Antisense L/D-Oligodeoxynucleotide Chimeras: Nuclease Stability, Base-Pairing Properties, and Activity at Directing Ribonuclease H†,‡

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ABSTRACT: Ultraviolet thermal denaturation studies substantiate our earlier hypothesis that substitution of a L-nucleotide residue for a D-nucleotide within a DNA duplex permits a stable structure in which *all* bases are paired through Watson–Crick hydrogen bonds (Damha, M. J., Giannaris, P. A., Marfey, P., & Reid, L. S. (1991) *Tetrahedron Lett. 32,* 2573–2576). This conclusion is also evident from the NMR work of Blommers et al. [Blommers, M. J. J., et al. (1994) *Biochemistry* (following paper in this issue)]. Our thermal denaturation studies indicate that, while weakening the interaction with target DNA and RNA, these substitutions allow for excellent cooperative binding. When the target is single-stranded DNA, the melting temperature of the complex is lowered by 4–5 °C per L-dU incorporation and by 0.4–2.6 °C when an internal D-dC is replaced by L-dC (1 M NaCl). When the target is RNA, the depression of T_m is also greater for L-dU substitutions (5–8 °C) than for L-dC substitutions (2–4 °C). The depressions of T_m caused by introducing A/C and G/T mismatches at the same positions were significantly greater. L/D-DNA chimeras were found to activate RNase H cleavage when hybridized to RNA. Furthermore, the stability of chimeric L/D-DNA against degradation by various commercial phosphodiesterases was found to be significant, as was their stability against digestion in human serum. These experiments establish that L/D-DNA chimeras serve as excellent models of antisense oligonucleotides.

One of the most exciting developments in nucleic acid chemistry has been the discovery of remarkably efficient routes to the synthesis of DNA (Lestinger & Lunsford, 1976; Beaucage & Caruthers, 1981) and RNA sequences (Ogilvie et al., 1988). These advancements allow for the production of any desired natural sequence as well as sequences having virtually any modification in the basic nucleotide structure. Many of these oligonucleotide analogs, particularly those having phosphate and sugar modifications (e.g., methyl phosphonato-DNA, α-DNA), are important not only for structural and biological studies but also for the development of new therapeutic agents (Zon, 1988; Cohen, 1989; Ulhmann & Peyman, 1990; Cook, 1991; Agrawal, 1992). Of particular importance in this regard are oligonucleotide analogs that interact selectively with RNA (the "antisense" strategy: Belikova et al., 1967; Miller et al., 1974; Paterson et al., 1977; Zamecnik & Stephenson, 1978), double-stranded DNA (the "antigene" strategy: Dervan, 1989; Hélène & Toulme, 1990), or peptide sequences (the "sense" strategy: Bielinska et al., 1990; Ma et al., 1993).

Our laboratory has recently focused upon chimeric oligodeoxynucleotides having both D- and L-2′-deoxyribose sugars (L/D-DNA) (Damha et al., 1991). The rapid degradation of unmodified D-oligodeoxynucleotides (D-DNA) by ubiquitous nucleases renders them unsuitable as effective therapeutic agents despite their well-known affinity for complementary RNA and double-stranded DNA. In marked contrast, enantiomeric L-DNA oligomers have enhanced resistance to the action of nucleases (Holý & Sorm, 1969, 1971; Tazawa et al., 1970) but have been found to hybridize either weakly or not at all with natural RNA and DNA. For example, oligomers of L-dU did not show any base pairing with D-poly(dA) (Anderson et al., 1984). Similarly, no interaction of L-dT and L-dU oligomers with the complementary deoxy- and ribo-D-homopolynucleotides was observed (Morvan et al., 1990; Asseline et al., 1991). L-$(dAp)_5$dA formed a complex with D-poly(rU), although this complex was substantially less stable than the natural D-$(dAp)_5$dA/poly(rU) complex (Fujimori et al., 1990). Interestingly, this hexamer showed preference for hybridization with complementary RNA (D-poly(rU)) rather than DNA (D-poly(dT)). In contrast with this result, L-$(dAp)_4$ covalently linked through its 3′-terminus with an acridine derivative formed complexes with both D-poly(rU) and D-poly(dT) (Asseline et al., 1991). The 3′-acridine derivatives of L-$(dUp)_8$ and L-$(dTp)_8$, like their nonsubstituted derivatives, did not interact with poly(rA) or poly(dA) (Asseline et al., 1991). Garbesi et al. (1993) failed to detect, by UV spectroscopy, circular dichroism, and mobility shift experiments on native gel electrophoresis, any interaction between L-oligomers containing all four natural base residues and complementary D-DNA or RNA sequences. Thus, contrary to a previous suggestion based upon hybridization experiments with L-$(dAp)_5$dA (Fujimoro et al., 1990), DNA oligomers constructed from L-nucleosides cannot form the basis of an effective antisense strategy.

In principle, it should be possible to retain nuclease resistance while improving hybridization properties using oligodeoxynucleotides containing *both* L- and D-deoxynucleotide residues. This possibility was explored first by our group (Damha et al., 1991), and independently by Capobianco et al. (1991), by preparing a series of L/D-oligodeoxynucleotides of mixed base composition. Our preliminary studies confirmed that chimeric

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‡ This paper is dedicated to the memory of L. S. Reid (Alleix Biopharmaceuticals, Mississauga, ON).

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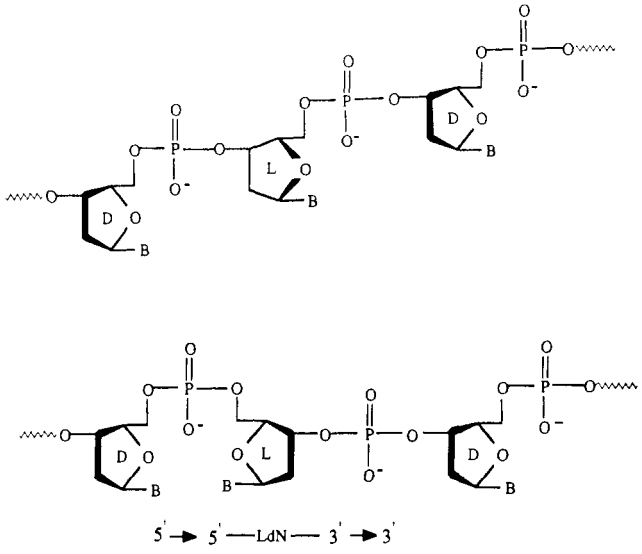

FIGURE 1: Structures of L/D-oligodeoxyribonucleotides containing (3',5')-L-dN-(3',5') and (5',5')-L-dN-(3',3') residues. Haworth formulas of sugar rings indicate absolute stereochemistry.

