A Parallel-Stranded DNA Triplex Tethering a Hoechst 33258 Analogue Results in Complex Stabilization by Simultaneous Major Groove and Minor Groove Binding

Jordi Robles, Sharanabasava B. Rajur, and Larry W. McLaughlin*

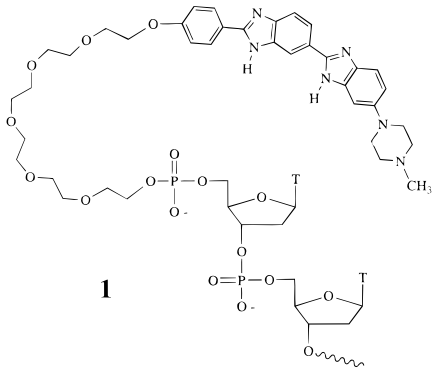
Department of Chemistry, Merkert Chemistry Center Boston College, Chestnut Hill, Massachusetts 02167

Received March 19, 1996

Pyrimidine oligodeoxynucleotides can bind to double-stranded DNA containing polypurine tracts in a parallel orientation by interaction with the Hoogsteen functional groups of the target purine residues.[1] The triplex strand is bound within the major groove of the target duplex DNA, and in this motif, base pair recognition occurs through the formation of T-A-T[2] and C^+-G-C base triplets.[3] A number of small ligands are also capable of binding to double-stranded DNA sequences, but typically they interact with the duplex by intercalation or by binding within the minor groove. Sequence selectivity can be observed by agents that function as groove binders such as netropsin[4] and distamycin[5] as well as the benzindole DAPI[6] and the benzimidazole Hoechst 33258.[7]

The stability of DNA triplexes has been shown to be enhanced by intercalators capable of binding to the three-stranded complex.[8] In addition to intercalators (designed for the duplex[9] or specific for the triplex[8]), polyamines, such as spermine[10] or polylysine,[11] can assist in stabilizing the DNA triplex. Conjugates possessing a ligand that is covalently attached to the third strand of the triple helix can exhibit two modes of binding and thereby enhance the stability of the triple helix structure relative to the corresponding nonconjugated species. Enhanced stability will be an important characteristic if such materials are to be developed into an important class of diagnostics or therapeutics.

The presence of A-T rich sequences in many parallel-stranded triplexes suggests the possibility that certain minor groove specific ligands might bind to the triplex structure.[12] Tethering a minor groove binding agent, such as Hoechst 33258, to the third strand of the triple helix (**1**, Figure 1) could result in enhanced DNA triplex stability by providing simultaneous binding in the major groove (by the third strand) and minor groove (by the A-T specific ligand). Hoechst 33258 is known to bind to A-T rich regions of duplex DNA,[13] and minor groove binding results in a strong fluorescent signal.[14] Model building studies indicated that for simultaneous major groove binding a tether must terminate in the major groove at the site of the 5′-hydroxyl of the third strand, then traverse the phosphoribose backbone, and still penetrate deep within the minor groove to permit binding by the bisbenzimidazole dye. Hexa(ethylene glycol) provides the requisite length for this function and is additionally hydrophilic in nature, an attractive property for studies in aqueous systems. Similar glycol-based linkers have been used in other studies involving DNA duplexes[15] and RNA complexes.[16]



1

Figure 1. Structure of the conjugate **1**, consisting of Hoechst 33258, a hexa(ethylene glycol) linker, and the first two residues of a 15-mer polypyrimidine strand of DNA.

After a Mitsunobu reaction between hexa(ethylene glycol) and *p*-cyanophenol, the resulting product could be condensed with a diamino intermediate to generate the Hoechst ring system tethering the hexa(ethylene glycol) linker. To couple the Hoechst derivative to the oligonucleotide, we removed the terminal DMT protecting group and then phosphitylated the terminal 5′-hydroxyl of a support-bound 15-mer. The Hoechst analogue, tethering the hexa(ethylene glycol) linker, was then added to the column in the presence of tetrazole. Although this procedure did not result in a high-efficiency coupling characteristic of phosphoramidite protocols, we were still able to perform this final coupling with yields near 75% as judged from HPLC analysis of the crude reaction mixture obtained after deprotection procedures (see supporting information).

