Novel Stabilizing Method for Antisense Oligodeoxynucleotides

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INTRODUCTION

The ability of synthetic oligodeoxynucleotides to bind and interfere specifically with complementary sequences of RNA and/or DNA provides a valuable tool by which cellular and viral gene expression can be manipulated (1–3). The specific inhibition is based on the specific Watson–Crick base pairing between the heterocyclic bases of the antisense oligodeoxynucleotide and the cellular or viral nucleic acid. However, antisense oligodeoxynucleotides are often unsuitable for therapeutic application since they are rapidly degraded by 3′-exonucleases in serum and cells (4–6). To prevent enzymatic degradation by various nucleases, chemical modifications of the phosphodiester backbone have been made in oligomers (7–9). These chemical modifications can provide enzymatic stability, but stereochemical (10) and specificity (11) problems have been recognized. Since the introduction of a substituent on the phosphorus generates a new center of chirality and our synthesizer cannot perform stereospecific synthesis, the resulting analogues are a mixture of stereoisomers. These modifications also affect the ability to achieve sequence-specific binding (12), resulting in T_m values lower than those of unmodified oligomers.

Another approach is to stabilize unmodified oligomers. We report herein data on a novel method of stabilizing unmodified oligodeoxynucleotides: Chemically modified oligodeoxynucleotides, which carry complementary sequences of unmodified antisense, can form a double-stranded complex with the ability to stabilize the unmodified oligomer. To determine the potential of this assumption, the stability of the double-stranded oligomer in human plasma is evaluated in this paper. Dissociation of unmodified antisense from the complex and reconstitution of the double-strand with an unmodified sense oligomer were also studied.

MATERIALS AND METHODS

Analytical Instrumentation

A high-performance liquid chromatography (HPLC) system, consisting of a pump with a linear-gradient system (LC-9A, Shimadzu, Kyoto, Japan), a programmable multiwavelength detector (Model 490, Waters, Milford, MA), and a 20-μL fixed loop injector (Model 7125, Rheodyne, Cotati, CA), was used. Ultraviolet absorption spectra were taken on a spectrophotometer (UV-265, Shimadzu).

HPLC Conditions

Chromatography was performed on an anion-exchange column (10 × 50 mm, LiChrospher 4000 DMAE, Merck, Darmstadt, Germany) in a water jacket (Superformance, Merck). The column was eluted first with 20 m*M* Tris–HCl buffer (pH 8.0) and then with a 1.0 *M* NaCl linear gradient from 10 to 100% in 60 min at 1.0 mL/min.

Synthesis and Purification of Modified and Unmodified Oligodeoxynucleotides

A 15-base sequence of the chloramphenicol acetyltransferase (CAT) gene was selected as the target sense. Both types of oligodeoxynucleotides were synthesized by the phosphoroamidite method using an automated synthesizer (Cyclon Plus, Millipore, Burlington, MA). The oligomers were obtained as a dimethyltrithyl (DMT)-on form and purified by an Oligo-Pack (Millipore) cartridge. The purity of the sample was estimated to be more than 97% from a single peak in the HPLC analysis. The samples were evaporated to dryness *in vacuo* and stored at −40°C until used.

Measurement of Melting Temperature (T_m)

Equimolar amounts of antisense and complementary sense (7.5 μ*M*) were hybridized in 70 m*M* phosphate buffer (pH 7.4) containing 70 m*M* NaCl. Melting experiments were carried out in a thermostatically controlled quartz cell (10 mm in thickness). The temperature of the cell was increased from 15 to 60°C at a rate of 1°/min. Dissociation (melting) of the hybridized oligodeoxynucleotides was monitored by simultaneous measurements of the absorbance at 260 nm and the cell temperature.

Hybridization of an Unmodified Antisense:Phosphorothioate Sense Complex with Unmodified Sense

The hybridized unmodified antisense with phosphorothioate sense at a concentration of 7.5 μ*M* in 70 m*M* phosphate buffer (pH 7.4) containing 70 m*M* NaCl was incubated with equimolar unmodified sense or nonsense oligodeoxynucleotides at 37°C. The mixture was cooled to 15°C, then subjected to the dissociation experiments described above.

Enzymatic Degradation of Oligodeoxynucleotides

Either double- or single-stranded oligodeoxynucleotides at a concentration of 15 μ*M* and human plasma (40%) were incubated in 70 m*M* phosphate buffer (pH 7.4) containing 70 m*M* NaCl at 37°C. The changes in concentration were followed by HPLC analysis of samples taken periodically from the mixture.

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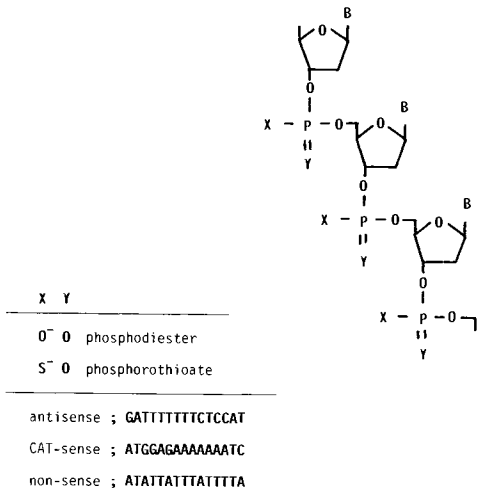

Fig. 1. Chemical structures of oligomers and their sequences.

