Synthesis and Properties of Second-Generation 2-5A-Antisense Chimeras with Enhanced Resistance to Exonucleases

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In order to stabilize 2-5A-antisense chimeras to exonucleases, we have synthesized chimeric oligonucleotides in which the last phosphodiester bond at the 3′-terminus of the antisense domain was inverted from the usual 3′,5′-linkage to a 3′,3′-linkage. The preparation of such analogues was accomplished through standard phosphoramidite chemistry with the use of a controlled pore glass solid support with a nucleoside attached through its 5′-hydroxyl, thereby permitting elongation at the 3′-hydroxyl. The structures of such terminally inverted linkage chimeras of the general formula pA_4-$[pBu]_2$-$(pdN_n3'$-$3'dN)$ were corroborated by a combination of snake venom phosphodiesterase digestion in the presence or absence of bacterial alkaline phosphatase. Most characteristically, the presence of the 3′-terminal-inverted phosphodiester linkage produced an unnatural dinucleotide of general composition dN3′p3′dM. These structures could be confirmed by independent synthesis and fast atom bombardment mass spectroscopy (FAB). 2-5A-Antisense chimeras of this structural class, pA_4-$[pBu]_2$-$(pdN_n3'$-$3'dN)$, were 5−6-fold more stable than their unmodified congeners, pA_4-$[pBu]_2$-$(pdN)_n$, to degradation by a representative phosphodiesterase from snake venom. In 10% human serum, the new 2-5A-antisense chimeras, pA_4-$[pBu]_2$-$(pdN_n3'$-$3'dN)$, possessed a half-life that was 28-fold longer than that of the unmodified chimeras. These results provide entry to a second generation of 2-5A-antisense chimeras.

Introduction

The decay of mRNA is of cardinal importance in the post-transcriptional regulation of gene expression and can be influenced by a variety of environmental and developmental factors.[1] The use of synthetic compounds to modulate the process of mRNA degradation is a promising strategy for the therapy of viral and neoplastic diseases that result from inappropriate expression or overexpression of specific mRNAs. The antisense technology approach[2,3] to the control of gene expression can involve, in addition to a steric blocking mechanism of action, the RNase H- or RNase III-catalyzed degradation of targeted mRNA species.[2,3] Recently, RNase L, a 2′,5′-oligoadenylate-activated nuclease associated initially with the interferon system, has been exploited for the directed degradation of a targeted mRNA.[4−8] In this latter approach, conjugation of an antisense oligonucleotide with the low molecular weight inhibitor of protein synthesis, 2-5A,[9,10] provides a chimeric molecule (2-5A-antisense) with unique properties. While the antisense domain of the chimera addresses a specific nucleotide sequence of a targeted RNA, its covalently linked partner, 2-5A, attracts and activates the constitutive RNase L[10,11] which degrades the selected RNA. Sequence-specific cleavage of a modified human immunodefiency virus RNA[4] and of mRNA encoding the dsRNA-dependent protein kinase (PKR)[6,7] has been achieved in cell-free systems, as well as ablation in intact HeLa cells of PKR mRNA, PKR protein, and the biologic function of PKR.[6]

The first generation of 2-5A-antisense chimeric oligonucleotides[5] entailed the covalent conjugation of 5′-monophosphoryladenylyl(2′→5′)adenylyl(2′→5′)adenylyl(2′→5′)adenosine [p5′(A2′p)$_3$A] through two 1,4-butanediol phosphate linker molecules to a 5′-phosphorylated 3′,5′-deoxyribonucleotide. The general formula for this novel oligonucleotide is p5′$(A2'p)_3$A2′$p(CH_2)_4$p-$(CH_2)_4$p$(5'N3'p)_m$N, where N is any nucleoside and *m* is any integer.

Initial biological studies were successful with such first-generation chimeras,[4,6] even though they bore no specific modifications to endow them with enhanced resistance to degradation in biological milieu. Three principal modes of biologic inactivation may be expected for such 2-5A-antisense oligonucleotides. First, phosphatase enzymes may remove the 5′-monophosphate moiety which is required for effective activation of the RNase L.[4,10,12] This mode of inactivation has been minimized by introduction of the 5′-thiophosphate group which is highly resistant to phosphatase action.[13] Second, endonucleases may cleave both the antisense, linker, and 2′,5′-oligoriboadenylate domains at internal phosphodiester linkages. Third, exonucleases may degrade the 2-5A-antisense chimeras chiefly from the 3′-terminus. Herein, we describe a second generation of 2-5A-antisense chimeras which have been modified to resist 3′-exonuclease.

Materials and Methods

Synthesis of 2-5A-Antisense Chimeric Oligonucleotides. The following generic oligonucleotide structural types were prepared for this study: (**I**) p5′A2′p(5′A2′p)$_3$-$[O(CH_2)_4O$p$]_2$-5′dN3′p(5′dN3′p)$_n$5′dN and (**II**) p5′A2′p(5′A2′p)$_3$-$[O(CH_2)_4$-Op$]_2$-5′dN3′p(5′dN3′p)$_m$5′dN3′p-3′pdN5′. The following procedures are illustrative of those employed to synthesize the 2-5A-antisense chimeric oligonucleotides above. In general, they follow the synthetic strategy developed in Lesiak et al.[5] and Xiao et al.[14]

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Reagents and Chemicals. For synthesis of oligonucleotides of structural type **I**, all DNA synthesis reagents, phosphoramidites, and long chain alkylamino-controlled pore glass solid supports (3′-lcaa-CPG 500) were purchased from Applied Biosystems (Foster City, CA).

