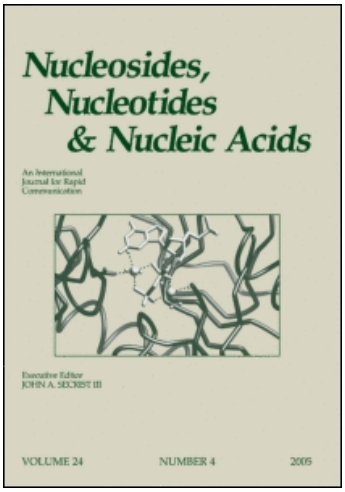

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TARGETED GENE CORRECTION: SYNTHESIS AND CHARACTERIZATION OF DOUBLE-HAIRPIN 2'-O-METHYL RNA/DNA CHIMERA OLIGONUCLEOTIDES

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ABSTRACT A series of 2'-O-methyl RNA/DNA chimera oligonucleotides were synthesized with a double-hairpin structural motif. Liposome formulated delivery of the chimeras effected targeted, high conversion of mutant alleles in mammalian cell culture. The chimera oligonucleotides were prepared with DNA and 2'-OMe RNA phosphoramidite nucleoside monomers on the ABI 394 synthesizer.

Oligonucleotides bearing 2'-O-methyl RNA and DNA nucleobases in a self-complementary, double-hairpin structure can effect targeted, point mutation correction.[1] After simple liposome formulation, a chimera oligonucleotide was introduced into lymphoblastoid cells homozygous for the hemoglobin β^S mutant allele. Up to 30% gene conversion to normal sequence was achieved.[2] Similarly targeted correction in a point mutation in the gene encoding for mammalian alkaline phosphatase on an extrachromosomal plasmid was demonstrated.[3] This gene therapy approach circumvents many of the problems associated with retroviral vector assembly and packaging of transduction genes, and eliminated the need for promoter and regulatory sequences that would otherwise be required. The chimera sequences are designed to adopt a duplex conformation with double hairpins of 2'-O-methyl RNA/DNA stems and poly-T hairpin loops. The 2'-O-methyl RNA regions flank a DNA target sequence. The chimeras hybridize at the

mutant locus and encode for the intended nucleotide change by an homologous recombination event. For example the 68mer chimera in Figure 1 was introduced to CHO (Chinese Hamster Ovary) cells previously transfected with p711, a plasmid containing an alkaline phosphatase cDNA insert with a single point mutation at position 711. At 11 nM, the chimera promoted the conversion of approximately 30% of the transfected cells from mutant to wild-type phenotype.[2]

The 2'-O-methyl ribonucleosides are present in many types of RNA, and are formed by post-transcriptional processing in bacteria and eucaryotes.[4] Oligonucleotides bearing 2'-O-methyl bases have been studied as ribozymes, nuclease-resistance antisense analogs, and other cellular mechanism probes.[5] Desirable features of 2'-O-alkyloligoribonucleotides include high chemical stability, substantial RNA- and DNA-nuclease resistance (including RNase H), and increased thermal duplex stability.

Efficient and selective 2' methylation of the ribonucleosides has facilitated the commercial availability of the 2'-O-methyl RNA phosphoramidite monomers.[6] Chimera oligonucleotides were synthesized with DNA (dA^{bz}, dG^{dmf}, dC^{bz}, T) and 2'-O-Me RNA (A^{bz}, G^{dmf}, C^{bz}, U) phosphoramidite nucleoside monomers (Figure 2).[7] The eight monomers are auto-diluted with dry acetonitrile (<50 ppm H_2O) and have useful lifetimes on the synthesizer of 2-3 weeks.[8] For each cycle at the .2 μmole scale, 40 μl of 0.1 M phosphoramidite nucleoside (ca. 3.5 mg) in acetonitrile was delivered concurrently with 120 μl of 0.5 M 5-H tetrazole in acetonitrile for coupling. Synthesis and cleavage from the solid support are automated without interruption, utilizing all 8 monomer positions and specific 25 second (DNA) and 4 minute (2'-OMe RNA) coupling times.