L/D-oligomers with *terminal* L-units provided adequate duplex-forming capability and excellent enzymatic stability. Furthermore, thermal denaturation (T_m) data and modeling studies suggested that the L-residues were involved in Watson–Crick base pairing and contributed to the cooperative melting of the duplex (Damha et al., 1991). Concurrently, Capobianco et al. (1991) reported that oligomers containing L-dA and L-dT units in the terminal or next to terminal positions were good inhibitors of SV-40 replication, while unmodified antisense strands or a modified sense strand produced a markedly lower inhibition. More recently, Shudo and coworkers (Hashimoto, 1993) reported on the synthesis of a nuclease-resistant dodecamer containing L-dA and D-dA residues in alternating sequence ("Meso"-DNA). Thermal denaturation profiles demonstrated that the affinity of this oligomer toward D-poly(rU) and D-poly(dT) was comparable to that of the natural D-dodecamer. L-RNA (van Boeckel et al., 1987; Ashley, 1992) and L/D-RNA dimers (Bhuta & Zemlicka, 1978) and their biological activities including resistance toward exonucleases have also been described. Also, recent studies with chimeric α-D-2'-deoxyribose/β-D-2'-deoxyribose (Koga et al., 1991), acyclic/cyclic sugars (Wilk et al., 1990; Augustyns et al., 1991), D-hexose/D-deoxyribose (Augustyns et al., 1992), 2'-*O*-alkyl-D-ribose/2'-D-deoxyribose (Lesnik et al., 1993), and internucleotide linkage combinations of phosphodiesters with methyl phosphonates (Tidd & Warenius, 1989), phosphorothioate (Stein et al., 1988), formacetal (Matteucci, 1990), sulfide (Kawai et al., 1993), amide (Idziak et al., 1993; Mesmaeker et al., 1994), and other derivatives (for a recent review, see Yogesh and Cook (1993)) indicate beneficial nuclease resistance characteristics.

One goal in this study was to incorporate L-2'-deoxyribonucleosides at multiple positions *within* a D-oligonucleotide chain and to evaluate the hybridization properties of such oligomers toward complementary DNA and RNA. We chose to incorporate L-residues within oligonucleotides in either the (3',5')-L-dN-(3',5') or the unnatural (3',3')-L-dN-(5',5') linkage motif (Figure 1). The decision to place L-residues within oligomers in either orientation was based primarily on the desire for high nuclease stability. Data available on dinucleotides linked by either 3',3'- or 5',5'-linkages (Nemer et al., 1985) and on oligomers carrying terminal 3',3'- and 5',5'-linkages (Selinger et al., 1991) seemed to show some enzymatic stability. Similarly, alternating α,β-oligothymidylates with alternating 3',3' and 5',5' internucleotidic phosphodiester linkages exhibited enhanced resistance to the action of nucleases and satisfactory hybridizing properties toward complementary DNA and RNA sequences (Koga et al., 1991). In addition, molecular models (Sigma) and preliminary energy minimization studies (QUANTA and CHARM21 parameter sets) did not reveal any obvious unfavorable steric interactions upon substitution of a D-unit by an L-unit in either the (3',5')/(3',5') or the (3',3')/(5',5') internucleotidic linkage motif. However, a better base-pairing geometry was achieved with the (3',5')-L-dN-(3',5') substitution. Other goals in the present study were to evaluate the behavior of L/D-oligonucleotides with respect to their stability in human serum and mammalian exonucleases, as well as to examine their ability to elicit RNase H activity. Our results suggest that L/D-DNA chimeras (or "heterochiral"-DNA) may show considerable utility as antisense agents due to their excellent nuclease stability and their ability to form stable hybrids with RNA that are susceptible to RNase H. The NMR studies by Blommers et al. (1993, 1994)and Urata et al. (1993) substantiate our earlier hypothesis that substitution of a L-nucleotide residue for a D-nucleotide within a DNA duplex permits a stable structure in which *all* bases are paired through Watson–Crick hydrogen bonds (Damha et al., 1991).

MATERIALS AND METHODS

Materials. 2'-*O*-*tert*-Butyldimethylsilylated RNA phosphoramidite monomers (U, A^{Bz}, C^{Bz}, G^{FMD}, and G^{iBu})[1] were obtained from Dalton Chemical Laboratories (Toronto, ON). DNA monomers (T, A^{FMD}, C^{iBu}, and G^{FMD}), reagents for DNA/RNA synthesis, and reversed-phase OPC cartridges were purchased from Applied Biosystems (Mississauga, ON). Reversed-phase C_{18} SEP-PAK cartridges were obtained from Waters. Polyacrylamide gel electrophoresis (PAGE) reagents were obtained from Bio-Rad (Mississauga, ON). HPLC grade solvents were purchased from Caledon (Toronto). Long-chain alkylamine controlled-pore glass (LCAA-CPG, 500 Å; CPG-Inc., NJ) with L-dU or L-dC attached through the 3'-OH or 5'-OH group was prepared according to the literature procedure (Damha et al., 1990; Pon, 1993). The nucleoside loading of these supports was in the range of 25–35 μmol/g. L-Deoxyuridine was a generous gift from Dr. D. Anderson (The Upjohn Company, Kalamazoo, MI), and detailed procedures for its preparation have been reported (Anderson et al., 1984).

Preparation of L-Nucleoside 3'-O-Phosphoramidite Monomers. The preparation of L-2'-deoxynucleoside phosphoramidite monomers has been described (Damha et al., 1991), and full experimental procedures as well as characterization (^{1}H-NMR, UV, ^{31}P-NMR, and MS) data of these compounds will be published elsewhere.

Preparation of Oligo-(L/D)-DNA and Unmodified Oligo-DNA and RNA. Oligo-(L/D)-DNA and unmodified oligo-DNA were prepared on an Applied Biosystems 381A DNA synthesizer using standard phosphoramidite chemistry, 0.2- or 1-μmol cycles, and LCAA-CPG as the solid support. The products with the 5'-DMT group were purified by reversed-

[1] Abbreviations: ADA, adenosine deaminase; AP, alkaline phosphatase; Bz, benzoyl; *i*Bu, isobutyryl; CMP, cytidine monophosphate; CPG, controlled-pore glass; CSPDE, calf spleen phosphodiesterase; ds, double stranded; DMT, dimethoxytrityl, FMD, formamidine; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ss, single stranded; SVPDE, snake venom phosphodiesterase; T_m, melting temperature; UV, ultraviolet.

phase chromatography using OPC cartridges (Applied Biosystems). RNA oligomers were assembled on the same instrument using the silyl phosphoramidite method (Ogilvie et al., 1988; Damha & Ogilvie, 1993). Following deblocking, the oligoribonucleotides with their 5′-hydroxyls free were purified by electrophoresis on 24 or 16% acrylamide/7 M urea gels followed by reversed-phase chromatography on C_{18} SEP-PAK cartridges (Damha & Ogilvie, 1993). ^{32}P-End labeling of RNA oligomer **24** (25 pmol) was carried out by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase. The radiolabeled oligomer was purified by Sephadex G-50 size-exclusion chromatography.