These following solid supports (all 1 μmol size) were obtained from Glen Research (Sterling, VA) and used to synthesize oligonucleotides with the reversed polarity 3′→3′ (structural type **II**) phosphodiester bonds: 3′-*O*-(dimethoxytrityl)-N^6-benzoyl-2′-deoxyadenosine-5′-lcaa-CPG, 3′-*O*-(dimethoxytrityl)-N^4-benzoyl-2′-deoxycytidine-5′-lcaa-CPG, and 3′-*O*-(dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine-5′-lcaa-CPG. Synthesis conditions were as previously described.[5,14]

For elongation of the DNA antisense chain of the oligonucleotides, the following phosphoramidites were used: 5′-*O*-(dimethoxytrityl)-N^6-benzoyl-2′-deoxyadenosine 3′-[2-(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite], 5′-*O*-(dimethoxytrityl)-N^4-benzoyl-2′-deoxycytidine 3′-[2-(cyanoethyl-)*N*,*N*-diisopropylphosphoramidite], 5′-O-(dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine 3′-[2-(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite], and 5′-*O*-(dimethoxytrityl)-2′-deoxythymidine 3′-[2-(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite] (Applied Biosystems). The linker to join chimeric domains was 2-(cyanoethyl)-*N*,*N*-diisopropyl-4-*O*-4,4′-(dimethoxytrityl)butylphosphoramidite which was synthesized by a modification of an earlier described procedure.[5]

For the synthesis of the 2′,5′-oligoadenylate domain of the chimera,[5,14] 5′-*O*-(dimethoxytrityl)-N^6-benzoyl-3′-*O*-(*tert*-butyldimethylsilyl)adenosine 2′-(*N*,*N*-diisopropylcyanoethylphosphoramidite) (ChemGenes Corp., Waltham, MA, cat. no. ANP 5681) was used. The phosphorylation reagent for the 5′-terminus of the 2′,5′-oligoadenylate domain of the chimera was 2-[[2-(4,4′-dimethoxytrityl)ethyl]sulfonyl]ethyl-2′-(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (Glen Research, Sterling, VA, cat. no. 10-1900-90).[5,14] A 1 μmol scale standard synthesis cycle was used. The cycle was modified by changing the coupling time (coupling of monomer) for each different region of the chimera. For the DNA antisense domain, 15 s was the coupling time, 300 s was used for the linker domain, 600 s was for the 2′,5′-oligoadenylate region, and 60 s was the coupling time for the 5′-phosphorylation. Conditions for synthesis and deprotection of oligonucleotides were as described previously.[5,14]

For oligonucleotide purification, a PRP-1 HPLC column (300 × 7 mm) was used with the following elution protocol:[15] solvent A was 10 mM tetrabutylammonium phosphate (TBAP), pH 7.5, in water; solvent B was 10 mM TBAP, pH 7.5, in acetonitrile/water (8:2, v/v); elution with a convex gradient of 5−80% solvent B in A in 45 min at a flow rate of 1.5 mL/min.

Fractions containing desired oligonucleotide were pooled and evaporated to about 1−2 mL and desalted by a C-18 Sep-Pak cartridge. The oligo-TBA salt was converted into its sodium salt form by the following procedure: 1 mL of Dowex 50W ion exchange wet resin (Na^+ form) was added to the oligonucleotide/water solution. The solution was stirred for at least 30 min at 4 °C. The resin was removed by passing the solution through a Poly-Prep chromatography column (Bio-Rad, cat. no. 731-1550), and the resin was washed with extra water until no oligonucleotide remained on the resin.

The dinucleotide p5′dC3′p5′dC was synthesized employing the above reagents and protocols used for the antisense domain of the chimeras. Terminal phosphorylation was accomplished using 2-[[2-(4,4′-dimethoxytrityl)ethyl]sulfonyl]ethyl-2′-(cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (Glen Research, Sterling, VA, cat. no. 10-1900-90).[5,14] Deprotection was as for the 2-5A-antisense chimeras except that TBAF deprotection was not required. Product was purified using a Dionex HPLC column.

After purification by HPLC, desalting, and ion exchange, the oligonucleotide (sodium salt) was dialyzed to remove small molecules and excess salt. The oligonucleotide was dialyzed against 0.02 M NaCl first for 4−6 h and then against water for 48 h. The oligonucleotide, after dialysis, was passed through a 0.22 μm Millex-GV filter unit (Millipore, cat. no. SLGV025LS) for sterilization. The resulting solution was quantitated as OD A_{260} by UV/vis spectrophotometry. The purities of 2′,5′-oligoadenylate/antisense chimeras were checked by Dionex HPLC and/or capillary gel electrophoresis using conditions already published.[5,14]

Base Composition Analyses of 2-5A-Antisense Chimeras. The nucleotide compositions of the chimeric oligonucleotides were analyzed by enzymatic digestion with snake venom phosphodiesterase (*Crotallus durissus*) (Pharmacia, cat no. 27,0821-01).[5,14]

Exonuclease Resistance of 2-5A-Antisense Chimeras. Sensitivity to 3′-exonuclease degradation was measured by hyperchromicity changes at 260 nm in the presence of snake venom phosphodiesterase (SVPD). 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 of nuclease-free water. To initiate the reaction, 15 μL of the 1.5 units/mL enzyme was added at 37 °C, and the ultraviolet absorption of the reaction mixture in a thermostatted cuvette held at 37 °C was monitored by a Varian DMS-200 spectrophotometer.