Synthesis efficiency was measured in real-time with the AutoAnalysis trityl conductivity monitor and generally exhibited >98% average stepwise yield.[9] High-cross link, 1000Å pore diameter polystyrene[10], loaded at 12 μmole 3' nucleoside/gm support, was used to generate about 40 crude odu of chimera at the .2 μmole scale (ca. 1.6 mg). Scale-up to 1 μmole gave 200 crude odu (ca. 8 mg) with 1000Å, 3' nucleoside CPG support. The nucleobase protecting groups for A, G, and C were selected for comparable deprotection rates in concentrated ammonium hydroxide (1 hour at 65 °C).[11] After cleavage and deprotection, chimeras up to 80 bases in length were routinely attained in high purity and yield.

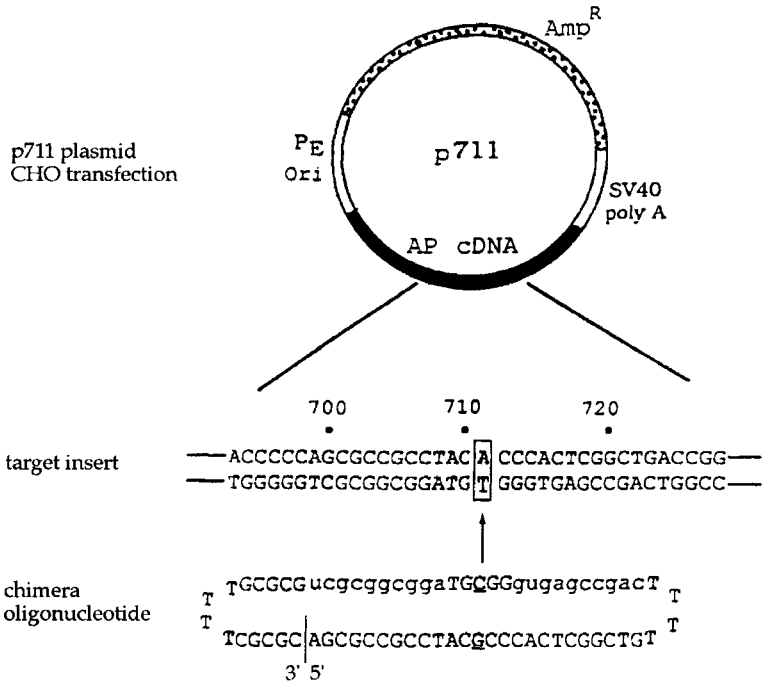

FIG. 1. Episomal targeting of CHO cells transfected with p711 containing point mutation at bp 711 in AP cDNA (solid box); SV40 early promoter (P_E), SV40 origin of replication (ori), polyadenylation addition site and small intron sequence for splicing (SV40 poly A) (open box); and pBR322 with origin of replication and β-lactamase (Amp^R) gene (dotted box). Conversion by the chimera oligonucleotide (small case letters represent 2'-OMe positions) to wild-type of the target insert was monitored by biochemical and DNA sequence levels, spectrophotometry, histochemical staining and analysis of Hirt DNA.[2]

Single-base resolution is challenging in the analysis and purification of such long oligonucleotides. The added complexity of multiple, stable conformations due to intramolecular hydrogen-bonding demanded denaturing conditions provided by elevated temperature, high pH, and/or denaturants. For example, under the non-denaturing conditions of reverse-phase HPLC, multiple conformations are evident in the trityl-off chromatogram (Figure 3) of a purified 68mer chimera. Slab polyacrylamide gel electrophoresis (PAGE) with 7 M urea was the method of choice for analysis and purification.[13] Several odu (≅ 100 μg) can be isolated from electrophoresing 10-20 crude odu on a 3mm thick gel, excising the band after visualization under UV light against a TLC plate, soaking in water overnight