Nuclease Resistance Assays. (A) Purified Mammalian Nucleases. These were obtained from Boehringer Mannheim (Quebec) except as indicated. (i) Calf spleen phosphodiesterase (CSPDE) was obtained as a suspension (2 mg/mL) in 3.2 M ammonium sulfate, pH *ca.* 6.0. (ii) Snake venom phosphodiesterase (SVPDE) from *Crotalus durissus* was obtained as a solution of 2 mg/mL in 50% (v/v) glycerol, pH *ca.* 6. (iii) Nuclease S1 from *Aspergillus oryzae* was obtained as a solution in 50% glycerol (v/v), 20 mM Tris-HCl, 50 mM NaCl, and 0.1 mM zinc chloride, pH *ca* 7.5. (iv) Alkaline phosphatase (AP) from calf intestine was obtained as a 2 mg/mL suspension in $(NH_4)SO_4$ (3.2 M), $MgCl_2$ (1 mM), and $ZnCl_2$ (0.1 mM), pH *ca.* 7.0. Incubation buffers for the enzyme digestions were prepared from autoclaved water and filtered through a sterile 0.2-μm membrane (Acrodisc, Gelman Sciences). Buffers: (i) CSPDE, 0.5 M $(NH_4)_2SO_4$, pH *ca.* 6.0. (ii) SVPDE, 50mM Tris-HCl and 10 mM $MgCl_2$, pH 8.0. (iii) Nuclease S1, 50 mM NaOAc, 1 mM $ZnSO_4$, 250 mM NaCl, and 50 μg/mL BSA, adjusted to pH 4.6 with AcOH. Typically, 0.3 OD unit of oligonucleotide was dissolved in *ca.* 20 μL of incubation buffer, to which the appropriate enzymes were added [5 μL of CSPDE (0.02 uhit); 2 μL of SVPDE (0.006 unit); or 0.2 μL of nuclease S1 (0.02 unit), 2 μL of AP (36 units), and 5 μL of ADA], and the mixture was incubated at 37 °C. Digestions with purified enzymes were analyzed by HPLC on a Waters instrument equipped with dual 501 pumps, a UK6 injector, and a 480 UV detector. The solvent gradient was controlled through a 740 data module. The column employed was a reversed-phase Whatman Partisil ODS-2 (10 μm, 4.6 × 250 mm) with a linear gradient 0–50% in B over 30 min (solvent A, 20 mM KH_2PO_4, pH 5.5; solvent B, methanol) and a 2 mL/min flow rate at room temperature. Injections of 5 μL (0.07 OD unit) were found to be adequate for determinations. Products were easily separated and identified in most cases by co-injection with authentic samples of nucleosides and nucleotides.

(B) Human Serum. A human blood sample, obtained from a healthy volunteer (L. Giannaris), was allowed to clot for 2–3 h, and the clot was removed by centrifugation. Oligonucleotides (0.2 A_{260} unit) were incubated at 30 °C with 100% human serum (20 μL). Aliquots (5 μL) were removed at appropriate times (after 5 min, 2 h, 3 h, 6 h, 12 h, and 48 h) and analyzed by HPLC. Conditions: DEAE 5PW ion-exchange column (Waters); mobile phase solvent A, water; mobile phase solvent B, 1 M sodium perchlorate; mobile phase solvent C, acetonitrile; gradient, 100% A to 65%A/25% B/10% C in 25 min; flow rate, 1.5 mL/min; temperature, 55 °C.

(C) Ribonuclease H. 5′-^{32}P-End-labeled oligoribonucleotide **24** (1 pmol) and complementary DNA or L/D-DNA oligomer (100 pmol) were added to a 1.5-mL test tube and lyophilized to dryness. Ten microliters of incubation buffer (40 mM Tris-HCl, pH 8.1, 4 mM $MgCl_2$, 1 mM dithioerythritol, 3 μg of calf thymus tRNA, 30 μg/mL BSA, and 50% glycerol) was added, followed by 0.5 unit of *Escherichia coli* RNase H (Pharmacia, Quebec), and the mixture was allowed to react at 37 °C for 15 min. A digestion ladder of **24** was obtained by incubating the oligomer in water (10 μL) at 80 °C for 5 min followed by partial digestion with nuclease S1 (2 units) at 37 °C for 2 min. All incubations were quenched by adding 9 M urea (10 μL) and cooling to −70 °C until analysis by denaturing 24% polyacrylamide gel electrophoresis. The gels were dehydrated by treatment with 20% acetic acid in methanol (10 min) followed by acetone (20 min) prior to analysis by autoradiography.

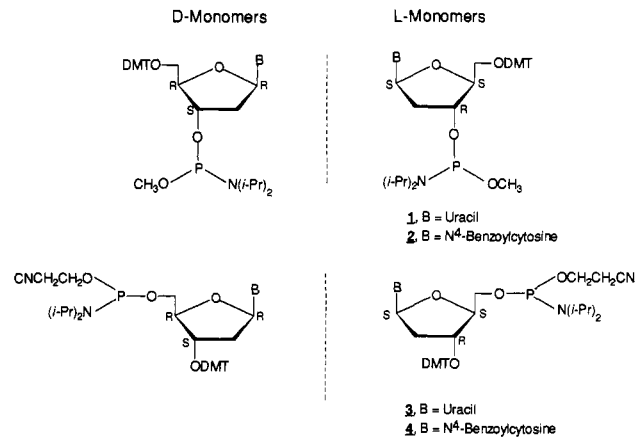

FIGURE 2: L- and D-2′-deoxynucleoside 3′-*O*-phosphoramidite building blocks for L/D-DNA synthesis. Absolute stereochemistry at C1′, C2′, and C3′ is indicated.

Melting Temperature Measurements. Melting temperatures, T_m, for an equimolar mixture of the oligonucleotide (normal or L/D-modified) and the complementary unmodified oligo-DNA and RNA strand were determined on a Varian-Cary 1 spectrophotometer equipped with a Peltier thermal unit controlled by software (release 3, Cary) from the manufacturer. The absorbance at 260 nm was measured at 0.5 °C intervals in 1-cm (1.5 mL) cuvettes with a temperature ramp of 0.5 °C /min. T_m's were calculated from first-derivative plots of absorbance versus temperature. Hyperchromicity (%*H*) values are reported as the percent increase in absorbance at 260 nm with respect to the initial absorbance. The solutions were 2.5 μM in each strand, pH 7.0, and contained 10 mM Na_2HPO_4 and 1.0 M or 200 mM NaCl. Oligonucleotide concentrations were obtained from the absorbance at high temperature, and extinction coefficients were calculated according to the method of Puglisi and Tinoco (1989). Samples were first heated to 40–80 °C for 20 min and then left at room temperature for 10 min and at 10 °C for 10 min prior to measurements.

