Phosphorothioate Oligonucleotide-Directed Triple Helix Formation†

Joseph G. Hacia, Barbara J. Wold, and Peter B. Dervan*

Divisions of Biology and of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

Received February 4, 1994; Revised Manuscript Received March 7, 1994®

ABSTRACT: Phosphorothioate oligodeoxyribonucleotides were tested for their ability to recognize double-helical DNA in two distinct triple helix motifs. Purine-rich oligonucleotides containing a diastereomeric mixture of phosphorothioate or stereoregular (all R_P) phosphorothioate linkages are shown to form triple-helical complexes with affinities similar to those of the corresponding natural phosphodiester oligonucleotides. In contrast, pyrimidine-rich phosphorothioate oligonucleotides containing a mixture of diastereomeric or stereoregular (all R_P) linkages do not bind to double-helical DNA with measurable affinity. These observations have implications for triple helix structure and for biological applications.

Oligonucleotide-directed triple helix formation is a powerful chemical approach for the sequence-specific recognition of double-helical DNA. Two distinct structural motifs are known to exist for triple helix formation. Pyrimidine-rich oligonucleotides bind in a parallel orientation to purine-rich strands in double-helical DNA through the formation of specific Hoogsteen hydrogen bonds (T·AT and C^+·GC base triplets) in pyrimidine–purine–pyrimidine triple helices (Moser & Dervan, 1987; Le Doan et al., 1987; Rajagopal & Feigon, 1989; de los Santos et al., 1989). Alternatively, purine-rich oligonucleotides bind in an antiparallel orientation relative to purine-rich strands in double-helical DNA through the formation of reverse Hoogsteen hydrogen bonds (G·GC and T·AT or A·AT base triplets) in purine–purine–pyrimidine triple helices (Beal & Dervan, 1991; Chen, 1991; Pilch et al., 1991; Durland et al., 1991; Radhakrishnan et al., 1991, 1993; Radhakrishnan & Patel, 1993a).

Phosphorothioate internucleotide linkages have been among the most widely examined oligonucleotide backbone modifications in both biophysical and pharmacological studies (Stein & Cheng, 1993). Although they possess many favorable attributes such as increased stability to cellular nucleases, phosphorothioate modifications introduce a chiral center at each internucleotide linkage that can lead to the formation of 2^n diastereomers per n internucleotide linkages. Interest in synthesizing oligonucleotides with stereoregular phosphorothioate linkages has been encouraged by biophysical studies that suggest phosphorothioate oligonucleotides exclusively containing all R_P or S_P diastereomer linkages have different affinities for single-stranded and double-stranded nucleic acids (Cosstick & Eckstein, 1985; LaPlanche et al., 1986; Latimer et al., 1989; Kim et al., 1992).

We describe the results obtained from quantitative DNase I protection studies examining the ability of oligonucleotides containing diastereomeric mixtures of phosphorothioate linkages to form triple-helical complexes in both the pyrimidine and purine motifs. Furthermore, we report a method for the enzymatic production of pure (R_P)-phosphorothioate oligonucleotides based on the work of Eckstein and co-workers and the effectiveness of these nonnatural nuclease-resistant oligonucleotides for triple helix formation.

MATERIALS AND METHODS

Materials. All DNA synthesis reagents, including tetraethylthiuram disulfide, were obtained from Applied Biosystems Inc. except for uridine phosphoramidite obtained from BioGenex. All restriction enzymes, T4 DNA ligase, and DNase I were purchased from Boehringer Mannheim. Sequenase 2.0 was obtained from U. S. Biochemicals. The S_P isomers of 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphates) were obtained from Amersham.