Triplex formation was assessed by examining absorbance vs temperature plots for a series of 15-mers tethering, or lacking, the pendant Hoechst derivative. A 40-mer DNA duplex that contained a 15-mer polypurine tract (Table 1) with three G residues was employed as the target sequence. The sequence on either side of the target was composed solely of G-C base pairs and thus provides no minor groove binding site for the Hoechst dye. T was used to recognize the A-T base pairs of the duplex, while both C and m^5C were employed for the recognition of the G-C target base pairs. Since triplex formation requires that the third DNA strand enter the major groove and be positioned between the charged residues of the two phos-

(1) (a) Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645−650. (b) Thuong, N. T.; Helene, C. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 666−690. For reviews, see: (c) Dervan, P. B. In *Oligonucleotides. Antisense Inhibitors of Gene Expression*; Cohen, J. S., Ed.; Macmillan Press: London, 1989; pp 197−210. (d) Praseuth, D.; Perrouault, L.; Le Doan, T.; Chassignol, M.; Thuong, N. T.; Lhomme, J.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1349−1352.

(2) All letter abbreviations represent the 2′-deoxynucleosides.

(3) (a) Strobel, S. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 7286−7288. (b) Syamichev, V. I.; Mirkin, S. M.; Frank-Kamenetskii, M. D.; Cantor, C. R. *Nucleic Acids Res.* **1988**, *16*, 2165−2178. (c) Francois, J. C.; Saison-Behoaras, T.; Thuong, N. T.; Hélène, C. *Biochemistry* **1989**, *28*, 9617−9626.

(4) Kopka, M.; Yoon, C.; Goodsell, D.; Pjura, P.; Dickerson, R. E. *J. Mol. Biol.* **1985**, *183*, 55−61.

(5) Coll, M.; Frederick, C. A.; Wang, A. H.-J. R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8385−8389.

(6) Larsen, T. A.; Goodsell, D. S.; Cascia, D.; Grzeskowiak, K.; Dickerson, R. W. *J. Biomol. Stereodynam.* **1989**, *7*, 477−491.

(7) Pjura, P. E.; Grzeskowiak, K.; Dickerson, R. E. *J. Mol. Biol.* **1987**, *197*, 257−271.

(8) (a) Mergny, J. L.; Duval-Valentin, G.; Nguyen, C. H.; Perrouault, L.; Faucon, B.; Rougee, M.; Montenay-Garestier, T.; Bisagni, E.; Hélène, C. *Science* **1992**, *256*, 1681−1684. (b) Pilch, D. S.; Martin, M.-T.; Nguyen, C. H.; Sun, J.-S.; Bisagni, E.; Garestier, T.; Hélène, C. *J. Am. Chem. Soc.* **1993**, *115*, 9942−9951.

(9) Thuong, N. T.; Asseline, U.; Moneney-Garestier, T. In *Oligodeoxynucleotides. Anti-sense Inhibitors of Gene Expression*, Cohen, J. S., Ed.; CRC Press, Inc.: Boca Raton, FL, 1989; p 25−52.

(10) (a) Glaser, R.; Gabbay, E. J. *Biopolymers* **1968**, *6*, 243−254. (b) Singelton, S. F.; Dervan, P. B. *Biochemistry* **1993**, *32*, 13171−13179.

(11) Potaman, V. N.; Sinden, R. R. *Biochemistry* **1995**, *34*, 14885−14892.

(12) (a) Durrand, M.; Thuong, N. T.; Maurizot, J. C. *J. Biol. Chem.* **1992**, *267*, 24394−24399. (b) Park, Y. W.; Breslauer, K. J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6653−6657.