RESULTS AND DISCUSSION

Figure 1 shows the general structure and sequences of the antisense, sense, and nonsense oligodeoxynucleotides. The 15-base antisense sequence targeted the initial methionine sequence (AUG) of CAT. The unmodified antisense (*N*-anti) formed a double-stranded complex with either unmodified sense (*N*-CAT) or modified phosphorothioate sense (*S*-CAT), and their T_m values were 50.7 and 43.1°C, respectively. This lower T_m for *S*-CAT suggests a decrease in specific binding ability, which may be attributable to the chemical modification. Figure 2 shows the stability of the oligonucleotides in 40% human plasma. The stability of the phosphorothioate derivative (*S*-anti) was about five times higher than that of the unmodified oligomer (*N*-anti), while the unmodified double-stranded complex (*N*-anti:*N*-CAT) displayed a stability comparable to that of the modified single strand (*S*-anti). The greatest stability was observed in the modified double-stranded complex (*N*-anti:*S*-CAT): approximately 40 times and 7 times higher than those of *N*-anti and *S*-anti, respectively.

Though the stability of the unmodified oligodeoxynucleotide was improved by the formation of a double-stranded complex with a modified complementary sequence (*S*-CAT), the unmodified single strands (*N*-anti) should be dissociated

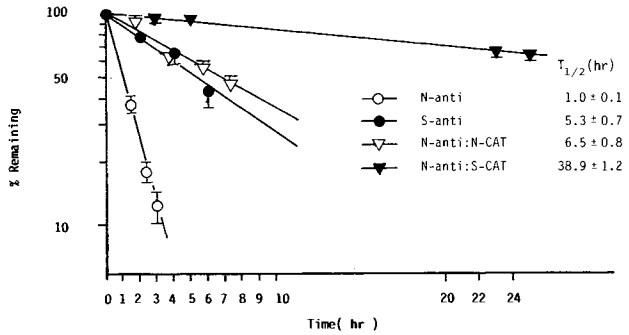

Fig. 2. Stability of anti-CAT oligonucleotides in 40% human plasma at 37°C (n = 3; mean ± SD). (○) Unmodified single strand (*N*-anti); (●) modified single strand (*S*-anti); (▽) unmodified double strand (*N*-anti:*N*-CAT); (▼) modified double strand (*N*-anti:*S*-CAT).

Table I. T_m Values of Double-Stranded Oligodeoxynucleotide Complexes

Complex	Single strand	Incubation	T_m (°C)
N-anti:*N*-CAT	—	—	50.7
N-anti:*S*-CAT	—	—	43.1
N-anti:*S*-CAT	*N*-CAT, equimolar	15°C, 1 min	48.1
N-anti:*S*-CAT	*N*-CAT, double-molar	15°C, 1 min	50.0
N-anti:*S*-CAT	Nonsense, equimolar	15°C, 1 min	43.0

from the complex and reconstitute the double-strand with its target sense to function as an antisense. Table I shows the T_m values of double-stranded complexes. The changes in T_m after incubation with unmodified sense (*N*-CAT) suggests regeneration of *N*-anti from the complex (*N*-anti:*S*-CAT) and subsequent sequence-specific reconstitution of unmodified double-strand (*N*-anti:*N*-CAT). The T_m of the double-stranded complex (43.5°C for *N*-anti:*S*-CAT) increased to 48.1°C in the presence of equimolar *N*-CAT. A further increase in T_m was observed when the molar quantity of *N*-CAT was doubled (50.0°C); this T_m is very close to that of *N*-anti:*N*-CAT as indicated above (50.7°C), though no change in T_m was observed in the presence of unmodified nonsense oligomer.

The above results demonstrate the ability of a modified oligodeoxynucleotide (*S*-CAT) to stabilize an unmodified complementary sequence (*N*-anti), which shows greater binding ability to the target sense than modified antisense. Dissociation of *N*-anti and reconstitution of double strands with unmodified sense (*N*-CAT) were suggested from the changes in T_m values *in vitro;* these phenomena may be attributable to the higher ability of specific binding of *N*-CAT than *S*-CAT to *N*-anti. Though the biological effect of the released modified sense (*S*-CAT) should be investigated in a further study, its effect, if it exists, cannot be specific to any gene expression. The modified oligodeoxynucleotide in this double-stranded complex was employed as a means of stabilization and may have no effect on the binding ability of unmodified antisense (*N*-anti) to the target sense. Further modification of the stabilizer to gain additional functions such as tissue targeting and/or carrier transmission through the cell membrane is an avenue of future research.

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