Synthesis of Oligonucleotides and Plasmid Construction. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer using β-cyanoethyl phosphoramidite chemistry. Oligonucleotides containing a diastereomeric mixture of phosphorothioate linkages were prepared using β-cyanoethyl phosphoramidite chemistry and oxidation with tetraethylthiuram disulfide (Vu & Hirschbein, 1991). Oligonucleotides were purified on 20% denaturing polyacrylamide gels. The plasmid pTER I was obtained by cloning the oligonucleotides 5′-pTCGACTTTTTTTCTTTTCTTTCT-TTTTTTTTTTTGGCGCATG-3′ and 5′-pCGCCAAAA-AAAAAAAAGAAAGAAAAAGAAAAAAAG-3′ into the large *Sal*I-*Sph*I restriction fragment of pUC19. The plasmid pTER 3 was obtained by cloning the oligonucleotides 5′-pGGGAAAAAGAGAGAGAGAGCTGCAGGCGG-CCGCCACTAGTGAGGGAGGGGAGG GGAGGGAG-CTGCT-3′ and 5′-pCTAGAGCAGCTCCCTCCCCTC-CCCTCCCTCACTAGTGGCGGCCGCCTGCAG-CTCTCTCTCTCTTTTTCCC-3′ into the large *Sma*I–*Xba*I restriction fragment of pTER I.

DNase I Footprinting. Either the 438-bp *Eco*RI-*Afl*III restriction fragment of pTER I or the 338-bp *Hin*dIII-*Nar*I restriction fragment of pTER 3 was 3′-end-labeled with deoxyadenosine [α-^{32}P]triphosphate (New England Nuclear) and chemically sequenced (Sambrook et al., 1989). Labeled DNA (30 000 cpm) and oligonucleotide were incubated in either association buffer I (Table 1) or association buffer II (Table 2) for 24 h at 25 °C. A 1-μL volume of DNase I (0.1 unit/μL in 10 mM $MgCl_2$, 10 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.0) was added to the reaction, and the mixture was incubated for 3 min at 25 °C before termination by ethanol precipitation and resuspension in formamide/TBE loading

† Supported by a predoctoral fellowship from the Howard Hughes Medical Institute (to J.G.H.) and by research grants from the NIH (AR40780 to B.J.W. and GM35724 to P.B.D.).

* To whom correspondence should be addressed at the Division of Chemistry and Chemical Engineering.

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

0006-2960/94/0433-5367$04.50/0

buffer. Samples were analyzed by electrophoresis on 8% denaturing polyacrylamide gels. Gels were dried and placed on film or photostimulable phosphorimaging plates and quantitated as described (Singleton & Dervan, 1992).

Synthesis of Stereoregular Phosphorothioate Oligonucleotides. DNA template **R1** (5′-ACCCACCCCACCCCAC-CCAAGGGTTGATGGAAGG-3′) or **Y1** (5′-AAAAAAA-GAAAAAGAAAAGGGTTGATGGAAGG-3′) (5 nmol) was added to 5 nmol of oligonucleotide primer **S1** (5′-CCTT-CCATCAACCCrUT-3′) in a volume of 20 μL of 1× sequenase buffer. The mixture was heated to 80 °C for 2 min prior to slow cooling to 37 °C. The reaction was brought up to a final volume of 100 μL in 1× sequenase buffer containing 4 mM DTT with the addition of 1 mM deoxyguanosine 5′-(α-thiotriphosphate) and 1 mM thymidine 5′-(α-thiotriphosphate) in the case of template **R1** or 1 mM thymidine 5′-(α-thiotriphosphate) and 1 mM deoxycytidine 5′-(α-thiotriphosphate) in the case of template **Y1**. Sequenase 2.0 (10 units) was added, and the reaction was allowed to proceed for 5 h at 37 °C. The reaction was terminated by ethanol precipitation, and the mixture was dissolved in 500 μL of ammonium hydroxide solution and incubated at 55 °C overnight. The DNA products were dried and resuspended in formamide/TBE loading buffer before purification on a 20% denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Two different structural motifs previously defined for phosphodiester oligonucleotides were considered in analyzing the effect of phosphorothioate internucleotide linkages on triple helix formation. The sequences of the triple-helical target sites are given in Figure 1. The effects of changing the backbone from phosphodiester to either mixed diastereomeric or pure (R_P)-phosphorothioate were tested for each sequence by measuring target occupancy with a quantitative DNase I footprinting assay.