(13) Abu-Daya, A.; Brown, P. M.; Fox, K. R. *Nucleic Acids Res.* **1995**, *17*, 3385−3392.

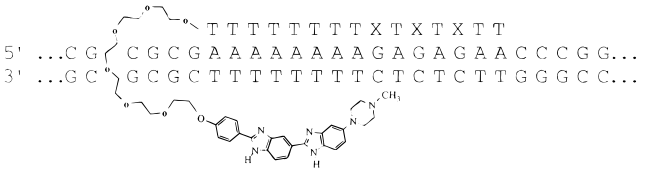
(14) Zimmer, C.; Wähnert, U. *Prog. Biophys. Mol. Biol.* **1986**, *47*, 31−112.

(15) Durand, M.; Chevrie, K.; Chassignol, M.; Thuong, N. T.; Mauizot, J. C. *Nucleic Acids Res.* **1990**, *18*, 6353−6359.

(16) (a) Ma, M. Y.-X.; Reid, L. S.; Climie, S. C.; Lin, W. C.; Kuperman, R.; Summer-Smith, M.; Barnett, R. W. *Biochemistry* **1993**, *32*, 1751−1758, (b) Benseler, F.; Fu, D. J.; Ludwig, J.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1993**, *115*, 8483−8484.

S0002-7863(96)00891-8 CCC: $12.00

Table 1. Effects of the Tethered Hoechst 33258 Groove Binder on DNA Triplex T_m Values (°C)[a]



				pH			
entry	conjugate **1**	15-mer[b]	[spermine][c]	6.4	7.0	7.5	8.0
i		X = C		22.7	11.3	*d*	*d*
ii	X = C			40.3	29.5	19.0	12.3
iii		X = m^{5}C		35.0	31.4	27.7	20.0
iv	X = m^{5}C			47.0	37.2	32.5	20.5
v		X = m^{5}C	0.5 mM	40.9	36.0	35.3	32.4
vi	X = m^{5}C		0.5 mM	50.2	43.3	41.9	44.6

[a] Thermal denaturation studies were performed in 10 mM PIPES, pH 6.4, 7.0 (or HEPES, pH 7.5, 8.0, 8.5), 10 mM $MgCl_2$, and 50 mM NaCl (with or without 0.5 mM spermine) at 1:1:1 strand ratios at low micromolar concentrations using a 40-mer as the target duplex: 5′d(CGCCGCGCGCGCG**AAAAAAAAGAGAGAA**CCCGGCGCG-CGC); 3′d(GCGGCGCGCGCGC**TTTTTTTTTCTCTCTT**GGGCCG-CGCGCG). [b] Adding 1 equiv of free Hoechst 33258 to the nonconjugated triplex had little effect upon the triplex transition and raised the transition of the duplex 40-mer by no more than 2 °C. [c] All duplex T_m values with or without spermine ranged from 83.1 to 85.9 °C. [d] No triplex transition observed.

phodiester backbones, we additionally examined the effects of the polyamine spermine which has been shown[10] to stabilize higher order nucleic acid structures, presumably due to its ability to limit charge−charge repulsion by shielding neighboring phosphates. The T_m values for the various triplexes measured over the pH range 6.4−8.0 are presented in Table 1. The control T_m values, those obtained for 15-mer triplexes lacking the pendant Hoechst derivative, exhibit the same trends that have been described in many other studies; the pH dependence resulting from the protonated C or m^{5}C residues results in decreasing triplex formation and stability with increasing pH. For example, the triplex transition for entry **i** (Table 1) drops from 22.7 to 11.3 °C when the pH is changed from 6.4 to 7.0. No triplex transitions were observed at pH values of 7.5 and 8.0. However, in the presence of the tethered groove binder, the T_m values at pH values of 6.4 and 7.0 are increased some 18 °C (**ii**, Table 1), while at pH values of 7.5 and 8.0 triplex transitions are now present, although still dependent on pH value of the solution.