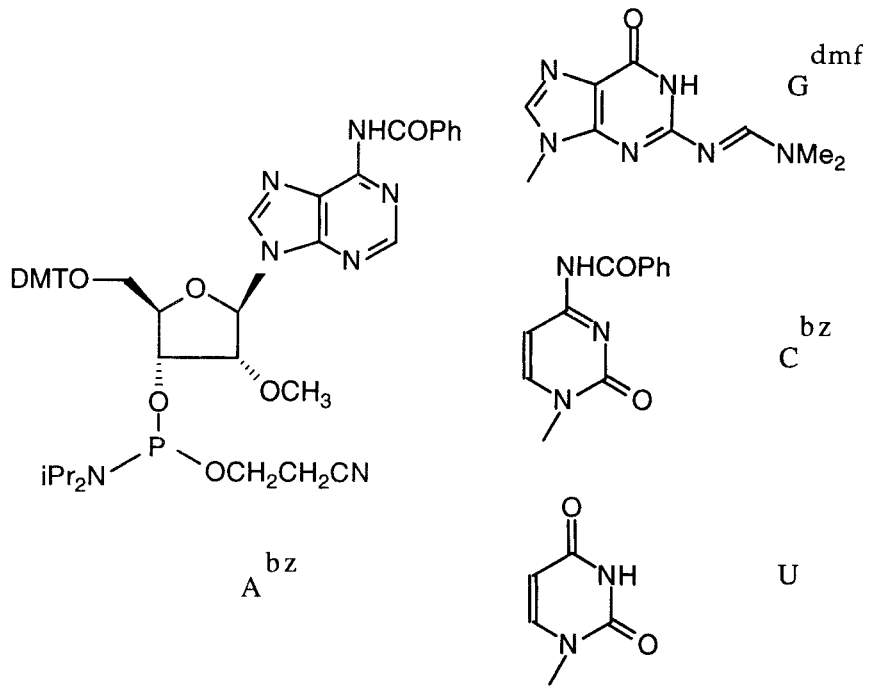

FIG. 2. 2'-O-methyribonucleoside phosphoramidite monomers

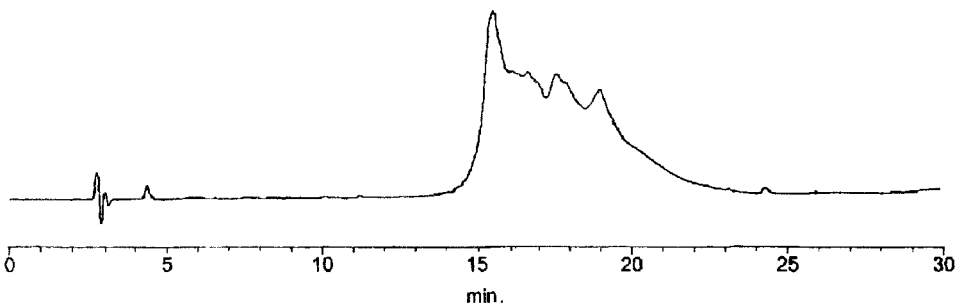

FIG. 3. Reverse-phase HPLC analysis of PAGE purified 68 chimera 5' ACC TGA CTC CTG AGG AGA AGT CTG CTT TTg cag acu ucu CCT CAg gag uca ggu GCG CGT TTT CGC GC 3' (lower case = 2'-OMe RNA). Applied Biosystems Aquapore RP-300 C-8, 4.1 x 220 mm. Mobile phase (pH 7): A - 0.1 M TEAA. B - CH_3CN. Flow rate: 1.0 ml/min. Gradient: 8% B at 0 min.; 24% B at 24 min.; 40% B at 30 min.

