DNA Triplex Stabilization Using a Tethered Minor Groove Binding Hoechst 33258 Analogue†

Jordi Robles and Larry W. McLaughlin*

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

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Abstract: Tethering the Hoechst 33258 fluorophore to the 5′-terminus of the polypyrimidine third strand of parallel-stranded DNA triplexes results in complexes with increased T_m values that vary with both the pH of the solution and the sequence of the target DNA. These pH/sequence effects are likely related to the presence of M^+-G-C base triplets, which can result in either charge−charge effects involving the base triplet and the charged piperazine ring of the ligand or changes in the nature of the minor groove binding site resulting from the introduction of the N^2-exocyclic amino group(s). As with DNA duplexes, sequence targets rich in A-T base pairs are most effective in taking advantage of such ligand-induced stability. A duplex sequence rich in A-T base pairs adjacent to the triple helix also appears to permit adjacent ligand binding; that is, the triplex is stabilized by the binding of the tethered minor groove ligand in an A-T rich duplex adjacent to the site of the triplex. The Hoechst ligand can be very effective in stabilizing G-T-A base triplets which are generally less effective in triplex formation, presumably as the result of a single interstrand G-T hydrogen bond. Stabilization may occur in part because the $(AATT)_2$ minor groove may offer the ligand a preferred binding site as has been documented for this sequence in DNA duplexes. Binding to the triplex results in an enhanced quantum yield for the fluorophore, the magnitude of which is dependent upon sequence effects. Stopped flow experiments have provided some insight into the nature of the process; rapid ligand binding to the duplex target is followed by a slower process, one interpreted to reflect the third strand binding to generate the conjugated DNA triplex. Although not conclusive, the experiments suggest that the Hoechst conjugated polypyrimidine strands bind to the target duplex by two simultaneous sets of interactions: (i) third strand binding in the major groove and (ii) tethered Hoechst 33258 ligand binding in the minor groove.

Introduction

In addition to the interstrand Hoogsteen (or reverse Hoogsteen) hydrogen bonds necessary for the formation of interstrand DNA triplexes, additional ligands, particularly those that have been conjugated to the third strand, can be very effective in providing additional complex stabilization. Attachment of intercalating agents such as acridine[1,2] strongly stabilized a pyrimidine−purine−pyrimidine DNA triplex as the result of intercalation of the acridine moiety into the duplex. A number of intercalating agents have been conjugated to DNA sequences, and the triplex−duplex junction at the 5′-terminus of the third strand appears to be a preferred site for intercalation by several agents.[3,4] The nonconjugated[5] (or conjugated[6]) intercalating agent benzo[*e*]pyridoindole appears to intercalate directly into the triplex to provide enhanced stabilization of DNA triple helix. Psoralen conjugates have also been effective in stabilizing these structures and thereby inhibiting transcription,[7] although the intercalation event appears to be a transient one, and in the presence of long-wave UV irradiation, cross-linking between the third strand and the target duplex occurs at the site of the psoralen ligand.

Cholesterol conjugates were prepared initially to enhance transport across the cell membrane and to provide stability against exonuclease digestion,[8] but additional studies have shown that the cholesterol ligand increases the stability of both duplex and triplex structures.[9] When present in triplexes formed from oligonucleotide "clamps", the terminal cholesterol ligand contributed significantly to the stabilization of such antisense complexes.[10] The effect of the cholesterol ligand may be to provide enhanced hydrophobic interactions, but the nature of this effect remains to be clarified. In addition to hydrophobic effects, polyamines such as spermine[11,12] are also known to stabilize DNA triplexes, presumably by charge−charge interactions that shield the potentially destabilizing phosphate−phosphate interactions. The conjugation of polyamines[13a] or polyamides[13b] to the third strand of DNA triplexes results in enhanced stability, and the presence of soluble polyamine or divalent metal in solution is no longer required.

* To whom correspondence should be addressed. Phone: (617) 552-3622. Fax: (617) 552-2705. E-mail: larry.mclaughlin@bc.edu.

† Abbreviations: M = 2′-deoxy-5-methylcytidine, C* = 4-amino-1-(β-2′-deoxy-D-*erythro*-pentofuranosyl)-5-methyl-2,6(1*H*,3*H*)-pyrimidinedione ($m^{5ox}C$).

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A number of studies have also examined the ability of nonconjugated ligands to interact with DNA triple helices. Minor groove binding ligands known to interact with duplex DNA can also bind to triplex structures. For example, netropsin binds to a pyrimidine−purine−pyrimidine triplex without disruption of the complex,[14−16] but the affinity for the triplex appears to be less than that for the corresponding duplex. However, this observation does not completely account for the interactions of netropsin with triple helices. Netropsin, as well as DAPI and berenil, has also been observed to induce the formation of hybrid triplexes involving both DNA and RNA sequences,[17] when such triplexes are normally not formed. Other minor groove ligands including Hoechst 33258 and distamycin appear to destabilize DNA triplexes,[18,19] at least under the conditions examined, while berenil will stabilize the DNA triplex in the absence of sodium chloride.[20] We have observed that upon tethering Hoechst 33258 to the 5′-terminus of the third strand by a hexa(ethylene glycol) linker that enhanced stability of a T-A-T rich triplex results.[21] Here we report on additional studies with various DNA−Hoechst conjugates.

Experimental Section

Materials. HPLC grade solvents were obtained from either Aldrich (Milwaukee, WI) or Fisher Scientific (Fair Lawn, NJ). 5′-Dimethoxytrityl nucleoside phosphoramidite monomers as well as all ancillary reagents for nucleic acid synthesis were obtained from Cruachem through Fisher Scientific or from Applied Biosystems, Inc. (Foster City, CA). Oligonucleotides were synthesized using nucleoside phosphoramidite derivatives and an Applied Biosystems 381A DNA synthesizer. High-performance liquid chromatography (HPLC) was carried out on an ODS-Hypersil column (0.46 × 25 cm, Shandon Southern, England), using a Beckman HPLC system. ^{1}H NMR spectra were obtained at 300 or 500 MHz on a Varian XL-300 or 500 multinuclear spectrometer. Absorption spectra were recorded using an AVIV 14DS UV/vis spectrophotometer. Emission spectra were collected on a Shimadzu RF5000U fluorescence spectrophotometer containing a Shimadzu DR-15 microprocessor and graphics display terminal. Mass spectra were obtained from the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, Urbana, IL. Stopped flow experiments were carried out in a Kintek Instruments System Model SF2001 with a temperature-controlled reaction chamber and a two-syringe pneumatically driven delivery system.