RESULTS AND DISCUSSION

Synthesis of L-dC and L-dU Phosphoramidite Building Blocks and Incorporation into Oligonucleotide Chains. The pyrimidine L-2′-deoxynucleosides dC and dU were chosen in this study since they can be prepared readily on a large scale from commercially available, naturally occurring L-arabinose (Anderson et al., 1984). Also, if desired, L-dU can be readily converted to L-dA and L-dG residues by transglycosylation procedures such as those described by Holý (1972) and Spadari et al. (1992). The protected L-dU and L-dC phosphoramidites **1** and **2** (Figure 2), which are suitable for direct use on an automated gene synthesizer, were prepared by standard protocols (Gait, 1984; Damha et al., 1991; full procedures

Table 1: Binding of L/D-DNA Oligomers with Complementary DNA and RNA[a]

	incorporation	5′	antisense oligomers											3′	T_m DNA **17**	T_m RNA **18**
5	3′,5′-D-dU-3′,5′	T	G	T	G	T	G	U	C	T	T	T	A	T	49.0	52.0
6	3′,5′-L-dU-3′,5′	T	G	T	G	T	G	U*	C	T	T	T	A	T	45.0	43.5
7	3′,3′-L-dU-5′,5′	T	G	T	G	T	G	U	C	T	T	T	A	T	37.6	40.9
8	A/C mismatch	T	G	T	G	T	G	$\overline{C}$	C	T	T	T	A	T	42.5	46.4
9	2 × 3′,5′-L-dU-3′,5′	T	G	T	G	U*	G	U*	C	T	T	T	A	T	39.0	35.9
10	2 × A/C mismatch	T	G	T	G	*C*	G	*C*	C	T	T	T	A	T	23.0	22.0
11	4 × 3′,5′-L-dU-3′,5′	U*	G	T	G	U*	G	T	C	U*	T	T	A	U*	38.0	30.4
12	7 × 3′,5′-L-dU-3′,5′	U*	G	U*	G	U*	G	U*	C	U*	T	U*	A	U*	*b*	*b*
13	all D-DNA	T	G	T	G	T	G	T	C	T	T	T	A	T	50.4	53.2
14	3′,5′-L-dC-3′,5′	T	G	T	G	T	G	T	C*	T	T	T	A	T	50.0	51.4
15	3′,3′-L-dC-5′,5′	T	G	T	G	T	G	T	$\underline{C}$	T	T	T	A	T	38.0	41.6
16	G/T mismatch	T	G	T	G	T	G	T	$\overline{T}$	T	T	T	A	T	41.0	*c*

[a] Hybridizations were carried out in 1 M NaCl and 10 mM phosphate, pH 7.0, with equimolar (2.5 μmol in each strand) strand concentrations. N* refers to 3′,5′-L-dN-3′,5′ residues; $\underline{N}$ refers to 3′,3′-L-dN-5′,5′; *C* refers to positions of D-dC substitutions for D-dT resulting in A/C mismatches upon binding. [b] No detectable T_m value. [c] T_m not measured.

Table 2: Hybridizations of L/D-DNA with Complementary HIV DNA and RNA Splice Site Sequences[a]

	5′	antisense oligomers																3′	T_m (0.2 M), DNA **23**	T_m (1.0 M) DNA **23**	T_m (1.0 M) RNA **24**
19	C	G	C	C	G	C	T	G	A	C	C	A	C	T	C	A	T	G	C	72.2	76.9	76.2
20	C*	G	C*	C	G	C*	T	G	A	C	C*	A	C	T	C*	A	T	G	C*	57.9	63.6	53.9
21	C*	G	C*	C*	G	C*	T	G	A	C*	C*	A	C*	T	C*	A	T	G	C*	48.7	52.7	ca.40
22	*T*	G	*T*	C	G	*T*	T	G	A	C	*T*	A	C	T	*T*	A	T	G	*T*	43.9	48.9	55.2

[a] Hybridizations were carried out in NaCl as indicated and 10 mM phosphate, pH 7.0, with equimolar (2.5 μmol in each strand) strand concentrations. C* refers to 3′,5′-L-dC-3′,5′ residues; *T* refers to positions of D-dT substitutions for D-dC resulting in G/T mismatches upon binding.

will be reported elsewhere). The polarity reversal in the alternating L/D-oligomers with alternating 3′,3′ and 5′,5′ linkages necessitated some steps of chemical synthesis in the 5′ to 3′ direction rather than in the conventional 3′ to 5′ direction (Van de Sande et al., 1988). The required 5′-phosphoramidite derivatives **3** and **4** were easily prepared from their free nucleoside precursors by standard protocols (Gait, 1984). The CPG supports with L- or D-units covalently attached at the 5′- or 3′-hydroxyl groups were prepared according to the method of Damha et al. (1990).

L/D-Oligonucleotides were obtained by automated solid-phase synthesis using building blocks **1**–**4** and commercially available D-deoxynucleoside phosphoramidites (Tables 1 and 2). Oligomers were prepared on a 0.2- or 1.0-μmol scale employing a "trityl on" synthesis cycle and the appropriate solid supports. After deprotection of the sugar and phosphate moieties, the terminal 5′-trityl group was cleaved with trifluoroacetic acid during purification with a reversed-phase OPC cartridge (Applied Biosystems). Purity of L/D-DNA oligomers was verified by analytical gel electrophoresis and was determined to be greater than 95%. Natural RNA (Damha & Ogilvie, 1993) and DNA (Gait, 1984) oligomers were prepared by standard means. After deblocking and purification by reversed-phase chromatography, the oligomers were subjected to nuclease digestion to confirm the sequences and to check for their enzymatic stability.

