Biochemical and Physicochemical Properties of Phosphorodithioate DNA†

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ABSTRACT: The biochemical and physicochemical properties of DNA oligomers containing phosphorodithioate linkages in various configurations were evaluated. Duplex stability studies, which were carried out by thermal denaturation analysis with complementary unmodified DNA, indicated a highly cooperative process similar to completely unmodified duplexes. Oligomers containing phosphorodithioate linkages were found to have reduced melting temperatures relative to unmodified duplexes, with the degree of T_m depression paralleling the percent phosphorodithioate composition of the oligomer. Relative to activation of RNase H, DNA oligomers containing up to 50% phosphorodithioate linkages were able to direct RNase H degradation with the same efficiency as unmodified DNA while those containing from 50 to 100% acted with somewhat reduced efficiency. At limiting concentrations, an oligomer containing alternating phosphorodithioate and phosphate linkages was able to direct RNase H degradation of the target RNA in an extended incubation, while an unmodified oligomer did not. The nuclease resistance of phosphorodithioate-containing oligomers was evaluated in HeLa cell nuclear and cytoplasmic extracts, in human serum, and with nucleases S1 and DNase I. Oligomers containing alternating phosphorodithioate and phosphate were highly resistant to degradation in all systems. However, oligomers having more than one unmodified linkage separating phosphorodithioates were degraded rapidly by DNase I, while demonstrating stability to degradation in all other systems tested. These results indicate that phosphorodithioate-containing DNA oligomers are highly nuclease-resistant, are able to form stable duplexes with complementary nucleic acid sequences, and efficiently direct RNase H degradation of target RNA.

The use of deoxyoligonucleotides as specific inhibitors of gene expression is a potentially powerful technique (Zamecnik & Stephenson, 1978). The ability to synthesize DNA oligomers complementary to a particular RNA species theoretically enables specific control of gene expression because an antisense oligomer can form a duplex with its target RNA sequence. Once this duplex forms, subsequent biochemical processes (such as pre-mRNA splicing, transport out of the nucleus, or translation) may be interrupted because of steric interference (hybrid arrest) with factors involved in these processing events. The RNA/DNA duplex may also be a substrate for RNase H, an enzyme that recognizes these hybrid duplexes and degrades the RNA portion. This RNase H mediated cleavage of RNA targets is an attractive method for control of gene expression since increased efficacy over hybrid arrest alone would be observed, especially if the DNA oligomer is stable to nucleases and directs the hydrolysis of many target RNA molecules. Effective antisense oligomers would therefore incorporate selectivity, activation of RNase H activity, and nuclease resistance. These characteristics would allow specific targeting and efficient, catalytic destruction of RNA.

The design of molecules possessing the above characteristics has centered on the synthesis of various phosphate-modified DNA oligomers. The primary goal of introducing phosphate modifications is to provide nuclease resistance, but still retain the desired characteristics of effective antisense molecules. Reported modifications include phosphorothioate (Stein et al., 1988), methylphosphonate (Ts'o et al., 1987), phosphoramidates (Froehler et al., 1988), and alkylphosphotriesters (Gallo et al., 1986; Marcus-Sekura et al., 1987; Summers et al., 1986). The phosphorodithioate linkage, an achiral, anionic mimic of natural DNA that contains an internucleotide phosphodiester group having sulfur at the nonlinking positions, has also been reported as a modification that confers nuclease resistance to deoxyoligonucleotides (Grandas et al., 1989; Porritt & Reese, 1990; Okruszek et al., 1995). Not all of these modifications have resulted in oligomers that possess the above characteristics. For example, methylphosphonates and phosphoramidates do not direct RNase H activity, but are resistant to nucleases (Agrawal et al., 1988, 1990; Maher & Dolnick, 1988; Quartin et al., 1989). To date, little information has been reported on the ability of deoxyoligonucleotide phosphorodithioates to direct RNase H activity.

The utility of phosphate-modified DNA oligomers as inhibitors of biological processes has been demonstrated in a number of applications including (1) the use of single-stranded DNA oligomers as viral inhibitors (Matsukura et al., 1987; Marshall et al., 1992), (2) as inhibitors of gene expression (Kulka et al., 1989; Marcus-Sekura et al., 1987; Storey et al., 1991), (3) as inhibitors of *in vitro* mRNA translation (Blake et al., 1985a,b; Cazenave et al., 1987, 1989), and (4) as inhibitors of sequence-specific DNA binding proteins when present in duplex form (Bielinska et al., 1990). In addition, phosphate-modified oligomers have recently been shown to be potent annealing, nonspecific inhibitors of HIV reverse transcriptase (Majumdar et al., 1989; Marshall et al., 1992; Marshall & Caruthers, 1993).

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In order to characterize some of the biochemical and physicochemical properties of phosphorodithioate (trivially, dithioate) containing oligomers, we have studied their DNA duplex stability, nuclease resistance, and ability to direct RNase H activity. We present evidence indicating that they possess characteristics desirable for effective antisense oligomers.

MATERIALS AND METHODS

Preparation of HeLa Cell Nuclear and S100 Extracts. HeLa cell S100 and nuclear extracts were prepared essentially as described (Dignam et al., 1983) with the following modifications. Nuclei were extracted with 6 mL of buffer C [20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT] per 10^9 cells. The final dialysis for both nuclear extracts and S100 fractions was against buffer D [20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.05 M NaCl, 0.2 mM EDTA, and 0.5 mM DTT] containing 50 mM KCl.

RNase H Assays. RNase H assays were carried out at 30 °C for 1 h or as indicated. Reactions were performed using 25 μL of nuclear extract, 1.5 μL of 0.1 M $MgCl_2$, 1.2 μL of 40 mM ATP, 3.4 μL of 0.3 M creatine phosphate, 10 μL of 13% polyvinyl alcohol, and the appropriate concentration of deoxyoligonucleotide in a total volume of 50 μL. After completion of each assay, RNA was purified from the snRNP particles by adding 150 μL of 0.1 M Tris, pH 7.5, containing 20 mM EDTA, 2% SDS, and 0.2 mg/mL proteinase K, incubating 10 min at 30 °C, extracting with phenol/chloroform, and precipitating with 3 volumes of ethanol. Samples were analyzed on 10% polyacrylamide gels containing 8 M urea by staining with ethidium bromide.

