain but to leave the 2−5A moiety of the chimera intact.[14] This observation was based on the fact that nuclease P1 can selectively degrade 3′,5′-phosphodiester bonds in the presence of 2′,5′-phosphodiester bonds. One of the 2−5A-containing resistant products formed in a nuclease P1 digest of a 2−5A-antisense chimera was the tetraadenylate still bearing the two butanediol linkers, p5′A2′(p5′A2′)$_3$pBupBu.[14] Co-injection of a such a nuclease P1-generated digestion product from 2−5A-antiPKR[14] with the spleen PDE digestion mixture of Figure 2 effected enhancement of the HPLC peak (Figure 2) with a 46 min retention time (results not illustrated). In accord with this finding, we tenatively have assigned the structure p5′A2′ (p5′A2′)$_3$pBupBu to the peak with a retention time of 44 min (Figure 2).

MALDI-MS also could be employed to characterize chimeric oligonucleotides. For instance, **2−5A-iso-antiPKR** gave a MALDI-MS putative molecular ion of 7406.6 [calculated for $C_{231}H_{303}N_{80}O_{152}P_{25}$ (M-1) 7403.2], an error of 0.045%. These results from MALDI-MS and enzyme digestion corroborated the oligonucleotide compositions as predicted by the solid-phase phosphoramidite methodology.

Ability of "Tail-to-Tail" 2−5A-Iso-antisense Chimera To Bind to Its Target Sequence As Determined by Melting Temperature. To determine if the reversal of the phosphodiester linkage of the antisense sequence of the 2−5A-iso-antisense chimera permitted the annealing of the chimera with its target sequence, the ultraviolet absorption−temperature profile, or melting temperature, of an equimolar mixture of a representative chimera with its DNA sense oligonucleotide was determined. The more readily available DNA sense oligonucleotide was used since we have shown that 2−5A-antisense chimeras can bind to either complementary DNA or RNA (W. Xiao, G. Li, and P. F. Torrence, unpublished T_m observations). Although the melting temperatures of such a chimera with complementary DNA or RNA oligonucleotides are different, an accurate measure of the effect of the chimera modification can be obtained by observing the stability of the interaction with the DNA sense strand. Table 1 gives the T_m's as obtained for **2−5A-anti-PKR**, **2−5A-anti-BCR**, **2−5A-iso-antiPKR**, and **2−5-iso-antiBCR** chimeras, each with its complementary sequence. It can be concluded from these data that the inversion of the direction of the phosphodiester bonds in the 2−5A-antisense chimeras has little or no significant effect on the stability of the helical complex formed between the chimera and its DNA target oligonucleotide. Thus, such "tail-to-tail" 2−5A-antisense chimeras would be expected to be just as effective in binding their targeted RNA sequences as the original "tail-to-head" formulation.

Table 1. Comparative Stabilities of Oligonucleotide Complexes

oligonucleotide	T_m (°C)[a] with complementary DNA[b]
antiPKR[c]	66.2
2−5A-antiPKR[d]	66.0
2−5A-iso-antiPKR[e]	65.4
antiBCR[f]	75.4
2−5A-antiBCR[g]	75.3
2−5A-iso-antiBCR[h]	73.6

[a] The buffer employed for the T_m measurements was 0.15 M NaCl, 0.01 M Na cacodylate, 0.001 M $MgCl_2$, pH 7.0. [b] d(5′CAG AAG CAG GGA GTA GTA C3′) for PKR or d(5′ATG GTG GAC CCG GTG GGC3′) for BCR. [c] d(5′GTA CTA CTC CCT GCT TCT G3′). [d] p5′A2′p5′A2′p5′A2′p5′A2′O(CH$_2$)$_4$OpO(CH$_2$)$_4$Opd(5′GTA CTA CTC CCT GCT TCT G3′). [e] p5′A2′p5′A2′p5′A2′p5′A2′O(CH$_2$)$_4$OpO(CH$_2$)$_4$Opd(3′ GTC TTC GTC CCT CAT CAT G5′). [f] d(5′GCC CAC CGG GTC CAC CAT C3)′. [g] p5′A2′p5′A2′p5′A2′p5′A2′O(CH$_2$)$_4$OpO(CH$_2$)$_4$Opd(5′GCC CAC CGG GTC CAC CAT3′). [h] p5′A2′p5′A2′p5′A2′p5′A2′O(CH$_2$)$_4$OpO(CH$_2$)$_4$Opd(3′TAC CAC CTG GGC CAC CCG5′).