Degradation of Oligonucleotides by Human Serum Components. Fresh human blood was obtained, allowed to clot, and then centrifuged. The supernatant serum was removed and used for the stability experiments. The concentration of human serum present in the reaction mixtures was 10% by volume in a buffer of 50 mM Tris, pH 7.8, 5 mM $MgCl_2$. The starting chimeric oligonucleotide concentration was 0.4 A_{260} unit/mL, and the temperature of the incubation was 37 °C. Reactions were stopped by freezing aliquots in dry ice and storing at −80 °C until analysis. Controls showed that this procedure halted degradation. Ion exchange HPLC was employed for analysis since the UV-absorbing chromophores in serum precluded the use of UV spectroscopy to follow degradation of the oligonucleotides. A typical chromatogram of 2′,5′-oligoadenylate/antisense was obtained under the following conditions: a Dionex NucleoPac PA-100 (4 × 250 mm) column was used with solvent A as 25 mM Tris/HCl, 0.5% acetonitrile (pH 7.0) and solvent B as 25 mM Tris/HCl, 0.5% acetonitrile containing 1 M ammonium chloride (pH 7.0). Elution was with a linear gradient of 10−80% B in A during 30 min followed by isocratic 80% for 10 min at a flow rate of 1 mL/min. Detection was at 260 nm. All HPLCs were run by coinjecting a standard with the sample to be analyzed. The standard was an 11-mer, specifically, d(5′ATC ATC ATC GG3′).

Results

Synthesis and Characterization of 3′-3′-Tailed 2-5A-Antisense Chimeras. The following 2-5A-antisense chimeric oligonucleotides with terminal 3′,3′-internucleotide phosphodiester bonds were prepared: pA_4-$[pBu]_2$-pd(gta cta ctc cct gct tct g3′-3′c), a chimera against PKR mRNA;[6] pA_4-$[pBu]_2$-pd(gcc cac cgg gtc cac cat3′-3′c), a chimera against *bcr-abl* mRNA; pA_4-$[pBu]_2$-pd(tgg gaa gct gtc act gta gag3′-3′c), a chimera directed against the signaling protein STAT mRNA; and pA_4-$[pBu]_2$-pd(aat ggg atc cat ttt gtc c3′-3′c), a 2-5A-antisense chimera against respiratory syncytial virus mRNA.

The purity of synthetic 2-5A-antisense chimeras could be determined by either Dionex HPLC method or capillary gel electrophoresis. Usually, both capillary gel electrophoresis and Dionex PA-100 ion exchange HPLC column could provide higher resolution and better peak shape compared with other HPLC chromatographic methods for the analysis of 2-5A antisense chimeras. The purities of synthesized 3′-3′-tailed chimeras were >90% determined by either Dionex HPLC or the capillary gel electrophoresis method.

A key step in the corroboration of the structure of synthetic 2-5A-antisense chimeras has been digestion of the composite nucleic acid with snake venom phosphodiesterase.[5,14] This procedure can yield valuable information not only about the DNA antisense sequence

but also concerning the presence of requisite amounts of butanediol phosphate linker, 2′,5′-linked AMP residues, and the presence of a 5′-monophosphate moiety. When a representative 3′-3′-tailed chimera, p5′A2′-(p5′A2′)$_3$-[pBu]$_2$-pd(aat ggg atc cat ttt gtc c3′-3′c), was digested under standard conditions[5,14] with snake venom phosphodiesterase, the HPLC of digestion products revealed seven major peaks (Figure 1A) with retention times of 10.8, 29.2, 31.1, 32.59, 33.4, 39.3, 40.2, and 41.5 min. These corresponded to, respectively, dCMP, dTMP, dGMP, rAMP, and pA2′pBupBu,[14] and at 40.2 min there appeared a new product not observed in a similar digestion of a standard, first-generation 2-5A-antisense chimera with no 3′-3′ linkage. Lastly came dAMP at t_R = 41.5 min. The overall ratio of above nucleotidic digestion products was 3:7:4:3:4 for dCMP:dTMP:dGMP:rAMP:dAMP, revealing an underabundance of dCMP from that expected from complete digestion. However, the peak with retention time of 40.2 min possessed a UV spectrum that could be generated by 1:1 addition of the spectra of two mononucleotides, 2 × dCMP (Figure 1A). Thus, the structure pdC3′p3′dC was assigned to this new peak. The structure of this product 3′-3′ dinucleotide was corroborated by comparison of the HPLC chromatogram of enzymatically digested product with synthetic dinucleotide. For example, the synthetic product pdC3′p3′dC was analyzed by fast atom bombardment mass spectra (FAB$^-$) and showed a mass of 595.1 with only 0.01% error. This synthetic pdC3′p3′dC product gave the same t_R (40.2 min) as the enzymatic digestion product.

Similar HPLC digestion patterns were obtained when other 3′-3′-tailed chimeras were digested with snake venom phosphodiesterase. For instance, pA$_4$-[pBu]$_2$-pd(gcc cac cgg gtc cac cat3′-3′c) gave the dinucleotide pdT3′p3′dC (t_R = 46.0 min), and pA$_4$-[pBu]$_2$-pd(tgg gaa gct gtc act gta gag3′-3′c) yielded pdG3′p3′dC (t_R = 44.4 min). Again, the 3′-3′ dinucleotides were assigned structures based on a comparison of their UV spectra with the calculated spectra from 1:1 summation of constituent mononucleotides.