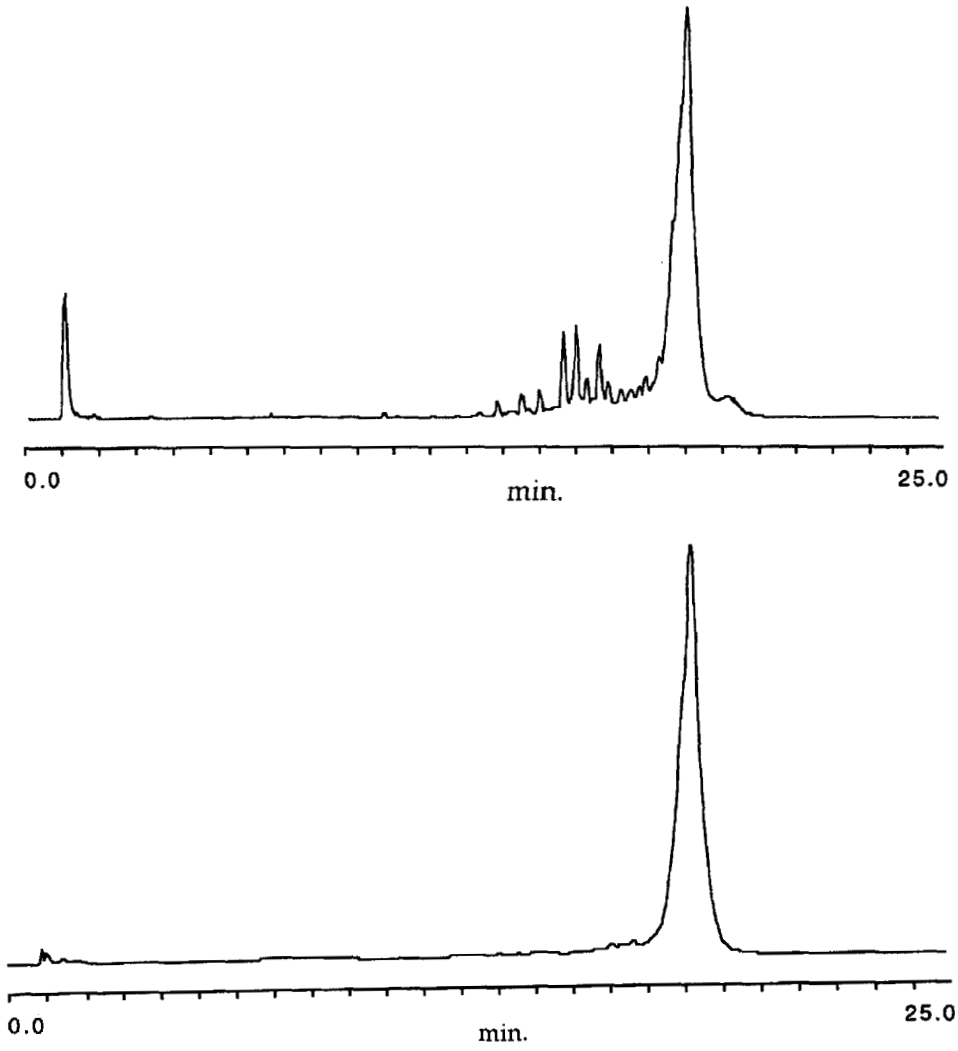

FIG. 4. Anion-exchange HPLC of crude (top) and PAGE purified (bottom) 68mer chimera. 5' ACC TGA CTC CTG AGG AGA AGT CTG CTT TTg cag acu ucu CCT CAg gag uca ggu GCG CGT TTT CGC GC 3' (lower case = 2'-OMe RNA). Dionex NucleoPac PA-100, 4 x 250 mm. Mobile phase (pH 12): A - 100mM NaCl, 10mM NaOH in 10% acetonitrile. B - 800mM NaCl, 10mM NaOH in 10% acetonitrile. Flow rate: 1.0 ml/min. Gradient: 0% B at 0 min.; 80% B at 25 min.