Methods. 4-[2-[Penta(ethylene glycol)]ethoxy]benzaldehyde (3). A mixture of 4-hydroxybenzaldehyde (**1**) (1.2 g, 10 mmol) and triphenylphosphine (3.9 g, 15 mmol) was suspended under nitrogen, in anhydrous dichloromethane (10 mL). When most of the solid was dissolved, hexa(ethylene glycol) (**2**) (3.7 mL, 15 mmol) was then added. The flask was cooled in an ice bath for 5−10 min, and then diethyl diazodicarboxylate was slowly added in three portions (total 1.8 mL, 15 mmol). The mixture was left to react for 1 h with ice cooling, after which time TLC analysis showed the complete disappearance of hydroxybenzaldehyde. The solvent was then evaporated, and the residue dissolved in ethyl acetate (50 mL) and washed with aqueous 5% sodium bicarbonate (2×) and water. The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by chromatography with silica gel using a gradient of methanol in ethyl acetate. The product eluted with approximately 10% methanol. The solvent was removed by evaporation to yield a pale yellow oil (1.14 g, 30%). TLC (ethyl acetate−methanol, 9:1): 0.40. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.88 (1H, s, −CHO); 7.83 (2H, d, J = 8.7 Hz, ArH); 7.03 (2H, d, J = 8.7 Hz, ArH); 4.24 (2H, t, J = 5.0 Hz, $-CH_2-$); 3.88 (2H, t, J = 5.0 Hz, $-CH_2-$); 3.46 (20H, m, $-CH_2-$); 2.86 (1H, br s, −OH) ppm. UV (methanol): λ_{max} = 269, 278 nm, λ_{min} = 231 nm. IR (film, $CHCl_3$): 3345, 2873, 1690, 1602, 1256, 1099 cm^{-1}. HRMS (FAB): calcd for $C_{19}H_{31}O_8$ 387.2019 (M + H^+), found 387.2024.

2-{4-[2-[Penta(ethylene glycol)]ethoxy]-1-phenyl-6-benzimidazolyl}-6-(1-methyl-4-piperazinyl)benzimidazole (5). The diamine **4**[22,23] (100 mg, 0.31 mmol) was dissolved in anhydrous methanol (5 mL) under dry nitrogen, and then the benzaldehyde **3** (120 mg, 0.31 mmol) was added and this mixture stirred at ambient temperature for 1 h. At this time, tBuOOH (40 μL, 0.36 mmol) was added. The mixture was left to react at room temperature under an inert atmosphere for five days. TLC analysis showed the complete disappearance of the diamine **4**. The solvent was evaporated, and the residue dissolved in a mixture of dichloromethane−methanol, 9:1, was applied to a preparative TLC plate, which was developed in a mixture of dichloromethane−methanol−triethylamine, 85:15:2. The portion of the plate that contained the product was removed, and the product was recovered by washing the silica with dichloromethane−methanol−triethylamine, 90:10:1 (50 mL). This solution was then evaporated to dryness, and the residue was precipitated after dissolving in hot acetonitrile (2 mL) by cooling first at ambient temperature, and then at 4 °C, to obtain the first fraction of solid product. Reduction of the supernatant yielded additional product (combined total 135 mg, 63%). Mp: 115−120 °C dec. R_f (dichloromethane−methanol−triethylamine, 85:15:2): 0.45 (in the same system, the diamine **4** showed an R_f = 0.20, and the aldehyde **3**, R_f = 0.90). ^{1}H NMR (300 MHz, CD_3OD): δ = 8.21 (1H, d, J = 1.5 Hz, ArH); 7.99 (2H, d, J = 9.0 Hz, Ar″H); 7.92 (1H, dd, J = 1.5, 8.4 Hz, ArH); 7.65 (1H, d, J = 8.4 Hz, ArH); 7.48 (1H, d, J = 9.0 Hz, Ar′H); 7.10 (1H, d, J = 2.1 Hz, Ar′H); 7.03 (3H, m, 2Ar″H, ArH); 4.16 (2H, m, $-CH_2-$ ethylene); 3.79 (2H, t, J = 4.8 Hz, $-CH_2-$ ethylene); 3.56 (20H, m, $-CH_2-$ ethylene); 3.21 (4H, m, $-CH_2-$ piperazine); 2.79 (4H, m, $-CH_2-$ piperazine); 2.42 (3H, s, CH_3-) ppm. UV (water): λ_{max} = 220, 264, 339 nm, λ_{min} = 245, 295 nm. IR (film, methanol): 3600−3300, 3250−3000, 2880, 1620, 1615, 1470, 1256, 1105 cm^{-1}. HRMS (FAB): calcd for $C_{37}H_{49}N_6O_7$ 689.3663 (M + H^+), found 689.3659.

DNA Synthesis. The 15-mers were synthesized using standard phosphoramidite protocols.[24] To attach the Hoechst derivative to the 15-mers through the hexa(ethylene glycol) linker, the following procedure was employed: After the final nucleoside coupling on the solid support, the DMT group was removed using 3% trichloroacetic acid in dichloroethane. After the support was washed with anhydrous acetonitrile, a solution of 1 M (2-cyanoethoxy)(diisopropylamino)-chlorophosphine and 1 M *N*,*N*-diisopropylethylamine in anhydrous acetonitrile was added to the support. After a reaction period of 30 min, the support was washed with anhydrous acetonitrile, and this phosphitylation step was repeated a second time. To complete the coupling, equal volumes of 0.5 M tetrazole/acetonitrile and a 0.1 M solution of the Hoechst derivative tethering the hexa(ethylene glycol) linker dissolved in anhydrous DMF were added to the support and the reaction was continued for 16 h. After the support was washed with acetonitrile, the acetylation and oxidation steps were performed in the usual manner. The conjugate was deprotected and removed from the support by treatment with concentrated aqueous ammonia at 50 °C for 16 h.

HPLC using a 9.4 × 250 mm column of ODS-Hypersil was used to purify the conjugated oligonucleotides using a linear gradient of 10−40% buffer B over 30 min (buffer A, 50 mM triethylammonium acetate, pH 7.0; buffer B, 50 mM triethylammonium acetate, pH 7.0, containing 70% acetonitrile). The conjugates eluted between 15 and 18 min under

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these conditions. An estimation of the efficiency of the final coupling step could be determined by comparing the ratio of the free 15-mer (failed coupling product) to the conjugated 15-mer during the HPLC isolation procedures. This final coupling step generally occurred with yields between 50% and 75%.