Nuclease Stability Studies. Deoxyoligonucleotides (20-mers) to be used for nuclease studies (HeLa cell nuclear and cytoplasmic extracts, human serum) were internally labeled. This was accomplished by first synthesizing the segment as two 10-mers, phosphorylating the 10-mer corresponding to the 3′-half of the full-length molecule with [γ-^{32}P]ATP and T_4 polynucleotide kinase (USB, Cleveland, OH), and joining this labeled segment to the 5′-half 10-mer using T_4 polynucleotide ligase (Promega, Madison, WI) and a complementary 10-mer DNA template. These internally labeled molecules were purified by polyacrylamide gel electrophoresis and ethanol precipitation.

Stability studies in nuclear extracts were carried out as described for RNase H assays at a deoxyoligonucleotide concentration of 0.1 μM. Similar studies with cytoplasmic extracts were completed at 0.1 μM deoxyoligonucleotide and 2 mg/mL protein in buffer D containing 50 mM KCl. Stability studies with human serum were completed in 20% serum, 0.2 μM deoxyoligonucleotide, and buffer D containing 50 mM KCl.

Studies with S1 nuclease were carried out using the oligomer series dKt (Marshall & Caruthers, 1993, Figure 1). Oligomers were 5′-end-labeled using 3′-phosphatase-free polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) and purified from unincorporated label by gel filtration on Sephadex G-25 (Pharmacia) using 50 mM triethylammonium bicarbonate in 30% acetonitrile as eluant. (We find that 3′-phosphatase-free T_4 polynucleotide kinase labels dithioate deoxyoligonucleotide analogs more efficiently than T_4 polynucleotide kinase; 30% acetonitrile facilitates the purification of phosphorothioates and phosphorodithioates over G-25 Sephadex.) Reactions were carried out at 1.0 μM oligomer in 30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM $ZnCl_2$, and 5% glycerol at 37 °C over 20 min using 0.08 unit of S1 nuclease per 50 μL reaction volume.

DNase I studies were performed using the oligomers described in Figure 1. Oligomers were labeled and purified as described for the S1 nuclease studies. Reactions were carried out at 1 μM oligomer in 40 mM Tris (pH 7.5) and 6 mM $MgCl_2$ at 37 °C over 20 min using 20 units of DNase I per 20 μL reaction.

In all studies, aliquots of the reaction mixture were analyzed directly on a 20% polyacrylamide/8 M urea gel without further purification. Quantitation of DNA degradations was accomplished using a Molecular Dynamics Phosphorimager. In all cases, the percent full-length deoxyoligonucleotide was determined by measuring the radioactivity corresponding to full-length deoxyoligonucleotide and dividing that number by the total radioactivity in a lane. Nuclease stability results were expressed as the percent of full-length segment remaining at a given time point.

Deoxyoligonucleotide Synthesis. Unmodified DNA was prepared using standard phosphoramidite chemistry (Caruthers, 1985). DNA segments containing phosphorothioate (Stein et al., 1988) and phosphorodithioate (Beaton et al., 1991a,b) internucleotide linkages were prepared according to published procedures. All DNA was synthesized on an Applied Biosystems Model 380A DNA synthesizer.

Melting Curves. Melting curves were generated on a Gilford 2400 spectrophotometer equipped with a Model 2527 thermoprogrammer. Absorbance values were recorded at 260 nm in 25 mM potassium phosphate (pH 7.0) and 150 mM NaCl (unless otherwise indicated) between 25 and 95 °C using a thermal ramping rate of 1 °C/min. Absorbance values were automatically recorded approximately 3 times a minute and corrected against a buffer blank. Duplexes were prepared by heating solutions to 90 °C for 5 min and cooling to room temperature. All duplexes were formed in a 1:1 ratio of the test oligomer with the unmodified, complementary strand at 4.5 μM. The concentration was determined by summing the molar absorptivity coefficients (pH 7.0) of the bases present, followed by the application of Beers' Law. Melting temperatures were determined as described (Markey & Breslauer, 1987) and are the result of at least three separate determinations. T_m values were calculated at $\alpha = 0.5$, where α is defined equal to zero when DNA is in the 100% duplex form, and α is equal to 1 when the DNA is in the 100% single-stranded form. From the absorbance vs temperature data, $\alpha = 0$ is represented by the lower base line (100% duplex), and $\alpha = 1$ is represented by the upper base line (100% single-stranded). The midpoint between the two base lines was taken to be $\alpha = 0.5$, and the T_m determined at this point. ΔH_{VH} values were determined as described (Markey & Breslauer, 1987) for non-self-complementary oligomers and are the result of at least three separate determinations. $\Delta H°$ values (ΔH_{VH}) were calculated by the following equation (Markey & Breslauer, 1987): $\Delta H_{VH} = (2 + 2n)R(T_m)^2(\delta\alpha/\delta T)_{T=T_m}$, where n = the molecularity of the reaction, R is the gas constant (1.9872 cal K^{-1} mol^{-1}), T = temperature (K), and α is defined as equal to 0 when the DNA is 100% duplex and 1 when the DNA is 100% single-stranded. $\Delta H°$ values and their associated standard deviations are shown in Table 1. The error associated with $\Delta H°$ values is ±4%. T_m values have an associated error of ±0.5 °C.