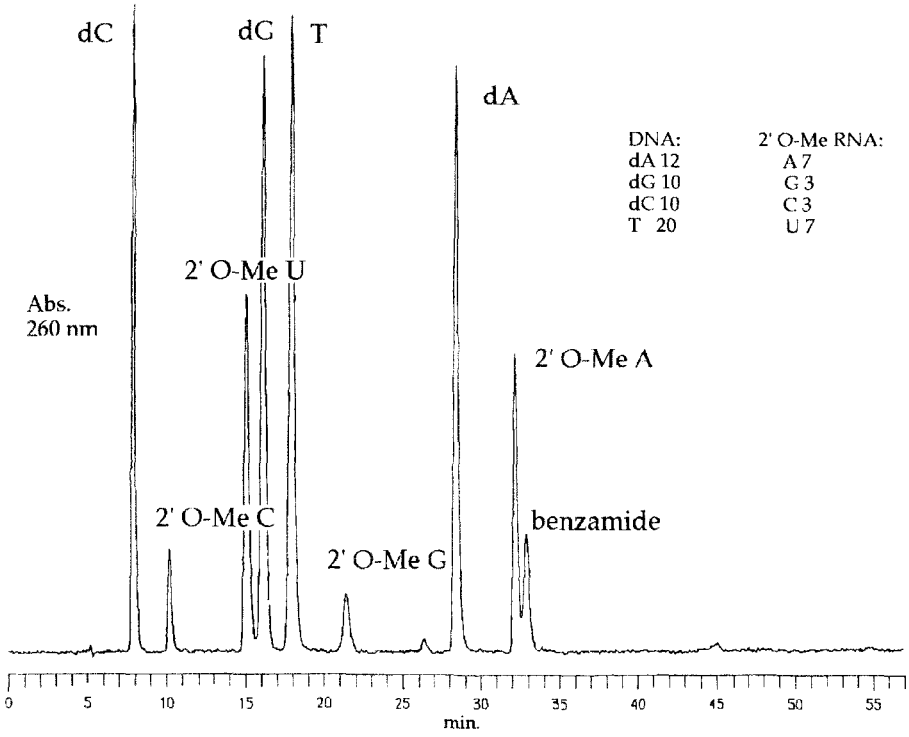

FIG. 5. HPLC - enzymatic digest analysis of crude 72mer chimera, Applied Biosystems Spheri-5™ C18, 4.1 x 250 mm. Mobile phase: A - 3% CH_3CN in 0.1M TEAA. B - 90% CH_3CN in H_2O. Flow rate: 0.5 ml/min. Gradient: 0% B at 10 min.; 10% B at 35 min.; 100% B at 60 min. Nucleoside composition of the chimera was assessed by digestion of the chimera (0.8 odu) with snake venom phosphodiesterase (0.1 U), bacterial alkaline phosphatase (0.7 U), 15mM $MgCl_2$, 30mM Tris, pH 7.5 at 37°C for 12 hours. The HPLC samples were ethanol precipitated twice, the supernatant dried thoroughly, and dissolved in H_2O.

at room temp., and desalting/concentrating on an OPC cartridge.[14] Scale-up purification by PAGE beyond this level is not feasible, so high-resolution, preparative HPLC methods have been developed. Anion-exchange HPLC on a polymeric adsorbent that endures high pH provides good resolution, predictable elution patterns, and reproducible retention times (Figure 4).[12]

Oligonucleotides were checked for incomplete deprotection, base modifications, and correct base composition by enzymatic digestion and HPLC separation of the constituent nucleosides.[13,15] All 8 nucleosides could be resolved and assigned by reverse-phase HPLC analysis (Figure 5). The quantitated base composition of the chimeras were in close agreement (<10%

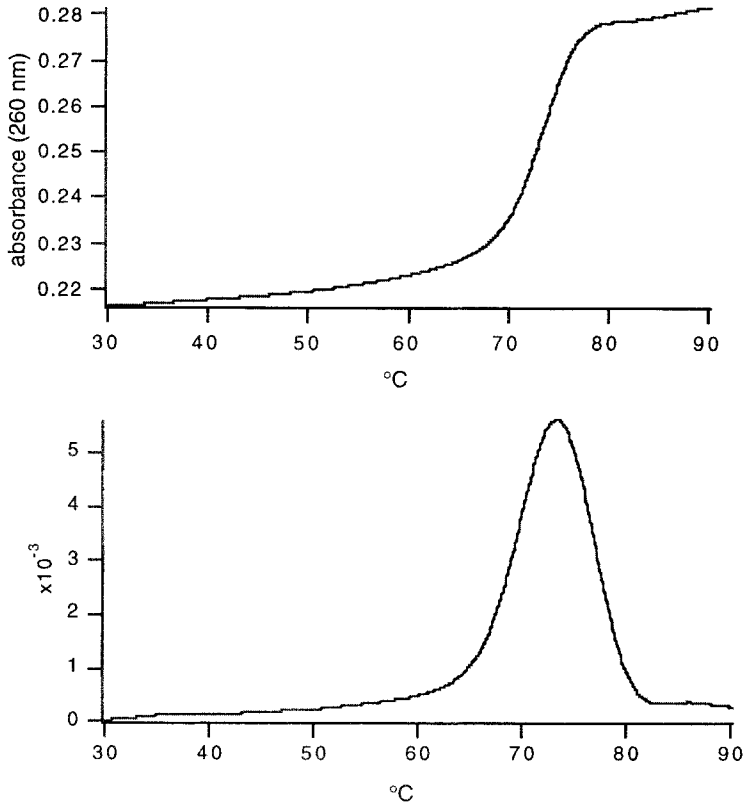

FIG. 6. Absorbance vs. temperature melting curve for a 68-mer chimera (top panel), and the corresponding first-derivative curve (bottom panel). Tm studies were conducted with a Perkin-Elmer Lambda 12 spectrometer equipped with a PC-controlled Peltier heating unit and operated with UV-WinLab and UV-TempLab software. The buffer contained 10 mM HEPES, pH 7.3, and 25 mM NaCl. Oligonucleotide concentration was in the low micromolar range. The helix-coil transition was monitored at 260 nm by heating from 30 - 90°C with a heating rate of 0.5 °C min^{-1}.

sum error) with the sequence using known and estimated extinction coefficients. There were no detectable base modifications or residual protected deoxynucleosides.