Stability of L/D-Oligodeoxynucleotides against Exonuclease Degradation. The nuclease stability of chimeric L/D-oligodeoxynucleotides is very high, as anticipated, since they are expected to interact poorly with nucleases. We have previously found that L/D-oligomers carrying terminal L-units exhibit resistance against mammalian exonucleases (Damha et al., 1991). For example, oligomers 5′-C*TTTTT-3′ and 5′-C*TTTTC*-3′ (where C* = L-dC) are totally resistant to digestion by calf spleen phosphodiesterase (CSPDE, a 5′-exonuclease) under conditions that lead to rapid degradation of unmodified strands. When the digests were run on L/D-oligomers lacking 5′-end protection, it was possible to observe the stepwise removal of individual D-units from the 5′-end of the chain (HPLC, PAGE). After 45 min of incubation, most of the L/D-hexamer 5′-TTTTC*C*-3′ was converted to the trinucleotide TC*C* (Figure 3). However, when a 20-fold greater concentration of enzyme was used, C*C* and Tp were obtained as the only products (data not shown). Under the same enzymatic conditions, 5′-TTTC*C*C*-3′ afforded Tp and C*C*C*.

Snake venom phosphodiesterase (SVPDE), a 3′-exonuclease, from *Crotalus adamanteus* accepts both L- and D-oligomers as substrates, but the rate of hydrolysis of phosphodiester bonds is dramatically slower for the L-oligomer than for the D-oligomer (Tazawa et al., 1970; Anderson et al., 1984). Observation of this effect, however, requires the presence of a high concentration of ammonium ion in the digestion buffer (Damha et al., 1991). For example, in the absence of NH_4^+, L-d(Cp)$_5$C was degraded by SVPDE to L-5′-dCMP and L-dC within 4 h, while D-d(Cp)$_5$C was completely hydrolyzed to D-5′-dCMP and D-dC within seconds under the same conditions. After 3 days of incubation in 0.2 M ammonium sulfate with a larger concentration of enzyme, L-d(Cp)$_5$C was partially degraded into a mixture consisting of L-d(Cp)$_5$C (15%), L-d(Cp)$_4$C (40%), L-d(Cp)$_3$C (40%), L-d(Cp)$_2$C (3%), and L-dCpC (2%), while the D-isomer was completely hydrolyzed within seconds. Evidently, chiral discrimination by SVPDE is markedly affected by the ammonium ion concentration.

Another interesting result in the SVPDE digestions was the observation that this enzyme cleaves 3′-terminally modified L/D-oligomers *endo*nucleolytically (this enzyme is known to hydrolyze natural oligomers from the 3′-end to produce only 5′-NMPs). Digestion of dTTTTTC* with SVPDE/AP/NH_4^+ (12 h, 37 °C) produced dT and a product with electrophoretic mobility consistent with that of the dinucleotide fragment dTpC* (data not shown). Under the same conditions,

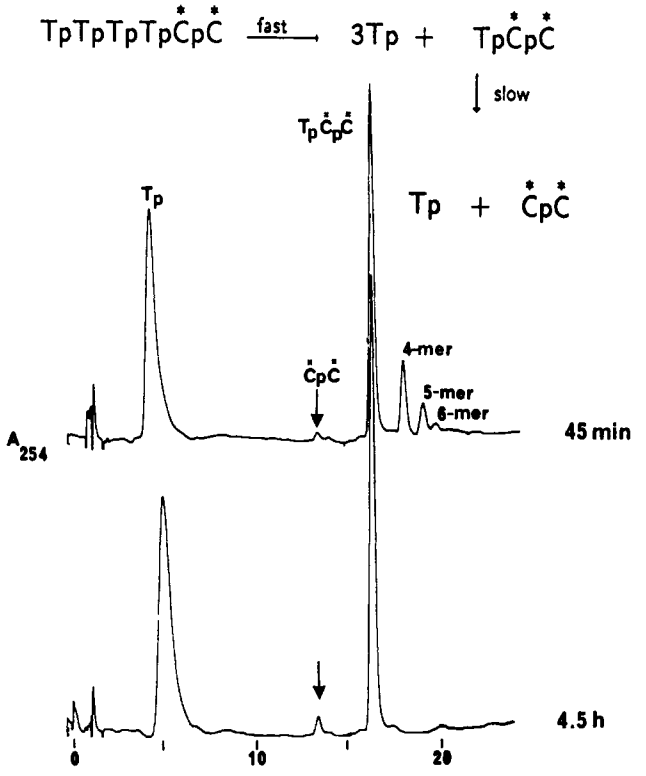

FIGURE 3: HPLC analysis of digestion of 5′-TTTTC*C*-3′ with calf spleen phosphodiesterase (C* = L-dC). Conditions are described in Materials and Methods.

dTTTTC*C* and dTTTC*C*C* were partially hydrolyzed by SVPDE/AP to give respectively dTC*C* and dTC*C*C* (PAGE). Again, the observation of this property required ammonium ions. When no NH_4^+ was added, the L/D-hexamers were completely degraded to monomers (12 h, 37 °C). We conclude from these observations that under ammonium conditions SVPDE "jumps over" a dDpL*...L* segment. This "endonuclease" activity of SVPDE has been recognized in earlier studies on oligonucleoside phosphotriesters (Miller et al., 1982), phosphorothioates (Uznanski et al., 1986), and methyl phosphonates (Agrawal & Goodchild, 1987) and more recently in studies on backbone-modified oligonucleotides containing either 1,3-propanediol (Seela & Keiser, 1987) or 1,3-butanediol moieties (Wilk et al., 1990) or an alkyl sulfide internucleoside linkage (Kawai et al., 1993).

A major difficulty associated with the use of oligonucleotides as therapeutic agents is their rapid degradation in vivo (Wickstom, 1986). Unmodified single-stranded oligo-DNA is rapidly degraded in serum by exonuclease (Hélène & Toulme, 1990; Eders et al., 1991; Hoke et al., 1991). Recent studies on hairpin and dumbbell oligo-DNA indicate that human sera also contain single-stranded endonucleases and less rapidly acting double-stranded exonuclease (Chu & Orgel, 1992). For studying nuclease sensitivity of L/D-oligonucleotides against sera, a small amount of unmodified oligomer **13** or L/D-nonadecadeoxynucleotide **20** or **21** was incubated with a fresh sample of human serum. Aliquots were then analyzed by HPLC and PAGE. L/D-19-mers **20** and **21** (Table 2) were chosen for these studies as they are complementary to a splice site donor site in HIV mRNA previously shown to be a good target for inhibition of HIV replication (Agrawal et al., 1988). Figure 4 illustrates the breakdown of the unmodified 13-mer **13** in neat serum. After 2 h of incubation at 30 °C, more than 50% of 13-mer **13** was converted to oligomers approximately 12–10 units long. The presence of these products and the lack of smaller fragments (PAGE) indicate that nucleolytic cleavage under the conditions used is mainly due to exonuclease activity. After a 6-h incubation, the 13-mer had been converted almost completely to the shorter fragments, whereas the L/D-19-mers **20** and **21** were found to be nearly intact (Figure 5). By 60 h, less than 10% of the L/D-oligomers **20** and **21** was degraded. Similar results were obtained for other L/D-oligomers. This study shows that incorporation of a few L-deoxyribonucleoside units at terminal and internal positions of a natural oligonucleotide chain can effectively stabilize phosphodiester bonds against serum exonucleases. Various other modifications of oligonucleotides at their 3′-ends prevent this problem (Tan et al., 1993).