Nucleoside Analyses. To 0.5 A_{260} unit of oligonucleotide in 100 μL of 50 mM Tris·HCl, pH 8.0, containing 100 mM $MgCl_2$ were added 0.003 unit of snake venom phoshodiesterase and 1 unit of alkaline phosphatase and the mixture was incubated for 2−6 h at 37 °C. The digestion products (approximately 0.2 A_{260} unit) were analyzed by reversed phase HPLC using a 4.6 × 250 mm column of ODS-Hypersil and a gradient of 5% buffer B for 10 min, followed by a linear gradient from 5% to 100% buffer B over 10 min (buffer A and buffer B were as described above). Under these conditions, the following retention times were observed (min): C, 3.1; G, 7.5; T, 8.0; A, 9.2; m^5C, 5.2; and Hoechst derivative **5**, 17.5 min.

Oligonucleotide Extinction Coefficients. Extinction coefficients at 260 nm were determined according to the method of Cantor and Warshaw,[25] and for the conjugates, the extinction coefficient for HO−$(eg)_6$−Hoechst 33258 at 260 nm (20 800 A_{260} units mM^{-1}) was added to the calculated value. Concentrations of sequences containing C, M, or C* were determined using the same extinction coefficient. The error in these extinction coefficients is estimated to be ±20%.

Sequence: 5′→3′		ε(A_{260} units mM^{-1})
TTTTTTTTXTXTXTT	X : C,M,C*	119,400
Hoechst-(eg)$_6$-TTTTTTTTXTXTXTT	X : C,M,C*	140,200
CGCCGCGCGCGCG**AAAAAAAAGAGAGAA**CCCGGCGCGCGC		385,400
GCGCGCGCCGGG**TTCTCTCTTTTTTTT**CGCGCGCGCGGCG		336,600
YTTTTTTTXTXTXTT	X : M Y : M	117,800
	X : M Y : C*	117,800
Hoechst-(eg)$_6$-YTTTTTTTXTXTXTT	X : M Y : M	139,600
	X : M Y : C*	139,600
CGCCGCGCGCGCG**GAAAAAAAGAGAGAA**CCCGGCGCGCGC		383,500
GCGCGCGCCGGG**TTCTCTCTTTTTTTC**CGCGCGCGCGGCG		335,700
XXTTTTTTXTXTXTT	X : M	116,900
Hoechst-(eg)$_6$-XXTTTTTTXTXTXTT	X : M	137,700
CGCCGCGCGCGCG**GGAAAAAAGAGAGAA**CCCGGCGCGCGC		381,600
GCGCGCGCCGGG**TTCTCTCTTTTTTCC**CGCGCGCGCGGCG		334,800
XTXTTTTTXTXTXTT	X : M	116,900
Hoechst-(eg)$_6$-XTXTTTTTXTXTXTT	X : M	137,700
CGCCGCGCGCGCG**GAGAAAAAGAGAGAA**CCCGGCGCGCGC		382,800
GCGCGCGCCGGG**TTCTCTCTTTTTCTC**CGCGCGCGCGGCG		334,800
TTTTXTXTXTXTXTT	X : M	117,600
Hoechst-(eg)$_6$-TTTTXTXTXTXTXTT	X : M	138,400
CGCCGCGCGCGCG**AAAAGAGAGAGAGAA**CCCGGCGCGCGC		384,000
GCGCGCGCCGGG**TTCTCTCTCTCTTTT**CGCGCGCGCGGCG		334,800
TTGGTTTTXTXTXTT	X : M	124,000
Hoechst-(eg)$_6$-TTGGTTTTXTXTXTT	X : M	144,800
CGCCGCGCGCGCG**AATTAAAAGAGAGAA**CCCGGCGCGCGC		379,600
GCGCGCGCCGGG**TTCTCTCTTTTGGTT**CGCGCGCGCGGCG		341,200
TTTGGTTTXTXTXTT	X : M	124,000
Hoechst-(eg)$_6$-TTTGGTTTXTXTXTT	X : M	144,800
CGCCGCGCGCGCG**AAATTAAAGAGAGAA**CCCGGCGCGCGC		379,600
GCGCGCGCCGGG**TTCTCTCTTTGGTTT**CGCGCGCGCGGCG		341,200
CGCCGCGC**AAAAA**GAGAAAAAGAGAGAA CCCGGCGCGCGC		400,100
GCGCGCGCCGGG**TTCTCTCTTTTTCTC**TTTTTTGCGCGGCG		334,100

Thermal Denaturation Studies. Thermal denaturation studies were performed in 10 mM PIPES, pH 6.4 or 7.0, 10 mM HEPES, pH 7.5 or 8.0, 10 mM magnesium chloride, and 50 mM sodium chloride (with or without 0.5 mM spermine) at concentrations of 1 ± 0.20 μM. (note, spermine-containing buffers were made fresh daily). Slight variations in concentrations arise due to the imprecision in determining extinction coefficients for oligonucleotide sequences. Absorbance (260 nm) and temperature values were measured with an AVIV 14DS UV/vis spectrophotometer equipped with digital temperature control. The temperature of the cell compartment was increased in 1.0 °C steps (from 0 to 95 °C), and when thermal equilibrium was reached, temperature and absorbance data were collected. T_m values were determined both from first-order derivatives and by graphical analysis to determine the midpoint of the transition directly from the absorbance vs temperature plots. Both techniques gave values that were within the experimental error (±1 °C) for the analysis. If unclear, the graphical analysis to determine the midpoint of the transition was relied upon. For some of the higher melting complexes, it was necessary to generate difference plots, in which the duplex component of the transition had been subtracted.

Fluorescence Studies. Fluorescence measurements were made in 10 mM PIPES, pH 6.4 or 7.0, 10 mM HEPES, pH 7.5 or 8.0, 10 mM magnesium chloride, and 50 mM sodium chloride (with or without 0.5 mM spermine) at triplex concentrations of approximately 1 μM. All measurements were performed with the following list of parameters: slit width, Ex/Em = 5 nm/5 nm; high sensitivity; medium speed. Samples were introduced into a 1.25 mL cell thermally isolated with a water jacket. Temperature was controlled with a recirculating water bath at 10 °C. Emission spectra were recorded with λ_{ex} = 354 nm.