Thermal UV melting experiments determined Tm values from the differentiated melting curves. All of the melting curves exhibited a single, sharp transition consistent with a simple two-state model (Figure 6). The double-hairpin structural motif contains two domains, or helical regions, that melt independently of each other. The shorter helix (5 bp) closed by a T-4

Entry	Sequence	DNA	2'-OMe	Delta
I	T T **GGAGGCCAGG**GTCGG**TCTCCGTT** T T T T CCTCCGGTCCCAGCCAGAGG CAA T	80.0	85.8	**+5.8**
II	T T **GGAGGCCAGG**GGTAG**TCTCCGTT** T T T T CCTCCGGTCCCCATCAGAGG CAA T	76.3	83.8	**+7.5**
III	T T **CTGGAACATG**TACACCTG**GGGGGAAAGCC**GCGCG T T T T GACCTTGTACATGTGGACCCCCTTTCGG CGCGC T T	71.3	76.8	**+5.5**
IV	T T **CTGGAACATG**TACCTG**GGGGGAAAGCC**GCGCG T T T T GACCTTGTACATGGACCCCCTTTCGG CGCGC T T	71.0	77.4	**+6.4**
V	T T **GACGGGGACT**CACAG**GAGATGCAGG**GCGCG T T T T CTGCCCCTGAGTGTCCTCTACGTCC CGCGC T T	73.5	80.8	**+7.3**
VI	T T **GACGGGGACT**CATAG**GAGATGCAGG**GCGCG T T T T CTGCCCCTGAGTATCCTCTACGTCC CGCGC T T	71.8	78.1	**+6.3**
VII	T T **AAACCAGAAT**GACAA**AAATGGAGAG**GCGCG T T T T TTTGGTCTTACTGTTTTTACCTCTC CGCGC T T	62.4	69.2	**+6.8**

FIG. 7. Comparison of Tm values for a series of chimeras and the corresponding all-DNA sequences. Bold letters represent 2'-OMe residues. Arrows indicate a nick in the bottom strand, with the 3'-terminus to the right of the arrow. All values are in °C and experimental conditions are as described in Fig. 6.

hairpin loop melts below room temperature in the indicated buffer system, whereas the longer helix (ca. 25 bp) closed by the same T-4 hairpin melts between 62 - 85 °C. The actual melting temperature is dictated by the sequence, base composition, and the identity of the carbohydrate (i.e., 2'-deoxy or 2'-OMe). The 2'-OMe substitutions increase the thermal stability of the duplex by altering the helix structure. The chimera provides a 5.5 - 7.5 °C stabilization over the corresponding all-DNA sequence (Figure 7). The greater stability of the 2'-OMe/DNA heteroduplex over the corresponding DNA/DNA homoduplex may be critical for high-efficiency gene correction during the homologous recombination event, where the 2'-OMe regions must hybridize with the target sequence. The relationship between the Tm of the chimera and its gene correction frequency remains to be determined.

TABLE 1. Comparison of measured and predicted Tm values for a series of chimeras and the corresponding all-DNA sequences. Predicted Tms assume 25 mM sodium, 1 μM oligonucleotide, and do not include the T-4 hairpin loops or the 5 bp region that melts independently. All values are in °C.

	Measured Tm		Predicted Tm		
Entry	DNA	2'-OMe	Maniatis	composition	stacking
I	80.0	85.8	60.7	70.0	58.6
II	76.3	83.8	58.6	68.0	53.3
III	71.3	76.8	60.5	88.0	64.1
IV	71.0	77.4	59.3	82.0	61.8
V	73.5	80.8	61.1	82.0	62.5
VI	71.8	78.1	59.5	80.0	59.6
VII	62.4	69.2	49.7	68.0	51.6

Unfortunately, the currently available Tm prediction methods are not suitable for forecasting an accurate Tm of the oligonucleotide chimeras. An accurate predictive method must account for the thermodynamic contributions of the stem loops and the self-complementary nature of the sequence in addition to each individual nearest-neighbor interaction. Although this information is available for DNA and RNA sequences, the necessary data matrices have not yet been compiled for analogs such as 2'-OMe RNA. Although the experimental approach is much more time consuming than inputting sequence information into a formula, it has the added benefit of allowing one to determine the true Tm in any given buffer, metal, or salt concentration. Table 1 compares the experimentally determined Tm values with three popular predictive methods.[16]