Evident from the above HPLC data was the fact that the retention times of the 3′-3′ dinucleotide products varied depending upon their composition. Critically, pdC3′p3′dC's retention time was close to that of other digestion products such as dAMP (t_R = 41.5 min) or p5′A2′pBupBu (t_R = 40.2). Under these conditions, accurate analysis of these key digestion products was problematic. In order to obviate this difficulty, digestion with snake venom phosphodiesterase was carried out with the addition of bacterial alkaline phosphatase. This resulted in the removal of all noninternucleotide phosphates so that digestion products consisted of nucleosides and the 3′-3′ dinucleotide. Thus, for instance, when the chimera pA$_4$-[pBu]$_2$-pd(aat ggg atc cat ttt gtc c3′-3′c) was digested with snake venom phosphodiesterase and bacterial alkaline phosphatase, the following products (with corresponding retention times) were obtained: dC (t_R = 25.6 min), dG (t_R = 42.8 min), dT (t_R = 44.8 min), dC3′p3′dC (t_R = 47.8 min), rA (t_R = 50.1 min), dA (t_R = 51.8 min), and A2′pBupBu (t_R = 54.9) (Figure 1B). Thus, this modified digestion procedure resulted in a shift in t_R values such that each individual product was well separated from the others. This additional digestion procedure is not always necesssary depending on the constitution of the dinucleotide product. It was found that most other 3′-3′ dinucleotides could be resolved very well from all nucleotides and could be easily identified by its on-line UV spectra and subsequently integrated.

Determination of the Stability of the Relative Stabilities of the 3′-3′-Tailed 2-5A-Antisense Chimeras to Enzymatic Degradation. 1. Degradation by Snake Venom Phosphodiesterase. Two representative 3′-3′-tailed 2-5A-antisense chimeras were compared to parent unmodified 2-5A-antisense chimeras for their susceptibility to degradation. Specifically, the rate of degradation of pA$_4$-[pBu]$_2$-pd(gta cta ctc cct gct tct g3′-3′c) was compared with that for the unmodified pA$_4$-[pBu]$_2$-pd(gta cta ctc cct gct tct g3′) (Figure 2), and in a separate experiment the rate of degradation of pA$_4$-[pBu]$_2$-pd(gcc cac cgg gtc cac cat3′-3′c) was compared to the degradation rate of the first-generation chimera pA$_4$-[pBu]$_2$-pd(gcc cac cgg gtc cac cat3′) (not shown). In both instances, a notable increase in stability toward degradation was observed with the 3′-3′-tailed 2-5A-antisense oligonucleotide. Thus, the 3′-3′-tailed chimera, pA$_4$-[pBu]$_2$-pd(gta cta ctc cct gct tct g3′-3′c), had a $t_{1/2}$ for degradation of 43 min, a 6-fold increase compared to the unmodified pA$_4$-[pBu]$_2$-pd(gta cta ctc cct gct tct g3′). The chimeric 3′-3′-tailed pA$_4$-[pBu]$_2$-pd(gcc cac cgg gtc cac cat3′-3′c) showed a $t_{1/2}$ of 100 min, a 5.5-fold stability increase compared to the parent untailed oligomer pA$_4$-[pBu]$_2$-pd(gcc cac cgg gtc cac cat3′).

For the above evaluations of stability toward snake venom phosphodiesterase, the UV spectrum of the oligonucleotide/enzyme reaction mixture was monitored as a function of time, and the increase in absorbance at the λ_{max} was assumed to be related to oligonucleotide degradation. The digestion was permitted to proceed overnight after which time there was no further increase in absorbance. This absorbance reading was assumed to represent 100% digestion. With the absorbance reading at t = 0 taken as 0% degadation, intermediate values of absorbance could be converted to percent degradation.

In the strictest sense, a linear relationship between hyperchromicity and oligonucleotide would not be expected since the hypochromicity per nucleotide residue tends to plateau for longer oligonucleotides while hypochromicity per nucleotide is less per nucleotide residue as chain length decreases from intermediate to shorter oligomers. For instance, the molar extinction coefficients per base for oligocytidylic acids are 6140 for (Cp)$_{29}$C and 6200 for both (Cp)$_9$C and (Cp)$_8$C but increases to 6340 for (Cp)$_7$C, to 6630 for (Cp)$_6$C, to 6820 for (Cp)$_5$C, and to 7250 for (Cp)$_3$C.[15a] Thus, a less dramatic increase in absorbance would be anticipated when a longer oligonucleotide, such as a 19-mer, undergoes a loss of one nucleotide. In that case, the product mononucleotide would show hyperchromicity, but the 18-mer product would not. However, when a shorter oligonucleotide, such as an octamer, is cut to yield a heptamer, the absorbance increase expected would be greater than in the case of a 19-mer since hyperchromicity contributions would arise now from both the shortened oligonucleotide as well as the severed mononucleotide. Thus, the half-life measured using this approach would be an apparent $t_{1/2}$ and may not represent the true $t_{1/2}$. Nonetheless, for oligonucleotides