Stopped Flow Analyses. Experiments were carried out in the stopped flow apparatus containing a temperature-controlled reaction chamber at 10 °C using two solutions: Solution A contained 20 μM target duplex in PIPES, pH 6.4, 7.0, or 8.0, 10 mM magnesium chloride, and 50 mM sodium chloride. Solution B contained 2 μM conjugate in the same buffer. Equal volumes of each solution were mixed in the reaction chamber (approximately 0.1 mL) by a pneumatic system. After the flow of solutions had stopped, the fluorescence emission (450 nm) was recorded as a function of time (λ_{ex} = 354 nm). Different experiments were performed to monitor the change in fluorescence over varying time periods. The fitted curves were the average of at least five experiments, with each experiment involving 400 data points. The averaged data were fitted to exponential curves from which kinetic constants were obtained.

Results and Discussion

DNA triplexes formed with the pyrimidine−purine−pyrimidine recognition motif typically contain a large number of neutral T-A-T base triplets. In these complexes the third strand occupies the major groove of the target duplex, and major groove binding ligands such as methyl green are excluded from binding to such DNA triplexes.[26] However, in these three-stranded complexes the minor groove remains largely unencumbered, and a variety of studies have shown that minor groove binding ligands can target DNA triple helices,[14−16,18−20] although in many cases the affinity of the minor groove agent for the triplex is often less than that observed for the corresponding duplex. Hoechst 33258 is known to bind to the minor groove of A-T rich duplexes and could, in principle, bind to DNA triplexes that are rich in T-A-T base triplets. By tethering the Hoechst derivative to the third strand, enhanced triplex stability could then occur. The third strand would be located in the major groove of the target duplex, and the Hoechst ligand would occupy the minor groove. In order for such a complex to benefit from both modes of binding, the linker used to tether the ligand to the DNA sequence must be long enough to have one end in the major groove—at the terminus of the third strand; it must then traverse the phosphoribose backbone, and finally penetrate deeply within the minor groove to permit binding by the Hoechst ligand. Model building studies indicated that a hexa(ethylene glycol) linker would span the distance necessary to permit simultaneous major groove and minor groove binding. Toward this end, we have incorporated hexa(ethylene glycol) into the terminal phenoxy ring of Hoechst 33258. In addition to the requisite length, this glycol-based linker is hydrophilic, and may tend to adopt a more extended form in aqueous solutions, rather than to fold back on itself as might happen with a more hydrophobic carbon-based linker.

Synthesis of the Conjugated Oligonucleotides. We were unable to incorporate the glycol-based linker directly into the Hoechst 33258 fluorophore, but could prepare the desired compound by synthesizing the bisbenzimidazole ring system as described[22,23] and simply altering the nature of the last

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cyclization step. Thus, hexa(ethylene glycol) (**2**) was used to alkylate *p*-hydroxybenzaldehyde (**1**) to generate the aldehyde derivative **3**. Preparation of the aldehydes allowed us to use an oxidative coupling procedure with the diamino derivative **4** (prepared using the described procedures[22,23]). Under oxidative conditions we obtained the Hoechst derivatives **5** in higher yields than with previously described procedures using the corresponding imidates.[27]

Fluorophores and other reporter groups can often be tethered to the 5′-terminus of oligonucleotides simply by preparing the agent as the corresponding phosphoramidites and coupling them to the DNA sequence as the last step of the synthesis.[28−31] Unfortunately, we could not prepare **5** as the corresponding phosphoramidite. However, this derivative could be successfully tethered to the terminus of the desired oligonucleotides by reversing the nature of the coupling step.[27] In this procedure, the DNA 15-mer was synthesized using standard protocols, and the final DMT-protecting group was removed in the normal fashion. The 15-mer was then phosphitylated to generate a phosphoramidite at the 5′-terminus of the 15-mer. The extent of this reaction could not be effectively quantified, so it was repeated in order to enhance the yield of the support-bound phosphoramidite. The Hoechst derivative **5** was then introduced to the support-bound phosphoramidite in the presence of tetrazole. Similar procedures were described previously for phosphotriester-based procedures.[32] After an extended reaction period (∼18 h) the sequence was deprotected (concentrated ammonia) and isolated (HPLC).

The extent of the final coupling reaction was determined by HPLC analysis. In these analyses two major species were typically present. The earliest eluting material corresponded to the nonconjugated 15-mer. It often appeared with a companion peak due to the variation in the terminal residue (± a terminal phosphate). The second peak was the major product and corresponded to the conjugated species, while the final minor peak represented the free fluorophore/linker. It is possible that this latter material represents some cleavage from the conjugated oligonucleotide during deprotection procedures. Alternatively, much of this material may simply be residual fluorophore that was nonspecifically bound to the solid support and subsequently released upon treatment with ammonia. The extent of conjugation using this procedure varied from 50% to 75%. Treatment of the major product with snake venom phosphodiesterase and alkaline phosphatase resulted in the correct ratios of nucleosides and the fluorophore linker derivative **5** (data not shown).

Thermal Stability of Hoechst-Conjugated DNA Triplexes. We initially examined a 15-mer triplex containing eight T-A-T base triplets at the 5′-terminus of the third strand, adjacent to the site of attachment of the poly(ethylene glycol) tether. The three G-C base pairs in the duplex region were targeted using 5-methylcytosine (M). As we have reported in an earlier paper,[21] the triplexes containing the tethered Hoechst dye exhibited significant increases in T_m values (see Figure 1), both with and without spermine present in the buffer (Table 1). But in all cases, due to the requirement for protonation of the M^+-G-C base triplets, the T_m values were observed to be dependent upon the pH values of the solution (see Figure 2). These pH effects can be eliminated by using an appropriate nucleoside analogue in which the protonation event is not required for base triplet formation (see Figure 2). We have previously described[33] such an analogue of C, which contains a carbonyl for C6 and a methyl group at C5. Triplexes formed from this C-analogue (C*; Table 1, Figure 2) do not exhibit the pH dependence of the M-containing triplexes. The stability of these triplexes can also be increased in the presence of spermine, but the combination of the tethered Hoechst dye and spermine results in roughly a 28 °C increase in triplex T_m at pH 6.4 relative to the nonconjugated duplex in the absence of spermine (Table 1).