Association of L/D-Oligodeoxyribonucleotides. The ability of L/D-oligodeoxynucleotides to hybridize to single-stranded nucleic acids is crucial for their use as antisense therapeutic agents. We have measured the melting temperatures (T_m's) of a series oligomers containing L-sugars at preselected positions hybridized to their complementary unmodified ssDNA and RNA strands (Table 1). For comparison, the T_m's of the unmodified duplexes were also determined. Sequence **5** is an unmodified 13-mer containing a single (3′,5′)-D-dU-(3′,5′) residue at position 7. Sequences **6, 7,** and, **8** are the same 13-mer except that instead of the D-dU unit they contain respectively (3′,5′)-L-dU-(3′,5′) (designated U*), (3′,3′)-L-dU-(5′,5′) (inverted linkage, designated $\underline{U}$), or (3′,5′)-D-dC-(3′,5′) (mismatched residue, designated *C*). Sequences **9, 11,** and **12** are similar L/D-13-mers with two, four, and seven L-dU residues. Sequences **13–16** are a similar set of 13-mers in which the substitutions are made at a single cytidine residue (position 8). The following conclusions can be made:

(1) Substitution in the middle of the sequence with L-units gave a reduction in melting temperature. The melting temperature of the duplex DNA is lowered by 4–5 °C for each L-dU incorporated into the strand (compare **5** with **6** and **9**) and by only 0.4 °C when an internal D-dC is replaced by L-dC (compare **13** with **14**). However, the apparent destabilization caused by L-units is significantly smaller than that created by mismatches at the same positions; e.g., compare **6** with **8** and **14** with **16** (Table 1). This strongly suggests that the L-residues in these duplexes retain classical base-pairing interactions. This conclusion is also evident from the work of Blommers and co-workers (1994). They have found very similar ΔT_m's (5 °C) for a heptamer DNA duplex incorporating a single L-dT residue in the middle (two-dimensional NMR revealed, unequivocally, that the base pair formed by the internal L-dT/D-dA residues is of the Watson–Crick type). As expected, the depression of T_m per substitution, ΔT_m, is greater when L-dU's are positioned in the middle of a sequence (4–5 °C) than when they are positioned at the termini (0.5 °C). This observation is particularly significant in view of the observed increase in resistance to nuclease digestion for oligomers with terminal L-units. We did not observe an interaction between oligonucleotide **12** (ca. 50% L content) and ssDNA. This indicates a limit to the amount of L-nucleoside substitution that a sequence can tolerate without impairing its ability to bind its target. However, the relatively small decrease in melting temperature by L-dC incorporation compared with that of its natural analog D-dC indicated to us that sequences containing several L-dC units, provided they are not too many or are strategically located, would still have sufficient hybridization properties to be used as antisense agents (*vide infra*).

(2) The depression of T_m per substitution is greater when the L/D-oligomer hybridizes to complementary RNA (**18**).

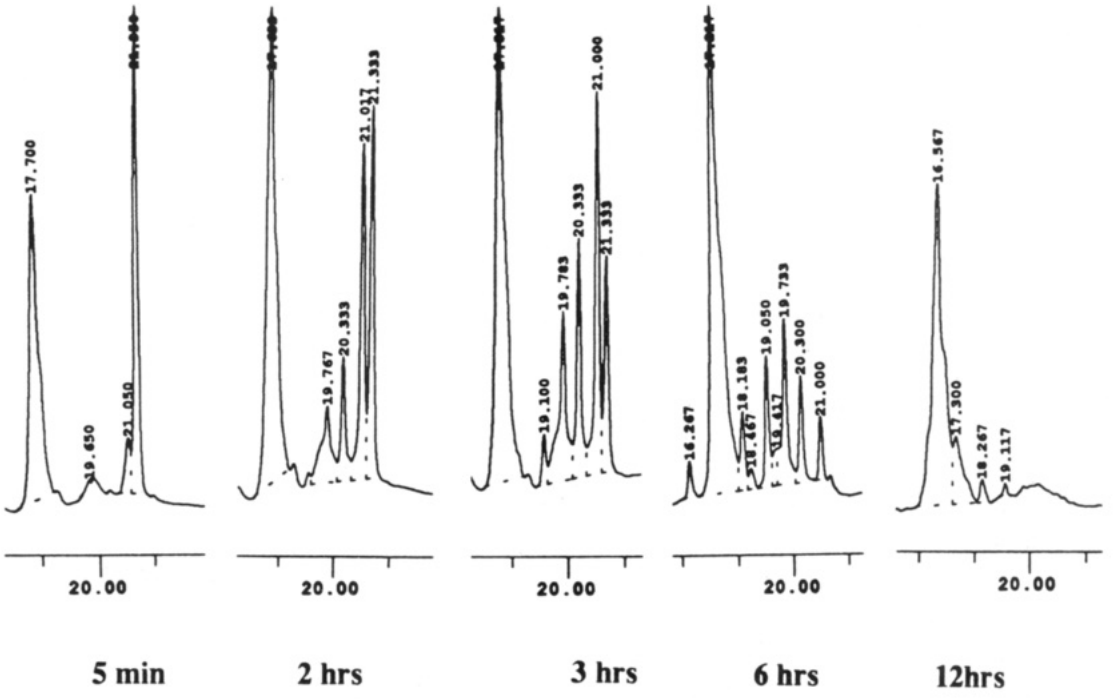

FIGURE 4: HPLC analysis of digestion of unmodified oligomer **13** in human serum. The peak near 17.0 min is a component of human serum and not a degradative product of the oligomer incubations (data not shown). See Materials and Methods for conditions.

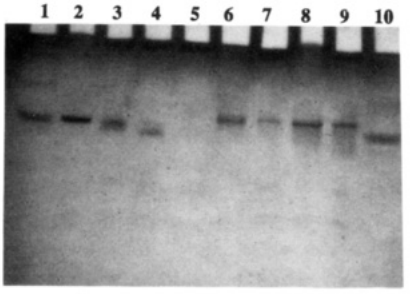

FIGURE 5: Comparative stability of D- and D/L-oligodeoxyribonucleotides in human serum. Following incubations (see Materials and Methods), the oligomers were analyzed by gel electophoresis on a 24% gel. Bands were visualized by UV shadowing. Lanes: 1 and 10, xylene cyanol and bromophenol blue dyes; 2–5, D-oligomer **19** at 0, 3, 12, and 60 h, respectively; 6 and 7, L/D-oligomer **20** at 0 and 60 h, respectively; 8 and 9, oligomer **21** at 0 and 60 h, respectively.