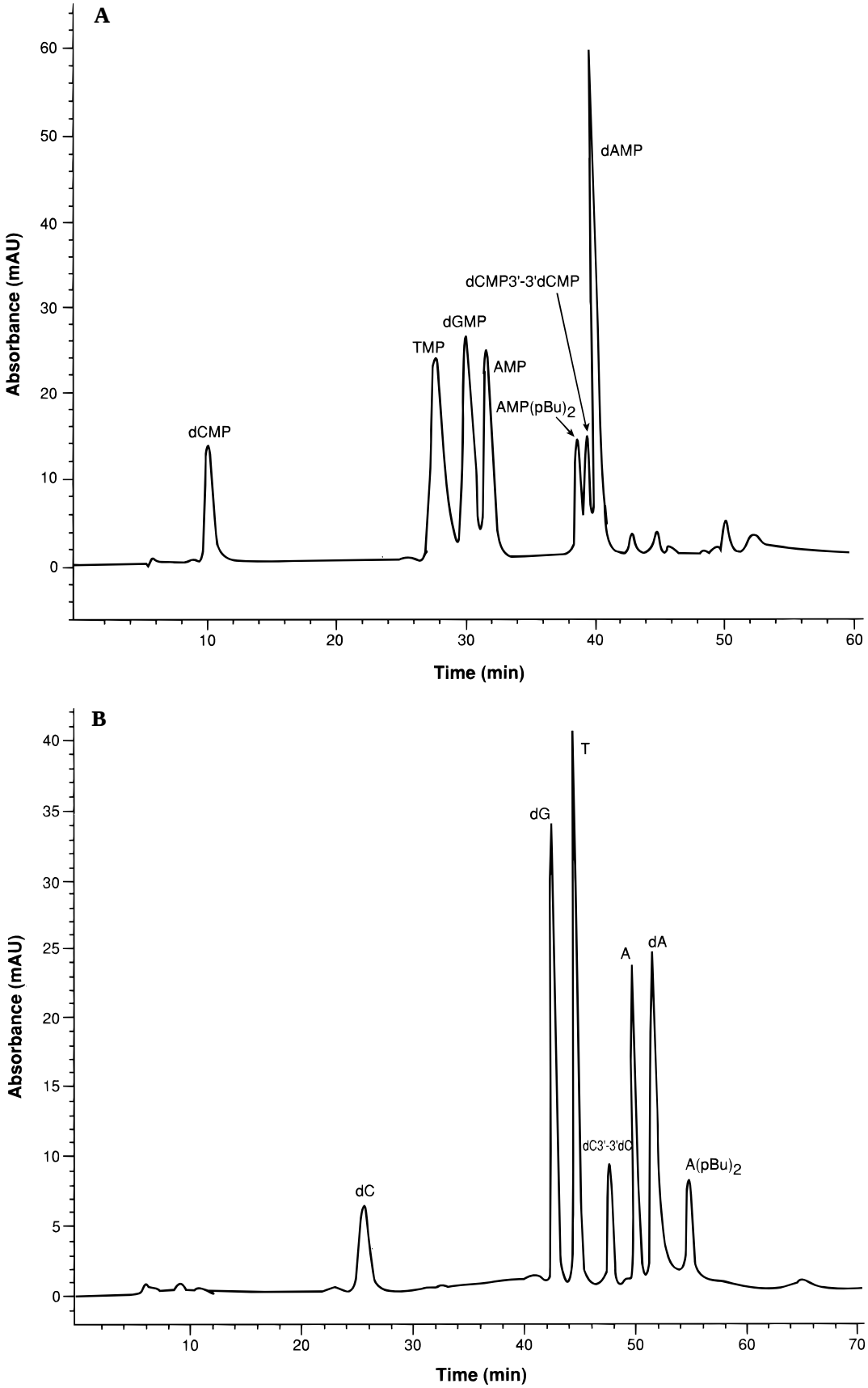

Figure 1. (A) HPLC analysis of products from snake venom phosphodiesterase-catalyzed hydrolysis of pA_4-$[pBu]_2$-pd(aat ggg atc cat ttt gtc c3′-3′c). After digestion, the sample was spin-rinsed at 12000*g* using a Microcon-10 concentrator according to the procedure described in ref 14. For separation using an HP1050 instrument with Chemstation software on a Ultrasphere (Beckman) ODS column (4.6 × 250 mm), the elution program was as follows: isocratic (1% solvent B for 20 min) at 0.5 mL/min, then a linear gradient from 1−45% solvent B in 30 min, followed by isocratic elution with 45% solvent B for 20 min at 0.5 mL/min. Solvent A was 100 mM ammonium phosphate (pH 5.5, in water), and solvent B was MeOH/water (50:50, v/v). (B) HPLC analysis of products from digestion of pA_4-$[pBu]_2$-pd(aat ggg atc cat ttt gtc c3′-3′c) with a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase. Sample preparation and HPLC conditions were as described above and in refs 5 and 14.