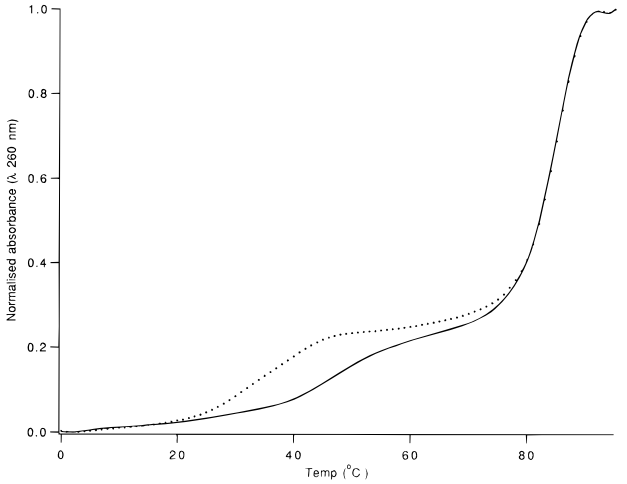

Figure 1. Absorbance vs temperature plots for the native (dotted line) and conjugated (solid line) triplexes at pH 6.4:

Hoechst-$(eg)_6$-TTTTTTTTMTMTMTT
CGCCGCGCGCGCAAAAAAAAGAGAGAACCCGGCGCGCGC
GCGGCGCGCGCGTTTTTTTTCTCTCTTGGGCCGCGCGCG

Table 1. T_m Values for m^5C- and $m^{5ox}C$-Containing DNA Triplexes by a Tethered Hoechst Derivative[1,a]

Hoechst-$(eg)_6$-TTTTTTTT**XTXTXT**T
CGCCGCGCGCGCGAAAAAAAAGAGAGAACCCGGCGCGCGC
GCGGCGCGCGCGCTTTTTTTTCTCTCTTGGGCCGCGCGCG

Entry	...XTXTXT... [3]	Third Strand	Spermine	pH Value[2] 6.4	7.0	7.5	8.0
1		15mer	-	34	24	18	10
2	...MTMTMT...	Hoechst-15mer	-	48	38	29	18
3		15mer	0.5 mM	48	34	25	18
4	...MTMTMT...	Hoechst-15mer	0.5 mM	62	53	47	30
5		15mer	-	25	25	24	23
6	...CTCTCT...	Hoechst-15mer	-	33	30	30	31
7		15mer	0.5 mM	29	29	28	27
8	...CTCTCT...	Hoechst-15mer	0.5 mM	40	40	39	40

[a] The superscript numbers are used to denote the following. (1) Values are listed in °C ± 1 °C. (2) Thermal denaturation studies were performed in 10 mM PIPES, pH 6.4, or 7.0, 10 mM HEPES pH 7.5, or 8.0, 10 mM $MgCl_2$, and 50 mM NaCl (with or without 0.5 mM spermine) at 1:1:1 strand ratios at approximately 1 μM. (3) M = 5-methyl-2′-deoxycytidine, C* = 4-amino-1-(*β*-2′-deoxy-D-*erythro*-pentofuranosyl)-5-methyl-2,6(1*H*,3*H*)-pyrimidinedione ($m^{5ox}C$).[33]

Effects of G-C Base Pairs. Although it is necessary for the tether between the third stand and the minor groove binding agent to traverse the phosphoribose backbone, it was unclear as to the nature of the minimum acceptable minor groove

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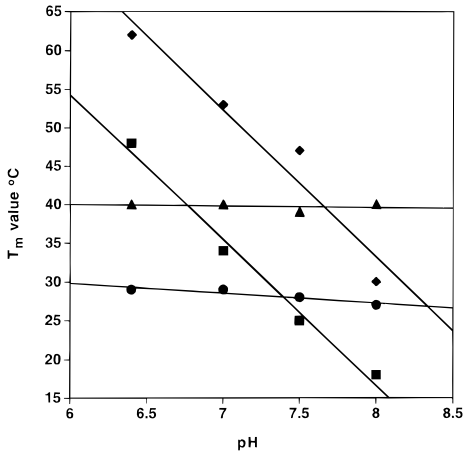

Figure 2. Comparison of the T_m values vs pH for the nonconjugated triplexes, entries 3 (■) and 7 (◆) of Table 1, with the corresponding conjugated triplexes, entries 4 (●) and 8 (▲) of Table 1.

Table 2. T_m Values for DNA Triplexes Tethering a Hoechst Derivative: Effects of Target Sequence[1,a]

Hoechst-(eg)$_6$-**XXXXXXX**TMTMTMTT
CGCCGCGCGCGC**YYYYYYY**AGAGAGAACCCGGCGCGCGC
GCGGCGCGCGCG**ZZZZZZZ**TCTCTCTTGGGCCGCGCGCG

Entry	-XXXXXXX... ...YYYYYYY... ...ZZZZZZZ...	Third Strand	pH Value[2] 6.4	7.0	7.5	8.0
1	-TTTTTTT...	15mer	34	24	18	10
2	...AAAAAAA... ...TTTTTTT...	Hoechst-15mer	48	38	29	18
3	-MTTTTTT...	15mer	38	28	18	nt
4	...GAAAAAA... ...CTTTTTT...	Hoechst-15mer	40	32	19	15
5	...C*TTTTTT...	15mer	32	25	18	10
6	...GAAAAAA... ...CTTTTTT...	Hoechst-15mer	42	32	20	15
7	-MMTTTTT...	15mer	38	26	15	nt
8	...GGAAAAA... ...CCTTTTT...	Hoechst-15mer	41	29	17	nt
9	-MTMTTTT...	15mer	38	26	15	nt
10	...GAGAAAA... ...CTCTTTT...	Hoechst-15mer	41	29	17	nt
11	-TTTTMTM...	15mer	47	35	26	nt
12	...AAAAGAG... ...TTTTCTC...	Hoechst-15mer	52	41	32	15

[a] The superscript numbers are used to denote the following. (1) Values are listed in °C ± 1 °C. (2) Buffer conditions (in the absence of spermine) as described in footnote 1 of Table 1.