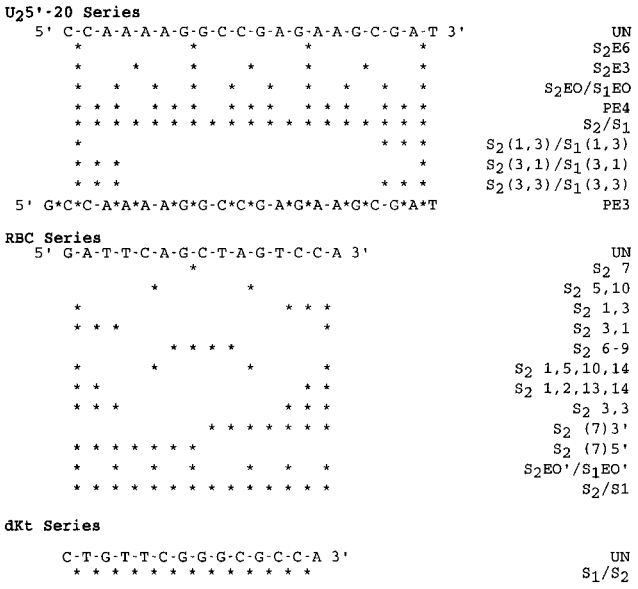

FIGURE 1: Deoxyoligonucleotide sequences and the distribution of modified internucleotide linkages. Modified linkages, either dithioate or thioate, are depicted by an asterisk while normal internucleotide linkages are represented by a dash. S_2 and S_1 represent phosphorodithioate and phosphorothioate linkages, respectively. UN refers to the completely unmodified oligomers having natural phosphate internucleotide linkages. Nomenclature is defined by the type of modified linkage followed by a description of the position(s) of this (these) linkage(s); i.e., S_2E3 or S_2EO are oligomers containing dithioate linkages at every third (E3) or every other (EO) position, and S_1(3,1) contains three phosphorothioate linkages at the 5′ end and one at the 3′ end; the remaining linkages are unmodified. Oligomers PE3 and PE4 contain normal phosphate linkages at every third and fourth position, respectively. The remaining linkages are dithioate. Symbols such as S_2EO/S_1EO depict two oligomers—one having dithioates at every other position, the other with thioates at every other position. An unmodified deoxyoligonucleotide complementary to these sequences was used for duplex stability studies.

RESULTS

In order to examine the stability of dithioate-containing DNA duplexes, model systems were constructed having one unmodified oligomer and an annealing partner with various combinations of phosphate and phosphorodithioate or phosphorothioate internucleotide linkages. These model duplexes, including their base composition, sequence, and distribution of various internucleotide linkages, are shown in Figure 1. The $U_2$5′-20 oligomers were complementary to the first 20 nucleotides at the 5′ terminus of the human U2 snRNA. The 15-mers contain a random base composition (RBC series) and were designed to be free of secondary structure. Representative thermal denaturation curves for duplexes prepared from $U_2$5′-20 oligomers are shown in Figure 2, panel A. Even with all linkages in the modified strand substituted with dithioates (S_2), the duplex formed with a complementary oligomer exhibits a transition curve essentially identical in shape to the corresponding unmodified duplex (UN). This observation suggests that phosphorodithioate-modified DNA forms duplexes that denature in a cooperative, two-state transition. Indeed, a comparison of the enthalpy for various transitions reveals some surprising results. First, most of the phosphorodithioate-containing 20-mer duplexes ($U_2$5′-20 series) have $\Delta H°$ values approximately 20 kcal/mol lower than the unmodified controls (Table 1). While one might expect a correlation between the number of dithioate linkages and the resulting decrease

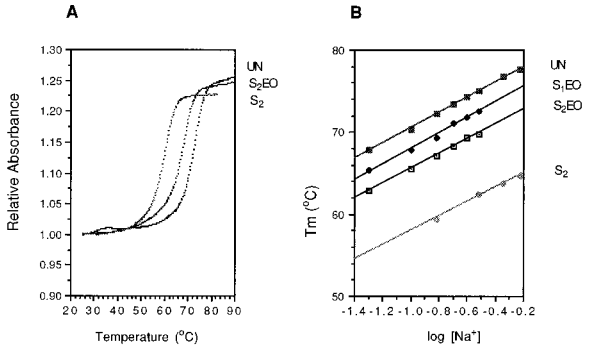

FIGURE 2: T_m profiles of modified DNA. (Panel A) Thermal denaturation curves for duplexes of the $U_2$5′-20 series. Denaturation curves are for oligomers S_2, S_2EO, and UN (left to right, respectively) in complex with unmodified, complementary DNA. (Panel B) Plot of T_m vs log [Na^+] for duplexes of the $U_2$5′-20 series. T_m's were determined at various NaCl concentrations for the indicated oligomers in complex with unmodified, complementary DNA.

in $\Delta H°$, this does not appear to be the case since duplexes containing between 37 and 100% phosphorodithioate modifications in one oligomer have essentially identical $\Delta H°$ values. Second, the duplex formed with S_2E6, an oligomer containing 21% dithioate linkages, has an associated $\Delta H°$ that is essentially the same as is observed for the unmodified duplex.

Phosphorodithioate linkages do not severely affect the shape of the T_m curve compared to phosphodiester linkages. This suggests that the cooperativity of dithioate duplex transitions is similar to unmodified DNA. In the $U_2$5′-20 series (Table 1), a duplex with an oligomer containing four of these modifications evenly distributed (S_2E6) has a T_m depression of 1.4 °C while other duplexes with oligomers having 53% and 100% dithioates have T_m depressions of 5.1 and 12.8 °C, respectively. Duplexes with oligomers containing clustered linkages at the 5′ and 3′ ends have slightly lower melting temperatures than those with evenly distributed modifications [compare S_2E6 with S_2(3,1) or S_2(1,3)]. In general, oligomers containing stretches of three or more dithioates exhibit a greater T_m depression as their corresponding duplexes than occurs when the modified linkages are distributed evenly. The T_m depression of duplexes in the RBC oligomer series (Table 1) also increases as the percentage of dithioate linkages increases. However, the effect of uneven distribution is more pronounced. Oligomer S_2(1,5,10,14) with four evenly distributed dithioate linkages forms a duplex that has a T_m depression of 2.0 °C while oligomers containing four unevenly distributed dithioate linkages [S_2(3,1), S_2(1,3), and S_2(6−9)] generate duplexes having T_m depressions of 4.4, 4.9, and 4.9 °C, respectively. For each series, the alternating dithioate−phosphate oligomers yield duplexes that have T_m depressions less than the all-phosphorothioate analogs (ΔT_m = 5.1 and 7.1 °C for the $U_2$5′-20 series; ΔT_m = 5.3 and 8.9 °C for the RBC series, respectively).