Stability of Oligonucleotides to Degradation by Snake Venom Phosphodiesterase, a 3′-Exonuclease. Snake venom phosphodiesterase was employed to measure the stability of the tail-to-tail 2−5A-antisense chimera or 2−5A-iso-antisense to a representative 3′-exonuclease, the general enzymic activity responsible for the degradation of oligonucleotides in biological milieu. The results of these experiments are shown in Figure 3. It is clear that the tail-to-tail 2−5A-antisense chimera was much more stable than the tail-to-head counterpart. The half-life for the degradation of the tail-to-head chimera was 7 min whereas the half-life for the degradation of the tail-to-tail chimera was 88 min, a greater than 10-fold increase in stability to the 3′-exonuclease, snake venom phosphodiesterase.

Stability Toward Degradation by Human Serum. Since the tail-to-tail modification showed greatly enhanced stability toward a typical 3′-exonuclease, its

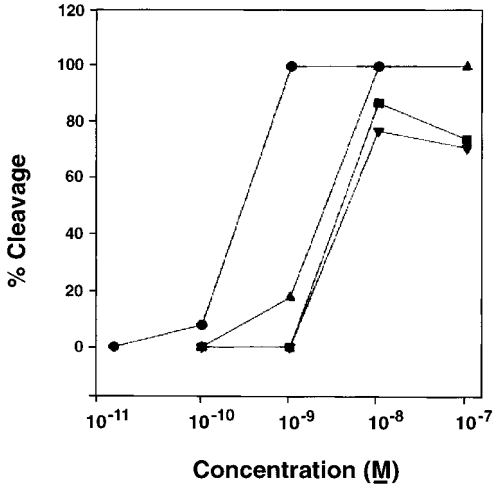

Figure 5. Effect of 2−5A or 2−5A-antisense chimeras on the ability of homogeneous recombinant RNase L to cleave the synthetic oligoribonucleotide [5′-^{32}P]-p$C_{11}U_2C_7$: ●, p5′A2′p5′A2′-p5′A2′p5′A; ■, **2−5A-antiBCR**; ▼, **2−5A-iso-antiBCR**; ▲, **2−5A-antiBCR3′-tail**.

stability in the presence of human serum was ascertained. The results of such experiments are shown in Figure 4. The half-life for the degradation of the unmodified **2−5A-antiPKR** chimera was approximately 12 h (not shown), the same as demonstrated previously under these conditions.[15]

In distinct contrast to the above situation, no trace of degradation of the tail-to-tail **2−5A-iso-antiPKR** chimera was observed even after 30 h of incubation in 10% serum at 37 °C (Figure 4). Thus, consistent with the results of the snake venom digestion, the tail-to-tail backbone modification endowed the **2−5A-antiPKR** chimera with greatly enhanced resistance to enzymatic degradation.

RNase L-Catalyzed Cleavage of the Synthetic Substrate [5′-^{32}P]-p$C_{11}U_2C_7$. Carroll et al.[18] have described cleavage of a short synthetic oligoribonucleotide by activated RNase L. Although this substrate would not anneal to any of the antisense sequences in the 2−5A-antisense chimeras that are the subject of this study, these assays permit a comparison of the abilities of the different oligonucleotides to effect the general activation of RNase L. The experiment of Figure 5 showed that while none of the 2−5A-antisense chimeras were as effective as parent 2−5A tetramer [p(5′A2′p)$_3$A] in causing cleavage of [5′-^{32}P]-p$C_{11}U_2C_7$, the three different backbone formulations, **2−5A-antiBCR**, **2−5A-iso-antiBCR**, and **2−5A-antiBCR3′-tail** were essentially equivalent in their ability to bring about substrate degradation.