Replacing the three C residues of the third strand by m^{5}C reduces the pH dependence (entry **iii**, Table 1) of the triplex transition as expected.[17] Addition of the tethered Hoechst derivative to this sequence results in an increased T_m of 12 °C at pH 6.4 for the triplex transition (entry **iv**, Table 1). Increases in T_m values of about 6 °C were observed at pH values of 7.0 and 7.5 (Table 1). Addition of the polyamine spermine results in further increases in the observed T_m values for the simple parallel-stranded triplexes, in agreement with previous studies[17] (compare entries **iii** and **v**, Table 1). The presence of the tethered groove-binding agent and the polyamine spermine results in a T_m value of over 50 °C at pH 6.4 (entry **vi**, Table 1) for the m^{5}C-containing triplex and significant increases in T_m at other pH values.

In addition to enhanced stability, as assessed from the T_m values, the triplexes formed with conjugate **1** are all fluorescent, as is the characteristic of Hoechst 33258 when bound in the minor groove of DNA duplexes. This observation is consistent with the fluorophore bound in the minor groove of the triplex structure while the polypyrimidine strand is present in the major groove, but we cannot, at this time, completely exclude the possibility that the Hoechst dye folds back into what remains of the triplex major groove. An examination of a fluorescence vs temperature plot for these complexes reveals a relationship that is sigmoidal in character (see supporting information), with the midpoint of the transition occurring near the T_m value for the complex (midpoint = ∼48 °C for complex **ii**, Table 1). These characteristics argue for the Hoechst derivative remaining largely bound to the three-stranded complex until the T_m for the triplex is reached.

That the fluorophore is bound to the triplex structure can be inferred both from the enhanced T_m values and the observed fluorescence characteristics. The present data stand in contrast to the conclusions of a previous study[18] with triplexes that involve the use of the Hoechst 33258 fluorophore in an untethered state. In those studies the groove-binding agent appeared to destabilize the three-stranded complex. This inconsistency can be explained in that the free fluorophore is known to interact with double-stranded DNA through multiple binding modes[19] (similar behavior could be expected also with the triple helix), and in the noted work relatively high ratios of dye/triplex were employed. By covalent tethering of the Hoechst derivative to the third strand, a single groove-binding agent is available for each three-stranded complex; under these conditions it appears that a single, preferred, ligand-binding mode results.

The described conjugate is easy to prepare using standard solid-phase based synthetic protocols, it results in major groove binding by a polypyrimidine strand in a parallel orientation while permitting simultaneous minor groove binding by the tethered ligand. The result of both types of binding is an overall increase in triplex stability. The use of such tethered ligands may contribute to the, thus far, elusive solution of the general targeting of duplex DNA by a third strand. Complex stabilization by tethered ligands may additionally permit the use of base triplets composed of analogue bases that would otherwise lack sufficient stability to generalize triplex formation at a wide variety of sequences.

Acknowledgment. We thank Dr. R. G. Kuimelis for suggesting conditions for the coupling reaction between the tethered Hoechst derivative and the support-bound oligonucleotide. J.R. is a fellow of the Ministerio de Educación y Ciencia of the Spanish Government. This work was supported by grants from the NIH (GM37065 and GM53201).

Supporting Information Available: Synthetic procedures, sample absorbance vs temperature plots, and fluorescent spectra (16 pages). Ordering information is given on any current masthead page.

JA9608914

(17) Lee, J. S.; Woodsworth, M. L.; Latimer, L. J. P.; Morgan, A. R. *Nucleic Acids Res.* **1984**, *12*, 6603−6614.

(18) Durand, M.; Thuong, N. T.; Maurizot, J. C. *Biochimie* **1994**, *76*, 181−186.

(19) Lootiens, F. G.; Regenfuss, P.; Zechel, A.; Dumortier, L.; Clegg, R. M. *Biochemistry* **1990**, *29*, 9029−9039.