Analysis of the T_m depression ($U_2$5′-20 series) reveals that oligomers containing less than 50% dithioate form duplexes where the melting temperatures are depressed by 0.4−0.5 °C per modification whereas the duplexes generated from oligomers containing greater than 50% dithioate have a T_m depression of 0.7 °C per linkage. This relationship does not hold for duplexes generated from the phosphorothioate-containing oligomers of this series as S_1EO and S_1 display T_m depressions of 0.4 °C per linkage. When introduced into

Table 1: Effect of Phosphorodithioate or Phosphorothioate Composition of Deoxyoligonucleotides on $\Delta H°$ and ΔT_m[a,b,c]

oligomer	% S_2/S_1	ΔH_{VH}	δ	$\Delta\Delta H°$	ΔT_m	ΔT_m per S_2 or S_1 linkage	phosphates between S_2 or S_1 linkages	no. of continuous S_2 or S_1 linkages
				U_25'-20 Series				
UN		128	5.0					
S_2E6	21	129	1.5	+1	1.4	0.35	5	1
S_2(3,1)	21	117	2.9	11	2.8	0.70	15	3
S_2(1,3)	21	119	2.1	9	2.0	0.50	15	3
S_2(3,3)	32	112	2.9	16	3.1	0.52	13	3
S_2E3	37	108	1.8	20	3.4	0.49	2	1
S_2EO	53	106	3.4	22	5.1	0.51	1	1
PE4	79	73	1.5	55	9.7	0.65	1	3
S_2	100	110	2.6	18	12.8	0.67	0	19
S_1EO	53	109	2.2	19	3.8	0.38	1	1
S_1	100	100	4.1	28	7.1	0.37	0	19
				RBC Series				
UN		110	7.5					
S_2(7)	8	109	14	0.5	1.6	1.6	6/7	1
S_2(5,10)	15	98.7	1.6	11	1.8	0.9	4	1
S_2(1,3)	31	90.5	3.6	19	4.9	1.2	10	3
S_2(3,1)	31	92.6	2.5	17	4.4	1.1	10	3
S_2(6−9)	31	98.2	1.4	12	4.9	1.2	5	4
S_2(1,5,10,14)	31	103	1.3	7	2.0	0.5	3	1
S_2(1,2,13,14)	31	101	1.5	9	1.6	0.4	10	2
S_2(3,3)	46	85.2	7.6	25	7.8	1.3	8	3
S_2(3′−7)	54	88.3	5.8	22	9.5	1.4	7	7
S_2(5′−7)	54	91.1	12	19	9.5	1.4	7	7
S_2EO	54	93.7	4.1	16	5.3	0.8	1	1
S_2	100	73.6	2.9	36	17.7	1.4	0	14
S_1EO	54	99.8	2.3	10	1.6	0.2	1	1
S_1	100	78.3	20	32	8.9	0.7	0	14

[a] Oligomer: The oligomer (see Figure 1) used to form a duplex with unmodified, complementary DNA. Due to insufficient material, the T_m for PE3 was not determined. [b] $\Delta\Delta H°$ values are expressed as kcal/mol and are the difference in $\Delta H°$ for duplexes containing a modified oligomer when compared to $\Delta H°$ obtained for a completely unmodified duplex. [c] ΔT_m values are the difference in the T_m for an unmodified duplex and the duplex having the same sequence but different numbers and/or location of phosphorodithioate or phosphorothioate internucleotide linkages. T_m for UN of the U_25'-20 series is 72.2 °C. T_m for UN of the RBC series is 59.7 °C.

the shorter RBC oligomers, dithioate-containing duplexes have a greater ΔT_m per linkage than observed in the U_25'-20 series. The T_m depression per linkage in duplexes generated from RBC oligomers containing three or more continuous dithioates is between 1.1 and 1.4 °C whereas it is 0.7 °C for the U_25'-20 Series.

The effect of ionic strength on T_m for duplexes prepared from oligomers S_1EO, S_2EO, and S_2 was compared to the natural duplex. A graph of T_m vs log [Na^+] shows that these modified duplexes respond to increasing sodium ion in the same manner as a totally unmodified duplex (Figure 2, panel B).

In order to investigate the ability of phosphorodithioate DNA to stimulate endogenous RNase H activity, a system was used that had been developed previously for studying human U2 snRNA (Krainer & Maniatis, 1985; Black et al., 1985). These investigators had shown that deoxyoligonucleotides complementary to the 5′ end of human U2 snRNA directed cleavage of this snRNA by the endogenous RNase H activity found in HeLa cell nuclear extracts. They further demonstrated that this RNase H cleavage occurred while the U2 snRNA was part of a U2 snRNP particle and thus protected against the nucleases found in this extract. Thus, RNase H-dependent cleavage of U2 snRNA could be monitored without the complication of nonspecific nuclease degradation of target. This system therefore was ideal for studies with phosphorodithioate DNA as it mimics *in vitro* the conditions presumed to be present within the cell. Thus, HeLa cell nuclear extract provided in one source a readily available target (U2 snRNA), an endogenous RNase H, and an environment rich in nucleases. Independently, and in an assay functional for RNase H activity, the stability of phosphorodithioate-containing DNA toward the nucleases present in this extract could also be monitored. A series of modified oligomers complementary to the 5′ end of the U2 snRNA (U_25'-20 series) was therefore prepared to examine these important properties.