binding site. Studies with DNA duplexes have indicated that a four base pair A-T rich minor groove site is required for effective binding by Hoechst 33258.[34−38] Hybridization studies with *duplexes* have indicated that a tethered Hoechst derivative can locate the requisite *double-stranded* A-T rich site at some distance from the site of the tether.[27] In the present work we examined the nature of the triplex minor groove binding site by preparing a series of three-stranded complexes that varied both the length and the position of the A-T rich minor groove. In the simplest alteration, we placed a G-C base pair at the end of the A-T rich binding site (entries 3 and 4, Table 2). This sequence alteration reduced the available binding site from eight A-T base pairs to seven, and moved it one base pair from the site of the tether. The addition of the additional M^+-G-C base triplet to this sequence increased the T_m for the nonconjugated triplex by 4 °C at pH 6.4 (entry 3, Table 2) relative to the triplex containing eight contiguous T-A-T base triplets, and this complex also exhibits the expected pH dependence. The incorporation of the tethered ligand to this triplex had relatively little stabilizing effect at pH 6.4, but was more effective at pH 8. The addition of the G-C base pair to the terminus of the duplex target site alters the nature of the minor groove (by the introduction of the N^2-amino group from the G residue). Additionally, third strand binding with the formation of a terminal M^+-G-C base triplet introduces an additional positive charge to the complex. In an attempt to separate these effects, we prepared a third strand sequence with a terminal C* residue, a neutral analogue of protonated cytosine.[33] With this sequence the minor groove still contains the N^2-amino group from the terminal G-C base pair, but no additional positive charge results at this site upon triplex formation. The triplex formed in this case (at pH 6.4) exhibits a 10 °C increase in the T_m value (compare entries 5 and 6, Table 2), similar to the 14 °C value observed for the complex containing an A-T rich terminal sequence (entry 2, Table 2). Two additional complexes (entries 7 and 9, Table 2) further shortened the A-T rich binding site, and moved it two and then three base pairs, respectively, from the site of the tether. The addition of the tethered ligand (entries 8 and 10, Table 2) increases these T_m values only marginally. A final sequence alteration is present with entry 11 (Table 2) in which the eight-residue A-T base pair binding site has been reduced to four (the minimum required for binding to duplex DNA), but it is located adjacent to the site of the tether. The 6 °C increase in T_m is more significant than that observed with corresponding complexes containing M^+-G-C base triplets within the putative binding site.

It is noteworthy that the elimination of the charged base triplet from the terminus of the three-stranded complex (entries 5 and 6, Table 2) results in a significant increase in the T_m value, relative to the complex of identical sequence, but containing a charged base triplet. One simple explanation for this effect is that, under the conditions of the assay, the piperazine ring of the Hoechst ligand also carries a positive charge, and ligand binding deep in the minor groove, in which the piperazine ring must approach the charged base pairs, may be modulated by charge−charge repulsion effects. On the basis of the comparison of the values for entries 1 and 2 with those for entries 5 and 6, such effects are clearly present. However, such an interpretation of the overall stability of the complexes with respect to pH is too simplistic. With two binding elements present in these complexes, the third strand and the minor groove ligand, the ΔT_m values will depend upon both the binding enthalpy of the third strand and the binding enthalpy of the ligand. While the T_m values for the third strands can be used to estimate relative enthalpy values for the nonconjugated triplexes, the binding enthalpies of the ligand are more complex. Tethering the ligand to the triplex may change the sequence specificity exhibited by the ligand and/or limit its ability to bind to a preferential binding site, or in the preferred binding orientation. The free energy of binding for these complexes will ultimately be determined by the competition of the ligand for its preferred minor groove binding site, and the third strand for its preferred major groove binding site—both mediated by the presence of the tether between the two binding entities.

Nevertheless, charge−charge effects appear to be present either directly or indirectly in virtually all of the triplexes containing additional M^+-G-C base triplets. As the pH of the

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Table 3. T_m Values for DNA Triplexes Containing Two Adjacent GTA Base Triplets and Tethering a Hoechst Ligand[1,a]

Hoechst-(eg)$_6$-TT**XXX**TTTMTMTMTT
CGCCGCGCGCGCGAA**YYY**AAAGAGAGAACCCGGCGCGCGC
GCGGCGCGCGCGCTT**ZZZ**TTTCTCTCTTGGGCCGCGCGCG

Entry	...XXX... ...YYY... ...ZZZ...	Third Strand	Spermine	pH Value[2] 6.4	7.0	7.5	8.0
1	...GGT... ...TTA...	15mer	-	31	23	15	nt
2	...AAT...	Hoechst-15mer	-	47	35	15	nt
3	...GGT... ...TTA...	15mer	0.5 mM	42	28	22	10
4	...AAT...	Hoechst-15mer	0.5 mM	62	45	27	11

[a] The superscript numbers are used to denote the following. (1) Values are listed in °C ± 1 °C. (2) Buffer conditions as described in footnote 1 of Table 1.

assay solution is increased, some loss of charged M^+-G-C base triplets occurs due to deprotonation effects. At pH 8 the triplexes are generally destabilized (note the odd-numbered entries of Table 2), and yet the conjugated triplexes often exhibit quite impressive T_m values. In all but one complex, the DNA triplex transition could be observed at pH 8.0 in the presence of the conjugated strand, while in four of the five complexes, no transition was observed for the nonconjugated triplex (the sole exception, entry 1 in Table 2, has the fewest pH-dependent M^+-G-C base triplets). In the conjugated complexes, the ligand may be more effective in binding to the minor groove structure at higher pH values; either due to the absence of charged base triplets or with the destabilization of the triplex, the minor groove becomes more duplex in character and the ligand can locate a preferred binding site. More effective binding by the ligand in the minor groove, from either effect, could stabilize the triplex by entropic means in the absence of protonation effects for base triplet stabilization. The only conjugated triplex that did not give a transition at pH 8.0 was entry 8 of Table 2. This complex has the A-T rich binding site moved furthest from the site of the tether and of the complexes tested is most likely to experience effects resulting from the ligand "reaching the end of its tether".