Oligonucleotides containing exclusively (R_P)-phosphorothioate linkages were made according to the scheme in Figure 2. All reported DNA polymerases accept only the S_P isomers of 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphates), producing the R_P linkage by inversion of configuration at phosphorus (Burgers & Eckstein, 1979; Romaniuk & Eckstein, 1982; Brody et al., 1982; Eckstein, 1985). A primer extension system was designed with a DNA primer containing a single ribonucleotide as the penultimate residue. Extension in the presence of 2′-deoxynucleoside 5′-*O*-(1-thiotriphosphates) yields a full-length duplex with one strand the DNA template and the other strand the elongated primer containing (R_P)-deoxyribophosphorothioate internucleotide linkages. Treatment of this chimeric duplex with base cleaves the labile ribophosphodiester linkage to separate the DNA primer from the stereoregular (R_P)-phosphorothioate oligonucleotide extension product. Denaturing polyacrylamide gel electrophoresis separates the three strands to isolate the stereoregular (R_P) phosphorothioate oligonucleotide.

Quantitative DNase I footprint titration experiments reveal that purine-rich stereoregular (R_P) or diastereomeric mixtures of phosphorothioate linkages had similar affinity for the purine target site relative to the phosphodiester oligonucleotide (Table 1). In sharp contrast, neither pure R_P nor diastereomeric phosphorothioate pyrimidine oligonucleotide had measurable affinity for the homopurine target site, which differs markedly from the energetics of the corresponding phosphodiesters (Table 2).

Although we find that triple helix formation by phosphorothioate oligonucleotides appears to be energetically favorable

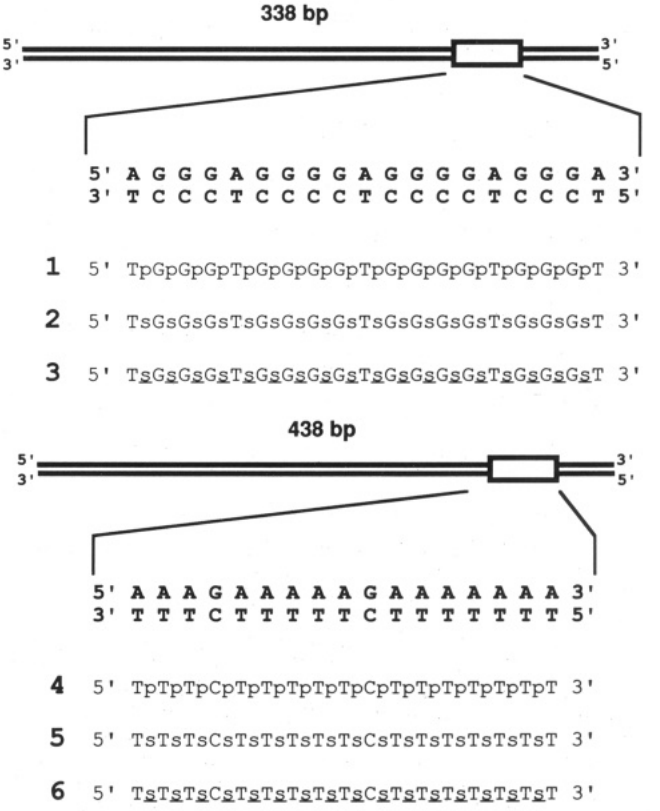

FIGURE 1: (A, top) Sequence of the 19-bp purine motif target site along with the relative position of the target site within the radiolabeled duplex and oligonucleotides tested. Key: p = phosphodiester, s = phosphorothioate mixed diastereomers, and s = phosphorothioate R_P diastereomer. (B, bottom) Sequence of the 17-bp pyrimidine motif target site along with the relative position of the target site within the radiolabeled duplex and oligonucleotides tested. Key: p = phosphodiester, s = phosphorothioate mixed diastereomers, and s = phosphorothioate R_P diastereomer.