To assess the ability of phosphorodithioate DNA to stimulate RNase H activity, aliquots of HeLa cell nuclear extracts were treated with variable concentrations of deoxyoligonucleotides and incubated for 1 h. The degradation of U2 snRNA was then monitored by polyacrylamide gel electrophoresis (Figure 3). At 0.5 μM, the unmodified oligomer promoted essentially complete degradation of U2 snRNA (Figure 3, panel A). Moreover, cleavage appeared to be specific since all other snRNAs remained intact. When a deoxyoligonucleotide having alternate phosphorodithioate and phosphate internucleotide linkages was tested, the results were essentially identical to those observed with the unmodified oligomer (Figure 3, panel B). Similar results (Table 2) were also obtained with most other phosphorodithioate- and phosphorothioate-linked DNAs. There were exceptions. For some oligomers, the concentration required to direct complete, specific degradation was only 0.2 μM. In contrast, other oligomers had to be used at higher concentrations for complete cleavage under the conditions of the assay. These less active oligomers had 70% (PE3), 75% (PE4), or 100% (S_2) phosphorodithioate internucleotide linkages.

The ability of phosphorodithioate DNA to direct RNase H cleavage during an extended time in HeLa cell nuclear extract was also examined. An oligomer concentration was chosen (0.1 μM) where U2 snRNA cleavage was detectable,

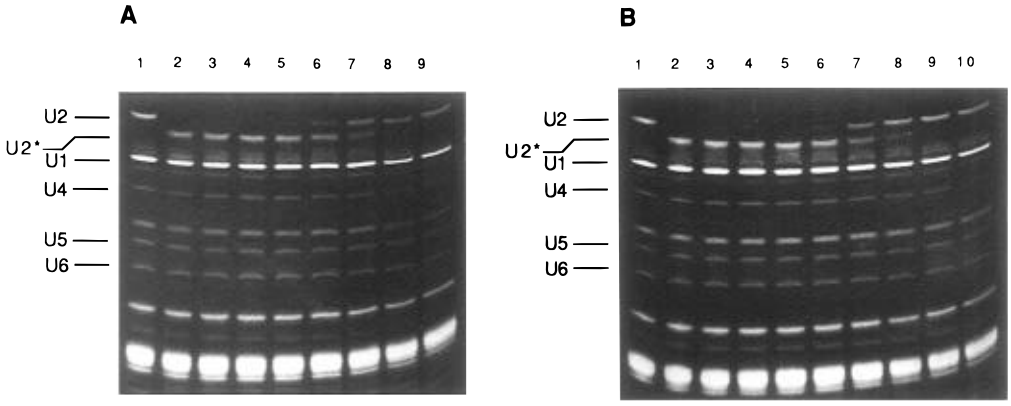

FIGURE 3: RNase H degradation of U2 snRNA. (Panel A) Titration of the unmodified $U_2$5′-20 oligomer in HeLa cell nuclear extract. Concentrations of added UN $U_2$5′-20 are as follows: lane 1, no oligomer added; lane 2, 2.0 μM; lane 3, 1.5 μM; lane 4, 1.0 μM; lane 5, 0.5 μM; lane 6, 0.2 μM; lane 7, 0.1 μM; lane 8, 0.05 μM; lane 9, 0.02 μM. U-snRNA's are as labeled. U2* is the product of RNase H degradation of U2 snRNA as directed by the added oligomer. (Panel B) Titration of $U_2$5′-20 S_2EO in HeLa cell nuclear extract. Concentrations of added $U_2$5′-20 S_2EO are as follows: lane 3, 2.0 μM; lane 4, 1.5 μM; lane 5, 1.0 μM; lane 6, 0.5 μM; lane 7, 0.2 μM; lane 8, 0.1 μM; lane 9, 0.05 μM; lane 10, 0.02 μM. Lane 1, no oligomer added. Lane 2, 2.0 μM UN $U_2$5′-20 oligomer.

Table 2: Deoxyoligonucleotide Concentrations Required To Direct Complete RNase H Degradation of U2 snRNA in 1 h

oligomer	concn (μM)	oligomer	concn (μM)
UN	0.5	S_2EO	0.5
S_2(3,3)	0.2	PE3	1.0
S_2(3,1)	0.2	PE4	2.0
S_2(1,3)	0.2	S_2	4.0
S_2E6	0.2	S_1EO	0.5
S_2E3	0.5	S_1	0.5

but incomplete during the 1 h incubation (Figure 3). Samples were then incubated for 21 h. Under these conditions, nuclease-resistant oligomers should direct RNase H-dependent cleavage of U2 snRNA over an extended time period and generate a larger fraction of cleaved RNA than observed in the 1 h assay. With unmodified oligomer, only partial degradation of U2 snRNA was observed (Figure 4, panel A). When a second aliquot of unmodified oligomer was added after 21 h, U2 snRNA degradation continues, which indicates that the endogenous RNase H was still active (data not shown). The band appearing just below intact U1 snRNA was found during extended incubations of HeLa nuclear extracts, even in the absence of any oligomer, and is unrelated to this study. Phosphorodithioate-containing oligomers were more active than the unmodified oligomer. Incubation with oligomers containing either phosphorodithioate modifications at every sixth linkage or three phosphorodithioate linkages at each terminus led to enhanced but incomplete cleavage after 21 h (data not shown). In contrast, oligomers having phosphorodithioate linkages at every third position (data not shown) or every other position (Figure 4, panel B) directed complete cleavage of U2 snRNA after 21 h. Complete cleavage was also observed for the phosphorothioate analog (data not shown). Although an attractive possibility, it is difficult to conclude from this study that the active dithioate or thioate oligomers catalyze multiple U2 snRNA cleavages. This is because we did not determine the concentration of U2 snRNPs in our extracts.