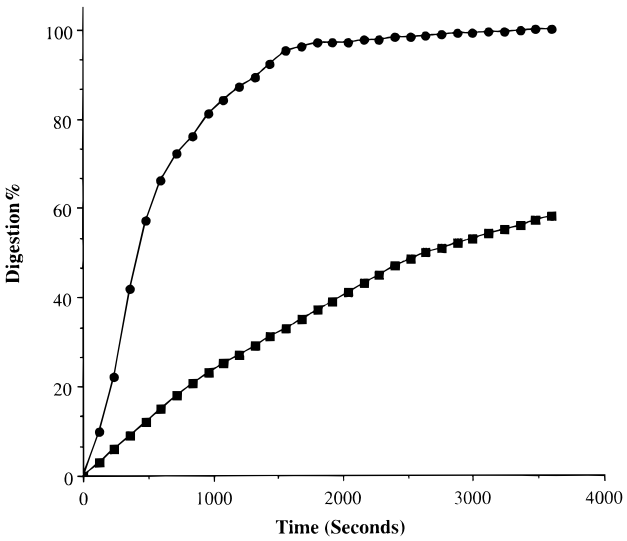

Figure 2. Comparison of stabilities of pA_4-[$pBu]_2$-pd(gta cta ctc cct gct tct g3′-3′c) (■) and pA_4-[$pBu]_2$-pd(gta cta ctc cct gct tct g3′) (●) toward degradation by snake venom phosphodiesterase. Digestion conditions were the same for both oligonucleotide reactions: 0.10 M NaCl, 0.10 M Tris (pH 8), 10 mM $MgCl_2$, 0.2 A_{260} unit/mL oligonucleotide, 0.025 unit/mL snake venom phosphodiesterase, $T = 37$ °C. The percent increase in absorbance during digestion relative to that at $t = 0$ was converted directly to percent degradation. Specifically, the digestion was permitted to proceed overnight after which time there was no further increase in absorbance. This absorance reading was assumed to represent 100% digestion. With the absorbance reading at $t = 0$ taken as 0% degadation, intermediate values of absorbance could be converted to percent degradation.

of identical sequence, with modifications only at the 3′-terminus, this analysis would still provide a valid measure of relative stability.

2. Degradation by Human Serum Enzymes. One of the above 2-5A-antisense chimeras, pA_4-[$pBu]_2$-pd-(gta cta ctc cct gct tct g3′-3′c), was chosen for evaluation of its stability to degradation in the presence of 10% human serum. In these experiments, either the 3′-3′-tailed oligonucleotide or its unmodified congener was incubated (37 °C) at a concentration of 7×10^{-6} M in 50 mM Tris buffer, pH 7.8, containing 5 mM $MgCl_2$ and 10% human serum. Aliquots were removed at indicated times and deproteinized with methanol, and the supernatant was analyzed by HPLC using a Dionex ion exchange column. The analytical procedure permitted visualization of loss of even one nucleotide from the intact initial 2-5A-antisense chimera. The oligonucleotide $n-1$-mer, $n-2$-mer, $n-3$-mer, etc., pattern of degradation is illustrated in Figure 3. It was evident that the unmodified chimera, pA_4-[$pBu]_2$-pd(gta cta ctc cct gct tct g3′), was degraded progressively with a $t_{1/2}$ of about 10 h under these conditions (Figure 3). In contrast, the 3′-3′-tailed congener, pA_4-[$pBu]_2$-pd(gta cta ctc cct gct tct g3′-3′c), showed little degradation even after 18 h incubation (Figure 4). Under these conditions, its apparent $t_{1/2}$ was in excess of 280 h, representing at least a 28-fold increase in stability compared to the parent 2-5A-antisense chimera (Figure 5).

Discussion

A wide variety of chemical alterations have been made to nucleic acids in an attempt to enhance their stability to endo- and exonucleases.[2,3,16−39] Antisense reagent analogues that involve alterations that encompass the entire nucleotide backbone such as phosphorothioate-substituted DNAs,[17] 2′-O-alkylated[18] or 2′-substituted RNAs,[19] α-anomeric oligonucleotides,[20] methylphosphonate-modified DNA,[21] unnatural L-nucleoside-based oligonucleotides,[22] and methylene(methylimino) (MMI),[23] to name just a few, confer greatly increased resistance to degradation. However, many such hypermodified nucleic acid backbones fail to confer the perceived requisite sensitivity of their resultant duplexes with an RNA target to RNase H cleavage activity. Inhibition of RNase H activity may even result, as is the case for phosphorothioates.[24] Limiting the extent and positioning of such modifications, however, may provide a useful compromise between extreme nuclease resistance and ability of derived hybrids to be substrates of RNase H. These difficulties emphasize the complexities the chemist must face in seeking chemical alterations that introduce resistance to one class of nucleic acid degradation enzyme while preserving the ability of another enzyme to act upon a derived complex thereof.

Synthetic alternatives that increase antisense oligonucleotide survival in biological fluids, but generally minimize disruption of RNase H action, are those that target one or both termini of the nucleic acids. These latter chemistries have often included alteration of one or a few phosphodiester linkages near (usually) the 3′-terminus of the oligo. Alternatively, the changes to increase stability have involved addition of various structural motifs to the nucleic acid terminus (or termini). For instance, Shaw et al.[26] found that oligonucleotides bearing phosphoramidite, methoxyethylamine phosphoramidite, or 3′-3′-linkages could provide resistance to the exonuclease activities of serum. Similar results were reported by Boutorine et al.[27a] and Ortgao et al.[27b] Gamper et al.[28] showed that a 2−3-fold increase in stability to degradation in hepatoma cell culture was obtained when oligos contained a 3′-phosphodiester-linked cholesterol, aminohexyl, hexanol, or acridine moiety. A 3′-phosphopropylamine oligonucleotide displayed sufficient stabilty in mice to undergo accumulation in tissue.[29] 3′-End-capping with terminal phosphorothioate linkages provided enhanced resistance to degradation in serum-containing medium but was degraded in HeLa cytoplasmic extract, unlike the fully thiophosphorylated oligonucleotide,[30] and this pattern of resistance also was reported by Ghosh et al.[31] Thomas and co-workers[32] used 3′-modification with a 1-amino-2-hydroxypropyl moiety to enhance resistance to serum degradation. Peyman and Uhlmann[33] relied upon a strategy of end-capping with phosphorothioates coupled with internal pyrimidine residue phosphorothioates to maximize resistance to nucleolytic enzyme attack. Additional stabilization paradigms have included conjugation of the oligonucleotide with polylysine,[34] polyornithine,[35] and biotin[36] and the construction of oligonucleotides that can form 3′-terminal hairpin structures.[37,3]