The first two entries in Table 1 exhibit particularly high Tm values. This could be due to the formation of a secondary structure other than a double-hairpin. An internal loop structure (bimolecular complex) is one alternative that must be considered (Figure 8). The distribution of each

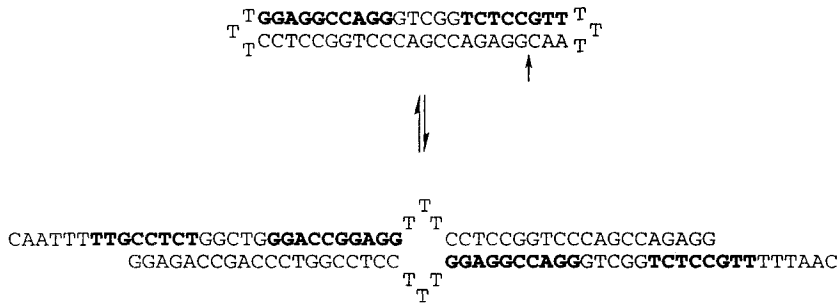

FIG. 8. Double-hairpin vs. an alternative internal loop secondary structure.

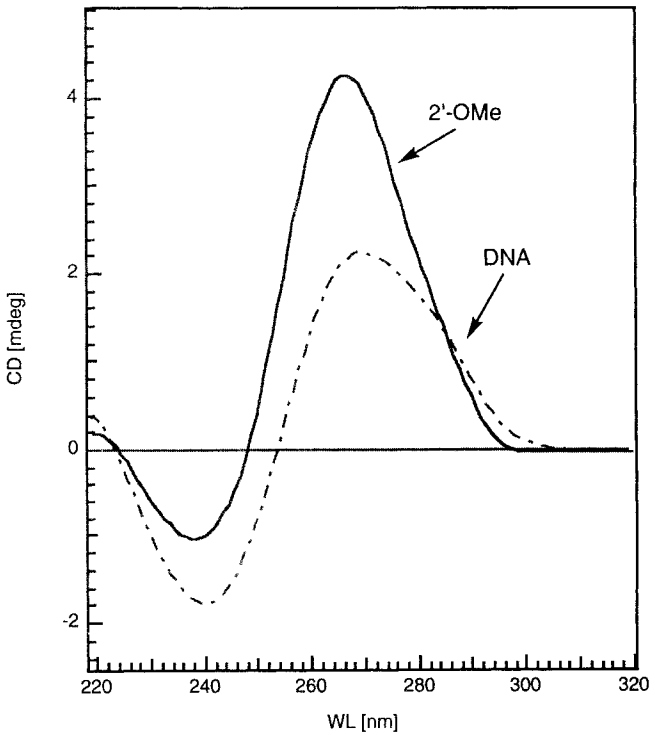

FIG. 9. CD spectrum of 68-mer chimera (solid line) and the corresponding all-DNA sequence (dashed line). Spectra were obtained with a Jasco J-600 spectrapolarimeter. Measurements were made with a 2 mm pathlength cell at 25 °C. Oligonucleotide samples were in 10 mM HEPES, pH 7.3, and 25 mM NaCl.

structure will be dictated by the relative thermodynamic stability of the single hairpin vs. the internal loop. Entries I and II contain T-3 hairpin loops as opposed to T-4 loops. The presence of the less stable T-3 hairpin loop (compared to a T-4 loop) could shift the structural equilibrium to favor the bimolecular complex (Figure 8). However, non-denaturing gel electrophoresis did not support this idea, and instead showed only a single species whose mobility was consistent with the double-hairpin structure.

Circular dichroism was used to evaluate the conformation of the double-hairpin motif. The CD spectrum of the all-DNA sequence exhibits features that are consistent with a B-type helix; namely, equal intensity positive and negative peaks at 270 and 240 nm, respectively (Figure 9).[17] In contrast, the spectrum of the 2'-OMe chimera shows features more in line with an A-type helix. It is well known that DNA/RNA heteroduplexes adopt an A-type helical structure due to the conformational restrictions imposed upon the carbohydrate by the 2'-OH group; the 2'-OMe substituent would impose similar restrictions.[18]

Systematic structural modifications of high-conversion chimeras are in progress. Nucleobase and internucleotide analogs which optimize self-complementarity have been synthesized and will be reported separately. Data from thermal melting experiments, CD spectroscopy, and other biophysical techniques will be used in an effort to correlate physical properties of the chimeras with their gene-conversion efficiency and to help elucidate the mechanism of homologous recombination.

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