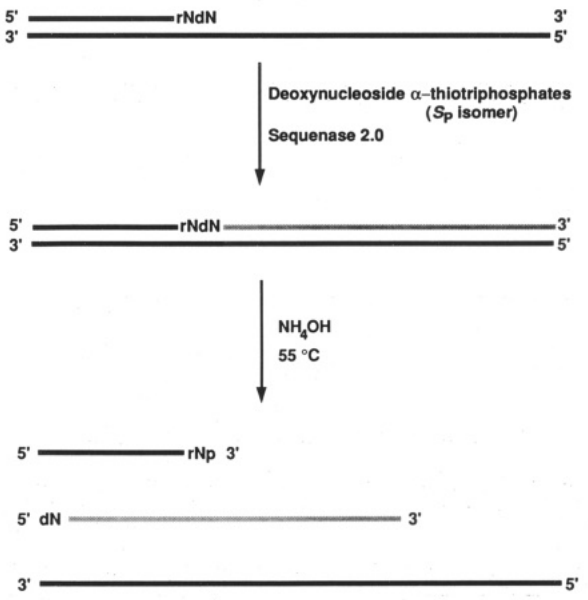

FIGURE 2: Synthetic scheme for the stereoregular (R_P) phosphorothioate oligonucleotide synthesis. N refers to any base complementary to the template.

for use in the purine motif, our results do not exclude the possibility that either a stereoregular (S_P) pyrimidine oligonucleotide or phosphorothioate oligonucleotides with modified pyrimidines that enhance affinity can bind double-helical DNA. However, other studies demonstrate that the introduction of a single (S_P)-phosphorothioate linkage in a

Table 1: Equilibrium Association Constants for Purine Motif[a]

oligonucleotide	K (M^{-1})	ΔG (kcal mol^{-1})
1	1.6 (±0.2) × 10^7	−9.8 (±0.1)
2	2.8 (±0.3) × 10^7	−10.2 (±0.1)
3	2.6 (±0.3) × 10^7	−10.1 (±0.1)

[a] Values reported in the table are mean values measured from four repetitions of DNase I titration experiments performed in association buffer I (20 mM NaCl, 100 μM spermine, 25 mM Tris–OAc, pH 7.2, 25 °C).

Table 2: Equilibrium Association Constants for Pyrimidine Motif[a]

oligonucleotide	K (M^{-1})	ΔG (kcal mol^{-1})
4	4.9 (±0.6) × 10^7	−10.5 (±0.2)
5	<1 × 10^5	
6	<1 × 10^5	

[a] Values reported in the table are mean values measured from four repetitions of DNase I titration experiments performed in association buffer II (20 mM NaCl, 20 mM $MgCl_2$, 100 μM spermine, 50 mM Tris-HCl, pH 7.2, 25 °C).

pyrimidine oligonucleotide dramatically decreases its ability to form a triple helix (Kim et al., 1992).

Model building and NMR studies suggest that the placement of the third strand oligonucleotide in the major groove of DNA differs for Pu·Pu·Py and Py·Pu·Py triple-helical structures. The Pu·Pu·Py third strand phosphodiester backbone appears to be only slightly displaced toward the purine strand of the Watson–Crick double helix (Radhakrishnan & Patel, 1993b) while the Py·Pu·Py third strand oligonucleotide shows a more pronounced displacement toward the purine-rich Watson–Crick strand. It is possible that the replacement of either the *pro-*R_P or *pro-*S_P oxygen atoms for a larger sulfur atom leads to steric crowding in the Py·Pu·Py triple helix. Alternatively, since hydrogen bonds to sulfur are likely weaker than those to oxygen, it is possible that the phosphorothioate modifications disturb specific backbone hydrogen-bonding interactions such as localized binding of water in the major groove (Cosstick & Eckstein, 1985). Furthermore, the charge distribution on the phosphodiester backbone is changed in the phosphorothioate linkages. The major resonance form of the phosphorothioate backbone appears to be one in which the phosphorus nonbridging oxygen bond order is close to 2 and sulfur carries a full negative charge (Frey & Sammons, 1985). Coulombic repulsion due to phosphorothioate substitutions could possibly be energetically destabilizing in the pyrimidine motif. Moreover, the increased polarizability of sulfur relative to oxygen may alter the specificity of stabilizing cations binding to the phosphorothioate internucleotide linkages. These structural implications are nonexclusive, and the relative contributions of each to triplex stability will require further studies. The distinction between the effects of the phosphorothioate backbone in the pyrimidine versus purine motifs is sufficiently large that we expect it to generalize to other target sequences and their corresponding oligonucleotides. However, the modest differences between phosphodiester and phosphorothioate oligonucleotides in the purine motif suggest that sequence composition effects must be examined further. In conclusion, these observations give guidance for the use of phosphorothioate oligonucleotides in *in vivo* biological settings.