We have provided evidence for a model of 2-5A-antisense action that posits a dual role for the antisense domain of the chimeric molecule.[4,6,7] Specifically, the antisense portion of the 2-5A-antisense conjugate not only provides the highly specific complementary binding site for the target RNA sequence but also positions the RNA substrate in proximity to the catalytically active

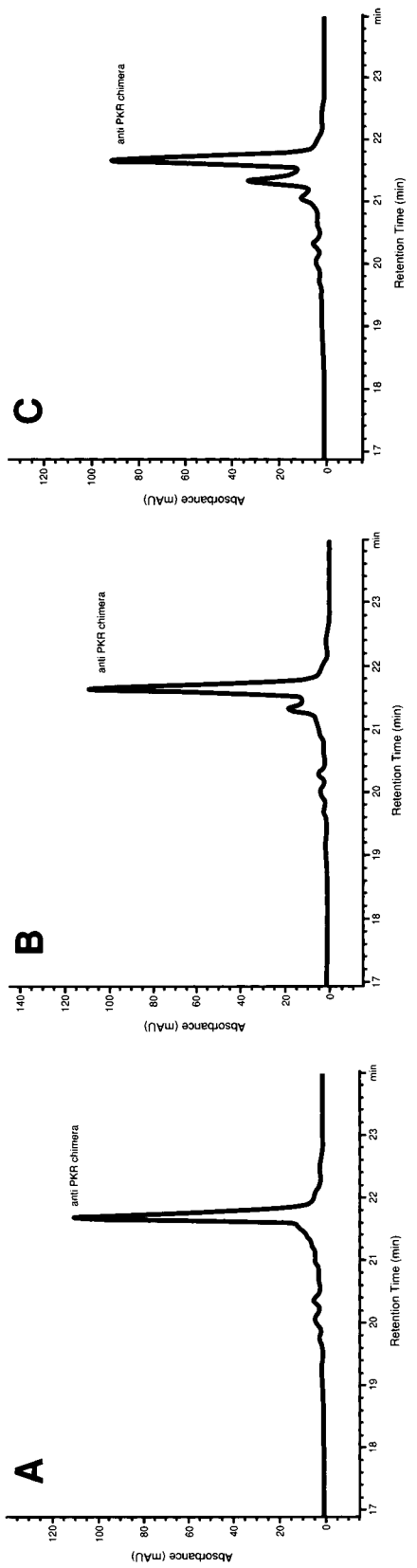

Figure 3. Time course of human serum-catalyzed degradation of pA_4-[pBu]$_2$-pd(gta cta ctc cct gct tct g3′) as analyzed by HPLC using a Dionex PA 100 column (4 × 250 mm) and a 10−70% gradient (30 min) of 25 mM Tris (pH 7.0), 0.5% acetonitrile into 25 mM Tris (pH 7.0), 0.5% acetonitrile in 1 M NH_4Cl. The reaction conditions were 0.4 OD_{260} unit of oligonucleotide/mL, 10% human serum, 50 mM Tris HCl, 5 mM $MgCl_2$, pH 7.8, $T = 37$ °C. Aliquots were removed at the indicated times and deproteinized with MeOH prior to analysis. HPLC detection was at 260 nm. The alphabetically labeled panels represent analyses of aliquots removed at the following times: A, $t = 0$ h; B, $t = 1$ h; C, $t = 3$ h; D, $t = 6$ h; E, $t = 18$ h.

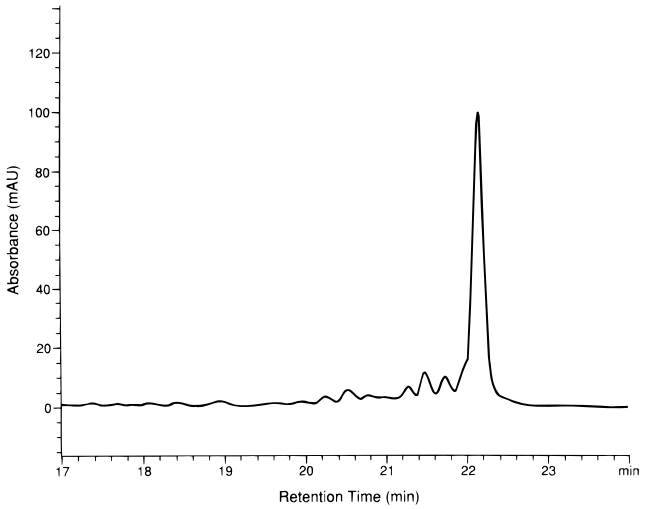

Figure 4. Chromatogram of pA_4-$[pBu]_2$-pd(gta cta ctc cct gct tct g3′-3′c) after 18 h exposure (0.4 OD_{260} unit/mL) to 10% human serum in 50 mM Tris HCl, 5 mM $MgCl_2$, pH 7.8, T = 37 °C. HPLC analytical conditions were the same as in the legend to Figure 3. The t = 0 h time point showed a homogenous peak at t_R = 22.2 min (not shown).