Cleavage of PKR mRNA by 2−5A-Iso-antiPKR. Previously, we ablated PKR mRNA and activity in HeLa cells using **2−5A-antiPKR** to this sequence.[7] **2−5A-Iso-antiPKR** was found to be an effective activator of RNase L with PKR mRNA as substrate (Figure 6). After a 30 min incubation, there was only 4.4% of the intact PKR mRNA remaining when the concentration of **2−5A-iso-antiPKR** was 25 nM. A 50% loss of intact PKR mRNA occurred between concentrations of 5 and 10 nM **2−5A-iso-antiPKR** (Figure 6). The autoradiogram of the dried gel and the PhosphorImager data are shown in Figure 6, parts A and B, respectively. These

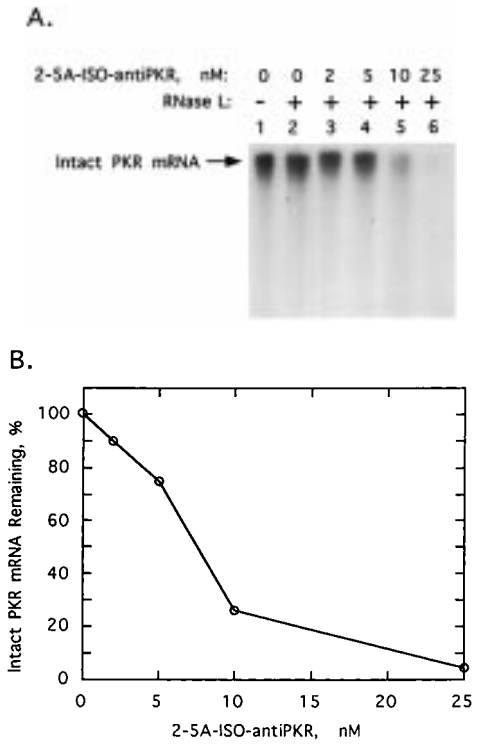

Figure 6. **2−5A-Iso-antiPKR** induced the degradation of PKR mRNA by RNase L. ^{32}P-end labeled PKR RNA and RNase L were incubated in buffer in the absence or presence of different amounts of **2−5A-iso-antiPKR** (*x*-axis of panel B). Panels A and B are the autoradiogram and a plot of the data obtained by PhophorImager analysis of the dried gel, respectively.

data confirm the potential of 2−5A-iso-antisense compounds as agents for destruction of mRNA molecules.

Specific Cleavage of a bcr RNA. Another potential target for application of 2−5A-antisense is the bcr/abl oncogene, implicated as the cause of chronic myelogenous leukemia (CML).[20,21] The 2−5A-iso-antisense approach now has been applied to the in vitro degradation of the related bcr RNA. The results show that **2−5A-iso-antiBCR** was able to activate RNase L to cleave the 5′-bcr RNA (Figure 7) containing the complementary sequence to the antisense domain of the chimera (Figure 8). After the incubation, only 22% of the 5′-bcr RNA remained intact. In contrast, we could measure no degradation of the bcr/abl RNA, encoding the noncomplementary (to the antisense region of **2−5A-iso-antiBCR**) fusion sequence, present in the same reaction mixtures. Similar findings were obtained using higher amounts (50 and 100 nM) of **2−5A-iso-antiBCR** (not shown). These findings demonstrate the activity of a 2−5A-iso-antisense compound against the bcr mRNA.

Discussion

2−5A-Antisense chimeras have exhibited biological activity in intact cells in tissue culture as such, without any other agent to enhance cellular uptake.[7,13] This has been an attractive feature of the 2−5A-antisense technology as many other antisense approaches require the use of compounds to facilitate transport into cells.[8,22−24] Nonetheless, in view of the known nucleic acid degradative enzymes present in cells and sera,[8,22−24] the biological activity of such first generation 2−5A-anti-

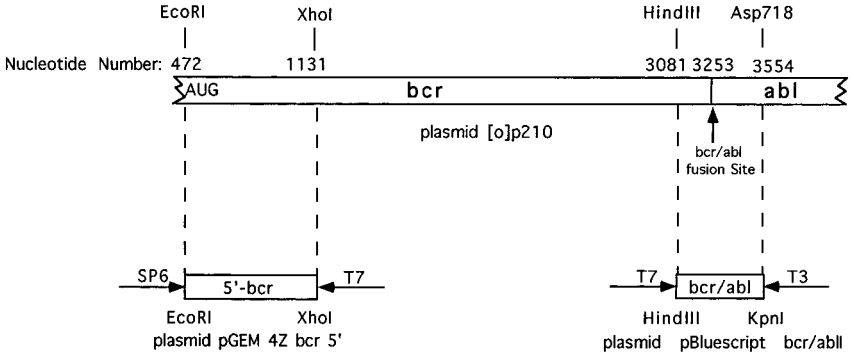

Figure 7. Construction of plasmids encoding the 5′-bcr region and the bcr/abl region for in vitro transcription reactions.