REFERENCES

Beal, P. A., & Dervan, P. B. (1991) *Science 251*, 1360–1363.

Brody, R. S., Adler, S., Modrich, P., Stec, W. J., Leznikowski, Z. J., & Frey, P. A. (1982) *Biochemistry 21*, 2570–2572.

Burgers, P. M. J., & Eckstein, F. (1979) *J. Biol. Chem. 254*, 6889–6893.

Chen, F.-M. (1991) *Biochemistry 30*, 4472–4479.

Cosstick, R., & Eckstein, F. (1985) *Biochemistry 24*, 3630–3638.

de los Santos, C., Rosen, M., & Patel, D. (1989) *Biochemistry 28*, 7282–7289.

Durland, R. H., Kessler, D. J., Gunnell, S., Duvic, M., Pettitt, B. M., & Hogan, M. E. (1991) *Biochemistry 30*, 9246–9255.

Eckstein, F. (1985) *Annu. Rev. Biochem. 54*, 367–402.

Frey, P. A., & Sammons, R. D. (1985) *Science 228*, 541–545.

Kim, S.-G., Tsukahara, S., Yokoyama, S., & Takaku, H., (1992) *FEBS Lett. 314*, 29–32.

LaPlanche, L. A., James, T. L., Powell, C., Wilson, W. D., Uznanski, B., Stec, W. J., Summers, M. F., & Zon, G. (1986) *Nucleic Acids Res. 14*, 9081–9093.

Latimer, L. J. P., Hampel, K., & Lee, J. S. (1989) *Nucleic Acids Res. 17*, 1549–1561.

Le Doan, T., Perrouault, L., Praseuth, D., Habhoub, N., Decout, J.-L., Thuong, N. T., Lhomme, J., & Helene, C. (1987) *Nucleic Acids Res. 15*, 7749–7760.

Moser, H. E., & Dervan, P. B. (1987) *Science 238*, 645–650.

Pilch, D. S., Levenson, C., & Shafer, R. H. (1991) *Biochemistry 30*, 6081–6087.

Radhakrishnan, I., & Patel, D. J. (1993a) *J. Am. Chem. Soc. 115*, 1615–1617.

Radhakrishnan, I., & Patel, D. J. (1993b) *Structure 1*, 135–152.

Radhakrishnan, I., de los Santos, C., & Patel, D. J. (1991) *J. Mol. Biol. 221*, 1403–1418.

Radhakrishnan, I., de los Santos, C., & Patel, D. J. (1993) *J. Mol. Biol. 234*, 188–197.

Rajagopal, P., & Feigon, J. (1989) *Nature 339*, 637–640.

Romaniuk, P. J., & Eckstein, F. (1982) *J. Biol. Chem. 257*, 7684–7688.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Singleton, S. F., & Dervan, P. B. (1992) *J. Am. Chem. Soc. 114*, 6957–6965.

Stein, C. A., & Cheng, Y.-C. (1993) *Science 261*, 1004–1012.

Vu, H., & Hirschbein, B. L. (1991) *Tetrahedron Lett. 32*, 3005–3008.