To study the nuclease susceptibility of phosphorodithioate-modified DNA, a series of internally labeled segments were prepared (see Materials and Methods) which eliminates the problem of endogenous phosphatases removing a 5′ end-labeled phosphate. When the unmodified, internally labeled 20-mer (0.1 μM) was incubated with nuclear extract under conditions used for RNase H directed cleavage of U2 snRNA, only 14% remained full-length after 30 min, and after 21 h, it was completely degraded. Based on the initial rate of degradation, this unmodified oligomer had a $t_{1/2}$ of 18 min (Table 3). In contrast, the internally labeled oligomer having phosphorodithioate internucleotide linkages at alternating positions remained 72% intact after 21 h. Similar results were obtained with the analog having all phosphorothioate internucleotide linkages where 63% of the oligomer remained intact after 21 h. These two oligomers had a calculated $t_{1/2}$ of 330 min based on extrapolations of the initial rates of degradation. The stability of these analogs toward endogenous nucleases was probably responsible for their extended activity in the RNase H directed cleavage assay of U2 snRNA over 21 h. However, the phosphorothioate oligomer having modifications at alternating positions was relatively unstable in nuclear extract as 66% and 15% remained intact after 30 min and 21 h, respectively ($t_{1/2}$ = 42 min). Capping each end of this oligomer with three phosphorothioate or phosphorodithioate linkages had significantly different effects on resistance toward endogenous nucleases. The phosphorothioate oligomer was marginally more stable in the nuclear extract than unmodified DNA ($t_{1/2}$ = 48 min), but the capped phosphorodithioate analog was significantly more stable with an initial half-life of 192 min.

The relative rates of degradation of all the oligomers studied in HeLa cell cytoplasmic extracts were found to mirror the results found with the nuclear extract (Table 3). Thus, the oligomers having either alternating phosphorodithioate and phosphate linkages or phosphorodithioate capped ends were again the most stable against endogenous nucleases with degradation rates 14- and 7-fold slower than unmodified DNA. The phosphorothioate capped oligomers were less stable toward these cytoplasmic nucleases.

Further studies were carried out to test the stability of these internally labeled oligomers to S1 nuclease, DNase I, and the nucleases found in human serum. Incubation of three oligomers in 20% human serum gave the same order (UN < S_1EO < S_2EO) of nuclease resistance as expected from the previous studies in HeLa extracts. For example, 78% of oligomer S_2EO and 7% of S_1EO remained full-length after 21 h. The $t_{1/2}$ values also reflect this trend (Table 3).

Incubation of the 5′ end-labeled dKt oligomer series [UN, S_1, S_2 (Figure 1)] with S1 nuclease resulted in nearly complete degradation of the unmodified oligomer in 20 min.

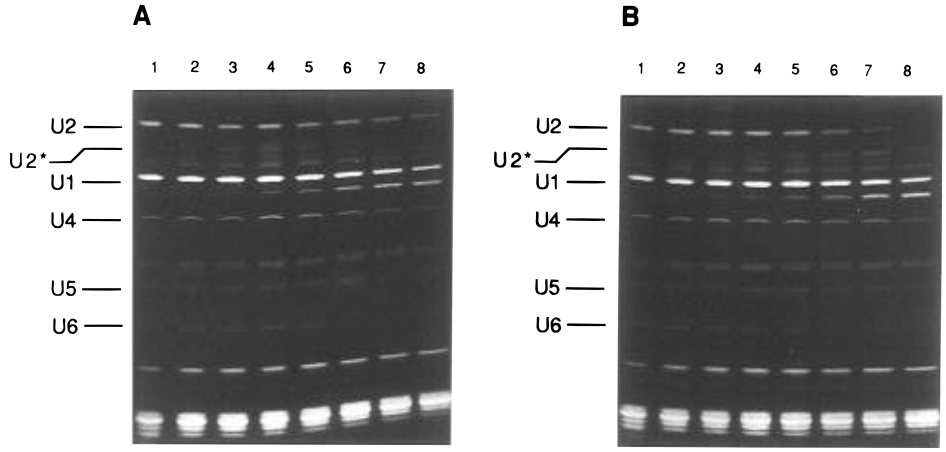

FIGURE 4: (Panel A) Time course for RNase H degradation of U2 snRNA. Extended incubation of UN $U_2$5′-20 (0.1 μM) in HeLa cell nuclear extract. Lane 1, 0 h; lane 2, 0.17 h; lane 3, 0.5 h; lane 4, 1.0 h; lane 5, 2.0 h; lane 6, 4.0 h; lane 7, 8.0 h; lane 8, 21.0 h. (Panel B) Extended incubation of $U_2$5′-20 S_2EO (0.1 μM) in HeLa cell nuclear extract. Lane 1, 0 h; lane 2, 0.17 h; lane 3, 0.5 h; lane 4, 1.0 h; lane 5, 2.0 h; lane 6, 4.0 h; lane 7, 8.0 h; lane 8, 21.0 h.

Table 3: Calculated Half-Lives of $U_2$5′-20-Modified Deoxyoligonucleotides in HeLa Cell Nuclear Extract, HeLa Cell Cytoplasmic Extract, and in Human Serum

oligomer	$t_{1/2}$[a] (min)	rel $t_{1/2}$[a]
	Nuclear Extract	
UN	18	1
S_1EO	42	2.3
S_1(3,3)	48	2.7
S_2(3,3)	192	10.7
S_1	330	18.3
S_2EO	330	18.3
	Cytoplasmic Extract	
UN	11	1
S_1(3,3)	22	2.0
S_1EO	24	2.2
S_1	50	4.5
S_2(3,3)	80	7.3
S_2EO	162	14.7
	Human Serum	
UN	21	1
S_1EO	56	2.7
S_2EO	228	10.9

[a] $t_{1/2}$ values are based on initial rates of degradation.