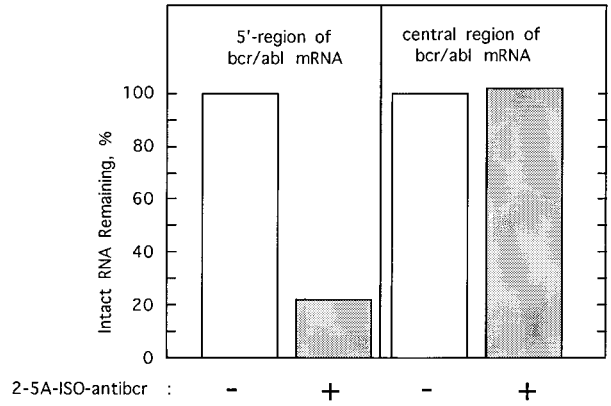

Figure 8. 2−5A-Iso-antiBCR induced the degradation of the 5′-region of bcr RNA in the absence of cleavage of the bcr/abl fusion region RNA. Two segments of the bcr/abl mRNA corresponding to the 5′-bcr region and the bcr/abl fusion sequence (see Figure 7) were ^{32}P-end labeled, gel-purified, and incubated together with **2−5A-iso-antiBCR** and purified RNase L (see taxt for details). Results were obtained by PhosphorImager analysis of the dried gel.

sense chimeras likely could be improved by stabilizing them to degradation. Such 2−5A-antisense structures described above have a DNA antisense domain which terminates with 3′-deoxyribonucleotide with a natural 3′,5′-phosphodiester bond.[5,6] As this oligonucleotide 3′-terminus is identical with the natural DNA, it would be expected to be a ready substrate for the degradative enzyme class of 3′-exonucleases. Indeed, as is demonstrated previously[15] and herein, such first-generation chimeras as listed above have a limited half-life when exposed to sera or pure snake venom phosphodiesterase, a prototypical 3′-exonuclease. Second-generation or 3′,3′-terminated 2−5A-antisense chimeras have shown increased resistance to nuclease attack[15] and significant antiviral activity.[13]

The principal class of enzyme(s) responsible for degrading unmodified oligonucleotides has a substrate specificity similar to that described for other 3′-exonucleases or phosphodiesterases such as the snake venom phosphodiesterase from *Crotalus atrox* sera.[8,22−24] Typically, 5′-exonucleases appear to be less evident as agents responsible for oligonucleotide degradation. Moreover, the 5′-terminus of 2−5A-antisense chimeras have two features which mitigate strongly against the action of 5′-exonucleases (such as spleen phosphodiesterase). Since 5′-exonucleaes require a free 5′-hydroxyl moiety on the 5′-terminal nucleoside,[3,4,9] 2−5A-antisense chimeras, constituted as above with a 5′-monophosphate group, would be poor substrates. Added to this is the significantly greater resistance of 2′,5′-phosphodiester bonds to enzymatic degradation in general.[2] Thus, the three 2′,5′-phosphodiester bonds of 2−5A-antisense chimeras would be relatively unreactive to 5′-exonuclease on this basis also. Evidence does exist for a phosphodiesterase which has a greater preference for 2′,5′-phosphodiester bonds than for the isomeric 3′,5′-bonds, but this enzyme degrades from the 2′-terminus of such 2′,5′-oligonucleotides.[25,26] We have previously demonstrated that the 2′,5′-oligoadenylate region of 2−5A-antisense chimeras is protected from phosphodiesterase action by the structural nature of such chimeras.[6] Finally, for protection of the 5′-terminus of 2−5A-antisense chimeras against degradation by 5′-exonucleases, we have synthesized chimeras with 5′-terminal thiophosphate instead of normal phosphate.[27] This 5′-thiophosphate alteration greatly enhanced resistance to phosphatase action,[27] and such novel 5′-thiophosphate 2−5A-antisense congeners retain the ability to activate the RNase L. As an added bonus, the 5′-thiophosphate, by reducing the ability of phosphatases to expose the necessary free 5′-hydroxyl, indirectly protects this class of 2−5A-antisense analogues from 5′-exonuclease degradation.