Enhancing the Stability of Weakly Bound DNA Base Triplets. Guanine can function in the parallel-stranded triplex motif to recognize a T-A base pair by the formation of a G-T-A base triplet.[39] Only a single hydrogen bond has been observed between the G residue of the third strand and the T-A base pair;[40] it has been proposed to involve the N^2-amino group of G and the O^4-carbonyl of T. With only a single interstrand hydrogen bond, the G-T-A base triplet is less stable than T-A-T or C^+-G-C base triplets. The tethered ligand approach could be advantageous in cases where less stable base triplets are involved by providing additional binding interactions to compensate for a weaker binding base triplet. To examine this possibility, we prepared a target sequence containing two adjacent T-A base pairs (entry 1, Table 3). By choosing this target sequence, the triplex minor groove provides a $(AATT)_2$ binding site. In DNA duplexes, the $(AATT)_2$ sequence represents one of the preferred binding sites for Hoechst 33258,[36] and similar preferences might exist in the minor groove binding sites of the DNA triplexes. The third strand used to form the DNA triplex contained three M residues (for targeting the G-C base pairs), ten T residues (for the A-T base pairs), and two G residues (for the T-A base pairs). The simple 15-mer triplex (entry 1, Table 3) exhibited a T_m value that was reduced from that observed for the triplex containing only T-A-T and M^+-G-C base triplets (see entry 1, Table 1). Introduction of the tethered Hoechst ligand results in variable increases in T_m values, most significantly from 12 to 16 °C at slightly acidic or neutral pH values. The addition of spermine results in moderate increases in the T_m values of the simple 15-mer triplex (entry 2, Table 3), and more dramatic effects with the tethered ligand (entry 4, Table 3). The combination of a tethered ligand and spermine results in triplexes that are significantly more stable than those prepared solely with T-A-T and M^+-G-C base triplets (compare entry 4, Table 3, with entry 4, Table 1). The combination of the ligand and the polyamine can enhance the T_m values for the triplexes by 20 °C or more. These enhanced T_m effects result from a combination of both ligand binding and triplex binding preferences and cannot at this time be detailed at the molecular level. Nevertheless, the use of the tethered minor groove ligand appears to enhance the stability of triplexes containing the less stable G-T-A base triplets.

Table 4. T_m Values for DNA Triplexes Containing an A-T Rich Ligand Binding Site Adjacent to the Triplex Target Site[1,a]

Hoechst-(eg)$_6$-MTMTTTTTMTMTMTT
CGCCGCGC**AAAAA**GAGAAAAAGAGAGAACCCGGCGCGCGC
GCGGCGCG***TTTTT***CTCTTTTTCTCTCTTGGGCCGCGCGCG

Entry	Third Strand	pH Value[2] 6.4	7.0	7.5	8.0
1	15mer	39	26	15	nt
2	Hoechst-15mer	58	45	35	20

[a] The superscript numbers are used to denote the following. (1) Values are listed in °C ± 1 °C. (2) Buffer conditions (in the absence of spermine) as described in footnote 1 of Table 1.

Effects of an Adjacent, Duplex, A-T Rich Ligand Binding Site. In the DNA triplexes initially examined, the sequence was designed such that the only minor groove A-T rich binding site available to the Hoechst ligand was that contained in the DNA triplex. An additional comparison could be made by providing a A-T rich binding site in the duplex region of the complex immediately adjacent to the triple helix (see Table 4). Two G-C base pairs were placed into the triplex target, generating a target sequence similar to that of entry 7, Table 2. This design should inhibit effective ligand binding to the triplex structure, but provide a five-base pair A-T rich binding site immediately adjacent in the duplex structure (see Table 4). At three of the four pH conditions used, the conjugated DNA triplex resulted in a 19−20 °C increase in the T_m value over the nonconjugated complex (compare entries 1 and 2, Table 4). The magnitude of these increased T_m values are similar to those we have reported previously[41] for the stabilization of DNA duplexes by a tethered Hoechst conjugate. The values obtained for this complex, with the A-T rich sequence in the duplex region of the complex, can be compared with two other complexes in this study. A comparison of the ΔT_m values for the conjugate in which the tethered ligand binds to the minor groove of an A-T rich DNA triplex (entry 2, Table 1), and the corresponding complex in which the ligand binds to an adjacent, A-T rich, DNA duplex (entry 1, Table 4), suggests that more effective binding occurs, as expected, to the duplex structure. This difference is more significant if the complex containing the identical triplex sequence (but no A-T rich target) is used for comparison (entry 10, Table 2).

We note that a previous paper[18] has concluded that Hoechst 33258, when present in an untethered state, destabilizes DNA triplexes. The differences in these earlier conclusions and the present ones may stem in part from variations in the experimental protocols. We have used a single molecule of Hoechst 33258 tethered to an oligonucleotide for each three-stranded

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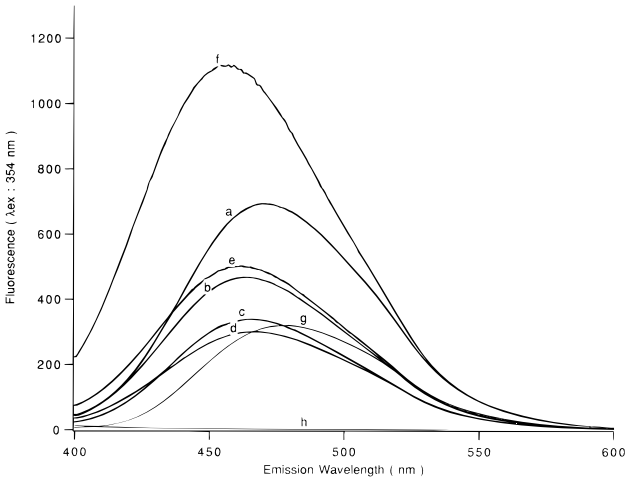

Figure 3. Fluorescence emission spectra for the triplexes described in Table 2: (a) entry 2, (b) entry 4, (c) entry 6, (d) entry 8, (e) entry 10, (f) entry 2, Table 4, (g) single-stranded conjugate of entry 2, Table 2, (h) Hoechst 33258. See the Experimental Section for details.

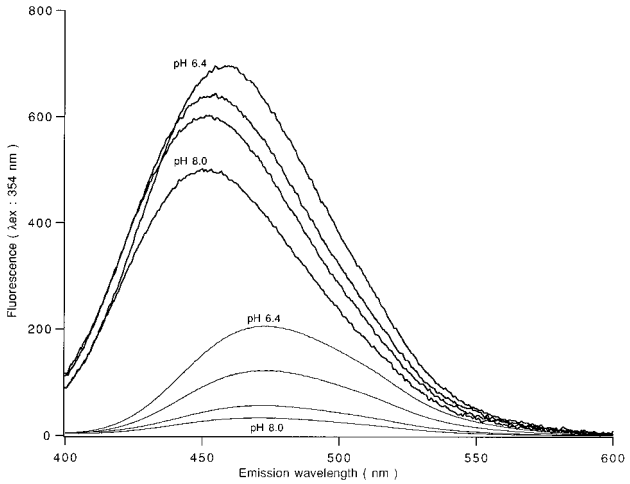

Figure 4. Fluorescence emission spectra for the triplex described in entry 2, Table 1, at pH values 6.4, 7.0, 7.5, and 8.0 (upper scans). Spectra for the single-stranded conjugate are illustrated in the lower scans.

complex; under these conditions the hybridization process then limits the ratio of ligand to triplex to 1:1. At this ratio, and with the dye tethered, we observe significant stabilization of the DNA triplexes. In the previous study, free untethered Hoechst 33258 was employed at a ratio of ligand to triplex in excess of 1:1. Since it is known that the Hoechst dyes have multiple modes of binding to DNA duplexes,[42] similar effects might be expected for the DNA triplex. Additionally, as triplex dissociation occurs with the conjugate described in this work, and the minor groove becomes more double-stranded in character, the ligand portion of the conjugate may remain effectively bound to the target sequence after dissociation of the third strand. With the ligand bound to the minor groove, and tethering the third strand, a high local concentration of third strand results and leads to an entropic stabilization of the conjugated triplex.