Again, L-dU substitutions were more destabilizing (ΔT_m, ca. 8 °C) than L-dC substitutions (ΔT_m, ca. 2 °C). The observation that a different degree of destabilization seems to be associated with the use of different L-units could be of importance to make the most favorable substitutions, and it forms the basis for future investigations by our research group.

(3) The oligonucleotides having a single L-unit connected in the (3′,3′)-L-dN-(5′,5′) linkage motif (designated N in Table 1) formed duplex molecules, although the T_m values were substantially lower than those of the unmodified oligonucleotides. In fact, the destabilization caused by a (3′,3′)-L-dN-(5′,5′) substitution appears to be greater (ΔT_m, ca. 12 °C) than that created by A/C and G/T mismatches incorporated at the same positions (ΔT_m, 6–9 °C). Oligomers possessing alternating L/D-residues with alternating 3′,3′ and 5′,5′ internucleotide linkages (namely, 5′-CACTCGCTCACAC-3′ and 5′-UAUTUCUGUGUGU-3′) did not form complexes with natural DNA or RNA at the temperatures and concentrations of these measurements. From these results, it seems unlikely that oligonucleotides containing alternating L- and D-residues in the (3′,3′)-L-dN-(5′,5′) linkage motif will be useful as antisense agents.

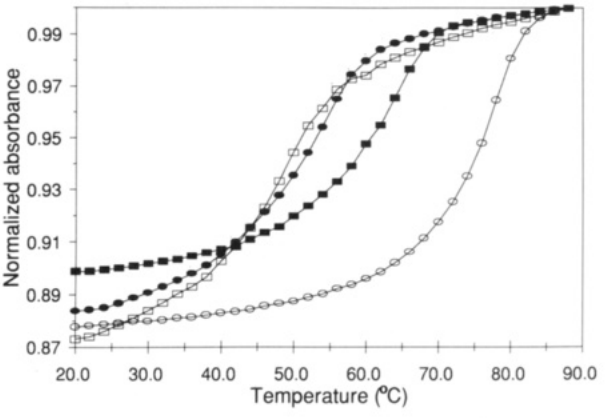

FIGURE 6: Melting temperature profiles of D- and D/L-oligodeoxynucleotides hybridized with complementary 19-mer HIV DNA in 1 M NaCl: DNA oligomer **23** with (○) **19**, (■) **20**, (●) **21**, and (□) **22**. Conditions are described in Materials and Methods.

In view of the encouraging results obtained with L/D-oligonucleotides incorporating L-dC residues in the normal (3′,5′),(3′,5′) mode, we investigated the binding properties of oligomers containing this nucleoside at several positions in low-salt (200 mM NaCl) and high-salt buffers (1.0 M NaCl). Figure 6 shows the normalized melting profiles for [19-mer L/D-DNA]/[19-mer DNA] duplexes and those of the corresponding all-D [DNA]/[DNA] (control) duplexes in 1.0 M NaCl. The sharp transitions observed in all cases are indicative of the purity and integrity of the oligonucleotide sequences. The melting temperature of the duplex DNA is lowered by *ca.* 2.5 °C for each L-dC incorporated into a strand, but the sharpness of the transition and the magnitude of the hyperchromic effect (%*H*) do not change significantly (Table 2). This indicates that even though there is a weaker interaction with target DNA, the L-dC residues are base paired and taking part in the cooperative melting of the duplex. Consistent with this hypothesis is the fact that the depression of T_m is greater for mismatch substitutions (ΔT_m = 5 °C per G/T pair) than for L-dC substitution (ΔT_m = 2.5 °C per G/L-dC pair).[2]

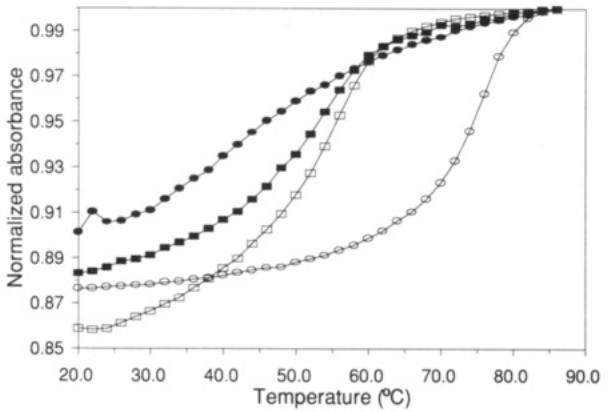

FIGURE 7: Melting temperature profiles of D and D/L-oligodeoxynucleotides hybridized with complementary 19-mer HIV RNA in 1 M NaCl: RNA oligomer **24** with (○) **19**, (■) **20**, (●) **21**, and (□) **22**. Conditions are described in Materials and Methods.

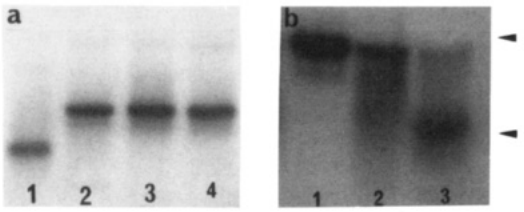

FIGURE 8: Mobility shift assay of L/D-DNA/RNA complexes (a) at 4 °C and (b) in RNase H incubation buffer at 37 °C. Mobilities of single-stranded **24** and duplexes are indicated. (a) Lanes: 1, **24**; 2–4, mixture of **24** with (2) **19**, (3) **20**, and (4) **21**, respectively. (b) Lanes: 1, mixture of **24** with **19**; 2, with **20**; 3, with **21**. Conditions are described in Materials and Methods.

Similar ΔT_m's were observed at the physiological ionic strength (Table 2). The interaction of L/D-DNAs **20** and **21** and the mismatched DNA **22** with complementary DNA was confirmed by mobility shift experiments on native PAGE at 5 °C (data not shown).

The lowering of the transition temperature resulting from L-dC incorporation is only slightly more pronounced for L/D-DNA/RNA complexes than for L/D-DNA/DNA complexes (ΔT_m = 3.7 °C/L-dC for **20**/RNA **24**, versus 2.2 °C/L-dC for **20**/DNA **23**; Table 2). However, mixtures of the most substituted L/D-DNA oligomer, **21**, and RNA **24** showed only a broad transition (T_m *ca.* 40 °C) of small hyperchromic shift indicative of weak hybridization under these conditions (Figure 7). This was somewhat expected since L/D-DNA **21** contains three more L-dC residues than L/D-DNA **20**. If the depression in T_m per L-dC substitution was an additive effect, one would predict a T_m of ca. 42 °C for complex **21**/**24**, which is in agreement with the experimental value. Mixtures of complementary ^{32}P-labeled RNA **24** plus (excess) L/D-DNA **20** and **21** and natural DNA **19** gave, in each case, the expected shift in mobility attributable to duplex formation (Figure 8a).