The original formulation[5,6] of 2−5A-antisense involved the linkage of the 2′-terminus of the 2−5A moiety through a linker to the 5′-terminus of an antisense deoxyribonucleotide [tail-to-head configuration]:

p5′A2′p[5′A2′p]$_3$-
[linker]p5′dN3′p5′dN3′p...5′dN3′p5′dN3′OH

The requirement of the 2−5A-dependent ribonuclease (RNase L) for a free 5′-mono- or polyphosphate group[2,3,4,9] precluded linkage of the 2′,5′-oligoadenylate in the reverse orientation [head-to-head configuration].

A5′p2′[A5′p2′]$_2$A5′p-
[linker]p5′dN3′p...5′dN3′p5′dN3′OH

However, there exists no known constraint to argue against the possibility to link the 2′-terminus of the 2−5A moiety to the 3′-terminus of the antisense domain [tail-to-tail configuration].

p5′A2′p[5′A2′p]$_3$-
[linker]p3′dN5′p3′dN5′p...3′dN5′p3′dN5′OH

This reversed linkage buries the once exposed 3′-terminus of the oligonucleotide deep within the structure, making it inaccessible to 3′-exonucleases.

In this paper, we have provided evidence that the latter tail-to-tail or 2−5A-iso-antisense chimeric oligonucleotide represents a new structural approach to impart resistance to enzymic degradation to the class of 2−5A-antisense chimeras for the following reasons.

1. The oligonucleotide prototype synthesized herein is a *composite* nucleic acid containing a first domain of 2′,5′-oligoadenylate which serves to activate the constitutive and latent RNase L and a second isomeric oligonucleotide domain with 3′,5′-phosphodiester bonds and which serves to bond to a targeted RNA with a specified complementary deoxyribonucleotide sequence. Thus, the composite or chimeric oligonucleotide consists of a 2′,5′-linked oligonucleotide connected through the 2′-terminus through a linker moiety to the 3′-terminus of a 3′,5′-linked oligonucleotide.

2. This new backbone oligonucleotide is endowed with the following properties: (A) It is capable of forming a complementary complex with an oligonucleotide of complementary sequence of stability (T_m) essentially undiminished from the complex formed from the prototypical tail-to-head 2−5A-antisense chimera, or the complex formed from the parent unmodified (no 2−5A moiety) oligonucleotide. (B) This novel tail-to-tail 2−5A-iso-antisense chimera can activate the RNase L to degrade an RNA. (C) By virtue of the polarity of the two different isomeric nucleic acid backbones, this tail-to-tail 2−5A-antisense chimera is significantly more resistant to degradation than the first generation *tail-to-head* 2−5A-antisense chimera, thereby extending its lifetime in biological milieu, but without adding any modified nucleotide which itself may carry with it additional biological properties. (D) In summary, such novel tail-to-tail 2−5A-iso-antisense chimeras provide a molecule with three vital properties of relevance to the therapeutic application of 2−5A-antisense technology; namely, ability to form a stable complex with a selected nucleotide sequence in a targeted RNA, capacity to activate RNase L which is responsible for targeted RNA degradation, and substantial resistance to enzymatic degradation.

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Supporting Information Available: Capillary gel electrophoretic analyses of **2-5A antiPKR**, **2−5A-iso-sensePKR**, **2−5A-iso-antBCR**, **2−5A-antiBCR**, **2−5A-antiBCR3′-tail**; snake venom phosphodiesterase digestion analyses of **2-5A-antiPKR**, **2−5A-antiBCR**, and **2−5A-antiBCR3′-tail**; and MALDI-TOF mass spectrometric analyses of **2−5A-iso-anti-PKR** (internal and external calibration (10 pages). Ordering information is given on any current masthead page.

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