In contrast, the phosphorothioate oligomer was degraded by approximately 25% and the phosphorodithioate oligomer remained essentially undegraded (Figure 5, panel A) in the same time period. The observed loss of approximately 5% of the labeled material with the dithioate oligomer in the absence of degradation products is thought to be due to removal of the 5′-$^{32}PO_4$ by a low-level phosphatase contamination. This is because the phosphorothioate oligomers did show observable degradation products when incubated with S1 nuclease (data not shown). There are other possible explanations for this 5% degradation. Due to the synthesis protocols, each internucleotide phosphorodithioate linkage contains approximately 2−3% phosphorothioate contamination. The small amount of degradation of the dithioate oligomer could therefore be due to the susceptibility of these phosphorothioate linkages to degradation by S1 nuclease. Of course the possibility also exists that the phosphorodithioate linkage is susceptible to degradation by S1 nuclease but at an extremely slow rate relative to the unmodified linkage.

Similar studies were completed using DNase I with the unmodified, phosphorothioate, and phosphorodithioate analogues of the RBC series. Under conditions that result in approximately 80% degradation of the full-length, unmodified oligomer, both phosphorothioate (S_1) and phosphorodithioate (S_2) RBC oligomers are undegraded (data not shown). To determine the resistance to an endonuclease as a function of the spacing of phosphorodithioate linkages, this study was extended to include oligomers in the $U_2$5′-20 series (UN, S_2E6, S_2E3, S_2EO). As shown in Figure 5, panel B, the resistance to degradation by DNase I increases very little relative to the unmodified oligomer when phosphorodithioate linkages are present at every sixth or every third linkage. However, with dithioate modifications present at every other linkage, there was virtually no degradation by this enzyme.

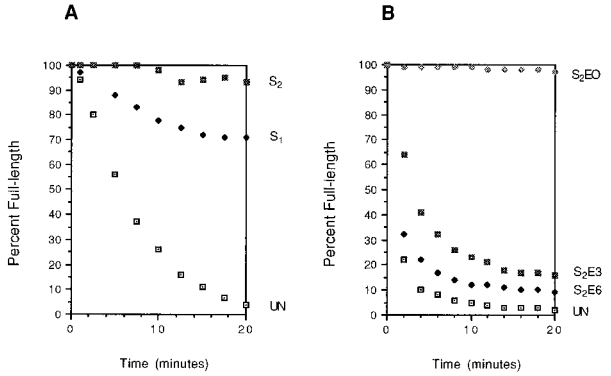

FIGURE 5: S1 and DNase I degradation of modified DNA. (Panel A) S1 nuclease digestion of dKt UN, S_1, and S_2. (Panel B) DNase I digestion of $U_2$5′-20 UN, S_2E6, S_2E3, and S_2EO.

DISCUSSION

Deoxyoligonucleotides containing phosphorodithioate linkages exhibit characteristics desirable for use as antisense agents. They form stable duplexes with complementary unmodified DNA, direct RNase H degradation of target RNA molecules in a sequence-specific manner, and are resistant to the nucleases present in HeLa cell nuclear and cytoplasmic extracts, as well as those present in human serum.

An indication of the stability of duplexes containing modified deoxyoligonucleotides is to study their melting profiles. All of the phosphorodithioate-modified oligomers examined form stable duplexes with complementary, unmodified DNA. A comparison of phosphorodithioate with phosphorothioate-containing duplexes indicates that the phosphorodithioate modification results in approximately a 2-fold decrease in ΔT_m. However, with the phosphorothioate

20-mer, a $\Delta\Delta H°$ of 10 kcal/mol less than the phosphorodithioate analog was observed. This suggests that the presence of multiple diastereomers in the case of a phosphorothioate modification may affect the cooperativity of duplex formation to a greater extent than any factors attributable to the dithioate analog.

The observation that dithioate-containing oligomers form duplexes with ionic strength dependencies equivalent to unmodified DNA is perhaps somewhat surprising given that dithioate DNA is not eluted from anion exchange columns under conditions characteristic of normal phosphodiester DNA (Marshall & Caruthers, 1993). However, in denaturing polyacrylamide gels, dithioate oligomers have gel mobilities that are similar to unmodified oligomers of the same length and sequence, indicating that they have an equivalent charge to mass ratio and one negative charge per internucleotide linkage. Internucleotide modifications that introduce hydrophobic groups at phosphorus and neutralize charge do not show an equivalent response to increasing ionic strength. Because dithioate oligomers also have an associated hydrophobic character relative to unmodified oligomers, one might expect, given these changes in physical characteristics, that dithioate oligomers might display ionic effects that differ from those of unmodified oligomers. As seen in Figure 2, panel B, the plots of T_m vs ionic strength for all duplexes tested have essentially the same slope.

All phosphorodithioate-containing oligomers that were complementary to the 5′ end of U2 snRNA were able to direct RNase H to completely degrade the target in a 1 h incubation. Generally, deoxyoligonucleotides containing up to 50% phosphorodithioate linkages were able to direct RNase H degradation of U2 snRNA at least as efficiently as the unmodified oligomer whereas those containing more than 50% dithioate were less reactive. Because the oligomer having 50% dithioate (S_2EO) is as active as normal DNA, it is difficult to conclude that endogenous HeLa cell RNase H inefficiently recognizes dithioate DNA/RNA duplexes. Perhaps these data reflect the same trend observed for dithioate DNA/DNA duplexes (Table 1)—specifically that high-percentage dithioate DNA/RNA complexes are simply less stable than the corresponding unmodified duplexes. The enhanced reactivities seen with deoxyoligonucleotides S_2-E6, S_2(3,1), S_2(1,3), and S_2(3,3) were likely due to their nuclease resistance combined with the presence of long stretches of unmodified linkages. The data supporting this conclusion are as follows. First, placement of phosphorodithioate linkages at the ends of a DNA segment significantly enhances its stability toward nucleases (Table 3; Grandas et al., 1989). Second, oligomers that have predominantly phosphorodithioate linkages are less efficient at directing RNase H activation than unmodified DNA (Table 2). The phosphorodithioate capped oligomer thus provides enhanced protection against nucleases while still providing a stretch of unmodified DNA for maximal RNase H activity. Further, the complete degradation of U2 snRNA by limiting concentrations of phosphorodithioate DNA in an extended time course was presumably due to its increased resistance toward the nucleases present in HeLa cell nuclear extracts since the corresponding unmodified deoxyoligonucleotides were unable to do so.