Activation of RNase H. The activity of the RNase H enzyme has been implicated in the antisense mechanism of some oligonucleotides which exhibit therapeutic utility (Walder & Walder, 1988). RNase H normally recognizes DNA/RNA duplexes and degrades the target RNA, freeing up the antisense DNA oligomer to hybridize another copy of RNA. Methyl phosphonates, phosphoramidates, and oligomers with complete sugar modifications such as 2′-*O*-methyls and α-sugars form heteroduplexes with RNA which do not elicit RNase H activity (Cohen, 1989). In contrast, Tidd and coworkers have recently reported that chimeric methyl phosphonodiester/phosphodiester oligomers show enhanced activity and specificity at directing ribonuclease H compared to all-phosphodiester oligomers (Giles & Tidd, 1992a,b). The increased efficiency of these analogs at activating RNase H correlated well with reduced antisense/RNA duplex stability. This was ascribed to the facilitated dissociation of the antisense/(cleaved product) RNA complex. Similar enhanced RNase H activity has been obtained with phosphorothioate DNA/RNA complexes (Stein et al., 1988). In view of this, we examined RNase H activity in L/D-DNA/RNA complexes where the L/D-DNAs were the anti-HIV sequences **20** and **21**. The cleavage of the ^{32}P-labeled target RNA was measured by PAGE (Figure 9a). Under the conditions used (37 °C, 15-min incubation), *E. coli* RNase H quantitatively cleaved the natural complex (**19**/**24**) at two points near the center of the RNA strand as shown in Figure 9. The L/D-DNA/RNA complexes **20**/**24** and **21**/**24** supported RNase H activity, although it is apparent that their rates of cleavage are slower than that of the control DNA/RNA and that the site of cleavage is dependent on the location of the L-dC residues along the oligonucleotide chain. Hybrid **20**/**24** was cleaved at a single point near the 3′-end of the RNA, whereas hybrid **21**/**24** was cleaved near the center at the same site observed for the natural **19**/**24** duplex (Figure 9). In order to estimate

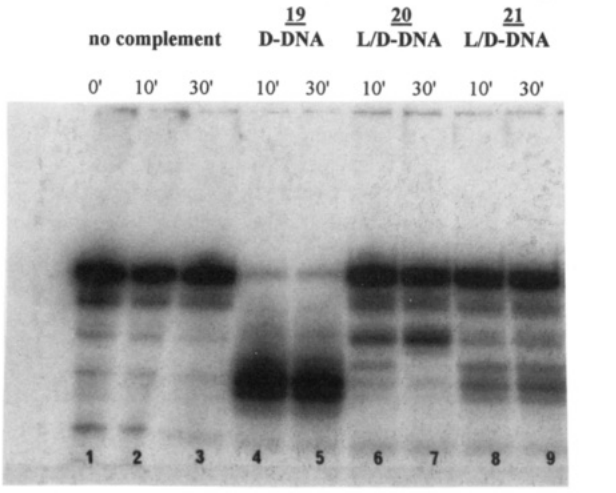

FIGURE 9: (a) 24% PAGE autoradiogram illustrating RNase H cleavage of L/D-DNA/RNA duplexes. Lanes: 1–3, **24** plus RNase H buffer at 0, 10, and 30 min, respectively; 4 and 5, **19**/**24** plus RNase H at 10 and 30 min, respectively; 6 and 7, **20**/**24** plus RNase H at 10 and 30 min, respectively; 8 and 9, **21**/**24** plus RNase H at 10 and 30 min, respectively. Conditions are described in Materials and Methods. (b) RNase H cleavage sites of L/D-DNA/RNA complexes. The sites of cleavage were estimated by comparing the mobilities of products to the ladder generated by digesting **24** with nuclease S1.

[2] The 5 °C drop in T_m observed per G/T mismatch is surprisingly low. A single G·C to G·T substitution within a duplex usually results in a drop of 10–20 °C in the T_m (Patel et al., 1982; Urata et al., 1990; Schneider & Benner, 1990; Hashimoto et al., 1993). It appears therefore that in the case of **22**/**23**, the G·T residues are forced to base pair ("wobble" base pairing) by the particular base composition of the duplex. Special stability of analogous G/U mismatches has been observed in transfer RNA and other RNA structures (He et al., 1991, and references therein).

the extent of duplex formation under these conditions, each of the DNA/RNA mixtures (**19/24**, **20/24**, and **21/24**) was incubated in RNase H incubation buffer, loaded on a native gel, and electrophoresed at 37 °C (Figure 8b). It is clear from Figure 8b that the mixture of natural DNA **19** and RNA **24** existed largely as a duplex, whereas the mixture of L/D-DNA **20** and RNA **24** had significant amounts of both duplex and single strands, and the mixture of L/D-DNA **21** and RNA **24** existed primarily in the single-stranded form. This result was expected since we had previously observed a progressive reduction in the DNA/RNA hybrid T_m's upon progressive L-dC substitution in the DNA chain. Therefore, in contrast to what is observed with chimeric methyl phosphodiester/phosphodiester analogs, reduced L/D-DNA/RNA hybrid stability correlates with decreased efficiency at eliciting RNase H activity. Since the depression in T_m arises from the L-dC substitutions, one may conclude that the reduced rate of cleavage observed among the DNA/RNA duplexes results in part from structural variations caused by the internal L-dC residues. This, in turn, restricts the interaction between RNase H and the L/D-DNA/RNA hybrid. The reduced RNase H activity observed can also be attributed to the lower concentration of L/D-DNA/RNA duplexes present under these conditions (e.g., complex **21/24**, Figure 8b).

The different sites of cleavage observed within the different DNA/RNA hybrids may demonstrate local conformational variations imposed by the L-dC inserts. The fact that the control duplex was cleaved at the same site observed for the L/D-DNA/RNA duplex **21/24** indicates that base sequence also plays a role in RNase H's cleavage specificity. Analogous oligonucleotide chimeras incorporating 2′-*O*-methylribonucleosides and other derivatives have been utilized for the specific cleavage of RNA by RNase H (Inoue et al., 1987; Schmidt et al., 1992; Kawasaki et al., 1993).

In summary, these experiments establish that oligodeoxynucleotides containing terminal as well as internal L-dC residues serve as excellent models of antisense agents that have enhanced resistance to the action of 3′-exonucleases, bind to nucleic acids through duplex formation, and elicit RNase H activity. As such, they should serve as valuable tools for studying and controlling gene expression in living cells.

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