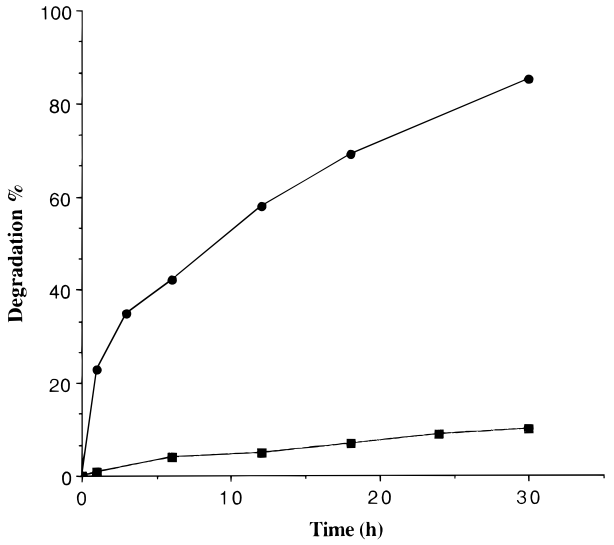

Figure 5. Comparative 10% human serum-induced degradation kinetics of pA_4-$[pBu]_2$-pd(gta cta ctc cct gct tct g3′) (●) and pA_4-$[pBu]_2$-pd(gta cta ctc cct gct tct g3′-3′c) (■) derived from HPLC analysis of the reaction mixture under conditions described in Figures 3 and 4. The percent degradation was determined by integration of associated peak area relative to coinjected standard.

site, thereby producing a proximity effect on catalysis.[7] According to this model, it may be expected that, due to the proximity of the antisense chain to RNase L and its likely interactions therewith, major alterations in overall antisense domain structure may not be tolerated as well as minor changes confined to the 3′-terminus. Coupled with this consideration were the previous reports that the predominate nuclease activity in some conditions corresponds to a 3′-exonuclease activity.[26,28,32,36] For these reasons, we chose to confine our initial investigations into nuclease-stabilized 2-5A-antisense chimeras as to what could be considered a minor change indeed: specifically, isomerization of the 3′-terminal internucleotide phosphodiester bond from 3′→5′ to 3′→3′.

We have found that the strategy of inverting the polarity of the 3′-terminal internucleotide 3′,5′-phosphodiester bond to a 3′-3′-linkage is entirely compatible with the synthetic chemistry of 2-5A-antisense oligonucleotides. Product oligonucleotides with 3′-3′-terminal linkages were obtained in comparable yields and purities to those for unmodified 2-5A-antisense. In addition, primary characterization of these products was effected by a simple addition of bacterial alkaline phosphatase to the snake venom phosphodiesterase protocol to provide constituent nucleosides and a 3′,3′-dinucleotide arising from the 3′-terminus.

As would be expected from earlier studies,[26,27] the 3′-3′-terminal linkage inversion increased significantly resistance to degradation by snake venom phosphodiesterase and the exonuclease activities present in human serum. Resistance to snake venom phosphodiesterase was enhanced by a factor of 5−6. The $t_{1/2}$ for individual 2-5A-antisense chimeras differed significantly. The 3′-3′-tailed chimera, pA_4-$[pBu]_2$-pd(gta cta ctc cct gct tct g3′-3′c), had a $t_{1/2}$ of 43 min, whereas chimeric 3′-3′-tailed pA_4-$[pBu]_2$-pd(gcc cac cgg gtc cac cat3′-3′c) showed a $t_{1/2}$ of 100 min. However, this relative difference was also present in the unmodified oligomers with the chimera pA_4-$[pBu]_2$-pd(gta cta ctc cct gct tct g3′) possessing a $t_{1/2}$ of 7 min, while the oligomer pA_4-$[pBu]_2$-pd(gcc cac cgg gtc cac cat3′) showed a $t_{1/2}$ of 18 min. The origins of these minor effects are not understood. Possible parameters that might be considered would be sequence dependence or composition dependence for the full-length oligonucleotide. Alternatively, the rate differences could be simply a matter of the nature of the first (3′-terminal) internucleotide bond as this differs for each pair, unmodified, and 3′-3′-tailed.

Importantly, when the 2-5A-antisense chimera, pA_4-$[pBu]_2$-pd(gta cta ctc cct gct tct g3′-3′c), was exposed to the nucleolytic activities of human serum, an even greater stabilization resulted since the $t_{1/2}$ showed at least a 28-fold increase relative to the parent unmodified chimera, pA_4-$[pBu]_2$-pd(gta cta ctc cct gct tct g3′). Although this observation confirms earlier experience on the predictive value of snake venom phosphodiesterase for extrapolating the resistance of antisense oligonucleotides to biological systems, it may also indicate some difference in the specificities of the snake venom 3′-exonuclease as compared to the 3′-exonuclease activity (or activities) of human serum. Alternatively, it could perhaps indicate a greater lability of the human 3′-exonuclease activity.

Incidental to the above observations was the demonstration that Dionex ion exchange HPLC can be a valuable analytical tool for following degradation of 2-5A-antisense chimeras and probably other oligonucleotides as well. More often, it has been the practice to employ gel electrophoresis of ^{32}P-labeled or unlabeled oligonucleotides to monitor degradation.[26,27,29,30,36−38] Capillary gel electrophoresis also has been used but is highly sensitive to high salt concentration so samples require a prior desalting or dialysis before analysis.[39]

The introduction of the 3′-3′-inverted phosphodiester linkage to stabilize 2-5A-antisense oligonucleotides to 3′-exonuclease attack has now been combined with a second modification,[13] 5′-thiophosphorylation, that blocks phosphatase removal of the critical 5′-monophosphate (or thiophosphate) which is responsible for RNase L activation.[4,8−10,12] Such doubly modified 2-5A-antisense chimeras, when targeted to specific regions of secondary

structure in the M2 mRNA of respiratory syncytial virus, provide an oligonucleotide with potent anti-RSV activity that results from specific degradation of the M2 mRNA.[40]

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