A comparison of oligomers containing phosphorothioate or phosphorodithioate linkages at equivalent positions indicates that dithioate linkages impart substantially more nuclease resistance. These differences are not totally surprising given that the R_p isomer of phosphorothioates has been shown to be susceptible to hydrolysis by snake venom phosphodiesterase and the restriction enzyme *Eco*RI (Bryant & Benkovic, 1979; Burgers et al., 1979a,b; Connolly et al., 1984a,b) while the S_p isomer is hydrolyzed by nuclease P1 (Potter et al., 1983). Oligomers containing phosphorothioate linkages are stable in *Xenopus* oocytes, but not in embryos (Cazenave et al., 1989; Woolf et al., 1990). Our studies as well as others indicate that dithioate linkages in deoxyoligonucleotides are not hydrolyzed by nucleases (Figure 5; Grandas et al., 1989; Porritt & Reese, 1990; Okruszek et al., 1995). These results are, however, substantially different from those of Ghosh et al., who claim that phosphorodithioate-containing deoxyoligonucleotides are as susceptible to DNase I as an unmodified control (Ghosh et al., 1993). In contrast, we have shown that the stability of deoxyoligonucleotides toward DNase I is clearly correlated with an increasing number of dithioate linkages (Figure 5, panel B), and the results of Okruszek et al. demonstrate that dithioate oligomers are not degraded by DNase I. In addition, Ghosh et al. report that phosphorodithioate-containing deoxyoligonucleotides are no more stable than an unmodified control in an MCF-7 cell nuclear fraction. In their study, 5′-[^{32}P]-phosphoryl-labeled deoxyoligonucleotides were used for quantitating the extent of oligomer degradation. This method renders the labeled deoxyoligonucleotide susceptible to enzymatic dephosphorylation. We found a high level of endogenous phosphatase activity in cellular extracts that precluded the use of a 5′ end-labeled molecule for accurate quantitation of nuclease degradation. Therefore, we presume that the results of Ghosh et al. reflect the ability of 5′-phosphorylated oligomers and oligomer analogs to act as substrates for phosphatase rather than indicate the susceptibility of the phosphorodithioate linkage to nuclease degradation.

Incubation of a completely modified phosphorodithioate oligomer with S1 nuclease results in no detectable degradation, while its phosphorothioate counterpart is degraded (Figure 5, panel A). Both uniformly modified phosphorothioate and phosphorodithioate oligomers are completely stable to degradation by DNase I (data not shown). Separation of dithioate modifications by more than one unmodified linkage results in a substantial loss of endonuclease resistance (Figure 5, panel B).

One interesting finding resulting from these studies is the radical difference in DNase I susceptibility of oligomers containing alternating dithioate linkages compared to those with every third linkage modified. These observations are consistent with the findings of Weston et al. (1992), whose crystallographic studies indicate that DNase I cleaves at a position one nucleotide removed from the binding site. Presumably a dithioate linkage at the binding site is not accepted by the enzyme—perhaps because it either does not chelate with magnesium or sterically does not fit into the binding pocket. If this is the case, then the minimum spacing for DNase I cleavage would be two successive internucleotide phosphate linkages followed by a phosphorodithioate.

Several factors must be carefully considered in the design of dithioate containing deoxyoligonucleotides having high specificity for a complementary sequence. Dithioate modifications may impart some stable, alternative secondary structure to the single-stranded oligomers (Piotto et al., 1990) that affects their ability to form duplexes with target RNA or DNA sequences. Therefore, the duplex-forming ability

may be dependent upon a competing equilibrium between annealing competent, single-stranded dithioate DNA and that which is locked in a nonproductive conformation. In addition, binding of dithioate-containing oligomers to proteins that interact with nucleic acids (Marshall et al., 1992) may decrease the concentration of oligomer available for duplex formation with its target sequence. Once formed, dithioate DNA/RNA duplexes may not be recognized by RNase H as readily as unmodified DNA. All of these pitfalls must be overcome in the design of the desired highly specific antisense agent.

One strategy in antisense oligomer design is to incorporate the minimal amount of modification that will introduce all of the beneficial effects of these substituents. Indeed, this approach for phosphorodithioates has led to a reduced ability of these oligomers to bind to proteins (Marshall et al., 1992). We and others have found that the primary nucleolytic activity found in serum and cellular extracts is by a 3′-5′ exonuclease (Shaw et al., 1991; Eder et al., 1991). Alternating dithioate and phosphate linkages impart substantial resistance to nucleases, but they are still slightly susceptible as S_2EO is degraded slowly with degradation products of 18-, 16-, 14-, 12-, and 10-mers (data not shown). This is consistent with degradation occurring at the unmodified linkage. Based upon this strategy, we have already discovered that an alternating dithioate−phosphate antisense oligomer is highly effective at down-regulating the erbB-2 oncogene (Vaughn et al., unpublished results). Other design strategies should also be examined. One would involve a nuclease-stable oligomer with 5′ and 3′ ends that are "capped" by two or more contiguous dithioate-modified linkages. While oligomers containing stretches of five or more unmodified linkages may be slightly more efficient at promoting RNase H activity, they lack sufficient nuclease resistance to survive extended incubation in nuclease-rich environments. Therefore, perhaps the capped configuration should be combined with alternating dithioate and phosphate linkages in the interior of the oligomer to impart total resistance to endonucleases. The interior portion of such an oligomer should still retain the ability to efficiently direct RNase H activity. The results presented in this study indicate that deoxyoligonucleotide analogs designed with the proper combination of dithioate and phosphate internucleotide linkages could result in antisense agents with favorable properties.

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