Fluorescence Characteristics of Hoechst-Conjugated DNA Triplexes. Fluorescence Studies. Upon binding of the DNA−Hoechst conjugate to the target duplexes, enhanced emission spectra result (Figure 3a). The magnitude of the attendant fluorescence signal, relative to the single-stranded conjugate, is not as dramatic as typically observed for double-stranded complexes, and is dependent upon the pH value of the solution. For example, the fluorescence emission spectra were measured under a variety of pH conditions for both the single-stranded conjugate and the corresponding DNA triplexes (Figure 4). As typified by the triplex corresponding to entry 1 (Table 2), a significant decrease in quantum yield with increasing pH was observed (Figure 4). We have therefore compared the emission spectra of the various conjugated complexes of Table 2 in Figure 3a−e at pH 6.4. In general, the increases in fluorescence quantum yield for the binding of the single-stranded conjugate to the target duplex was relatively moderate (compare parts g and a of Figure 3), largely due to the relatively strong emission signal from the single-stranded conjugate (compare parts g and h of Figure 3). The magnitude of the emission spectra, for triplexes of varying sequence, appeared to be related to the ease in targeting regions of contiguous T-A-T base triplets that define the minor groove binding site (compare parts a−e of Figure 3). The most fluorescent complex (Figure 3a) was that containing a region of eight T-A-T base triplets (entry 2, Table 2). As M^+-G-C base triplets were located near the site of the tether (entries 4, 6, and 8, Table 2), the ligand must reach across these residues to locate the T-A-T rich sequence. The reduced emission effects (Figure 3b−d) may reflect less than optimal binding by the ligand. However, when the ligand was provided an T-A-T rich sequence adjacent to the tether, but only four residues in length (entry 10, Table 2), a moderate increase in quantum yield (relative to entries 4, 6, and 8) was observed (Figure 3e).

Enhanced quantum yield effects for these bisbenzimidazole fluorophores appear to be related to the absence of nonradiative relaxation processes, largely collisional effects with solvent water molecules.[43] When bound deep in the minor groove of duplex structures, such effects are reduced and radiative relaxation effects predominate. When the tethered Hoechst derivative is directed to the minor groove of the DNA triplex containing eight contiguous T-A-T base triplets, the most significant quantum yield enhancement was observed, although this value was still less than that observed with the corresponding duplex (see the next section). The various sequence effects observed may again reflect charge−charge interactions between the protonated Hoechst dye and the nearby M^+-G-C base triplets, or the presence in the minor groove of the guanine N^2-amino group, and such effects may ultimately prevent the ligand from reaching the desired binding site, or prevent deep penetration by the dye into the groove structure.

We also examined the fluorescence characteristics of the complex in which the A-T rich binding site was present in the duplex region adjacent to the DNA triplex (see entry 2, Table 4). In this complex the tethered Hoechst dye is presented with a minor groove target in the *duplex* portion of the complex. The triplex portion of this complex contains two M^+-G-C base triplets to inhibit binding by the Hoechst derivative to the triplex portion of the minor groove. The conjugate of Table 4 exhibited an enhanced emission spectrum whose magnitude was more pronounced than that of any of the triplex-bound dye conjugates (Figure 3f). These differences, illustrated in Figure 3, most likely reflect variations in minor groove structure for the triplex and duplex, and suggest that while minor groove binding by the Hoechst conjugates to the triplex minor groove does increase overall stability and enhance the fluorescence emission spectra, the corresponding effects, when the duplex minor groove in involved, are more dramatic. It appears that the presence of

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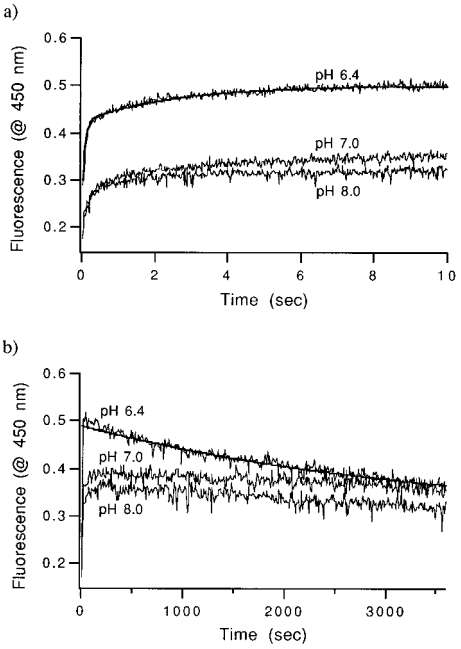

Figure 5. Stopped flow experiment for the formation of the triplex described in entry 2, Table 1: (a) Initial binding process as measured by an increased fluorescence signal over a 10 s time period, (b) second process as measured by a decreased fluorescence signal over a 3600 s time period. Fitted curves have been added to the experimental data collected at pH 6.4 in both cases.

the third strand in the major groove of the DNA duplex results in some modulation of the minor groove structure and reduces the effectiveness of minor groove binding ligands as has been noted in numerous other studies.

The Order of Ligand and Triplex Binding. Formation of the Hoechst-tethered DNA triplexes is likely to occur by one of two ordered processes: (i) the DNA triplex is formed first and ligand binding then proceeds or (ii) the ligand binds to the A-T rich duplex, and this event is followed by DNA triplex formation. It should be possible to discriminate between the order of these two processes on the basis of the nature of the fluorescent signal generated. In the first case, the fluorescence signal characteristic of complex formation should increase as the result of a first-order unimolecular process, while in the second case, this signal would increase as the result of a second-order bimolecular process. Additionally, with the first process the fluorescence signal would build up to some maximum value, characteristic of the triplex/fluorophore complex, while in the second process, the initial signal for the duplex/fluorophore complex should reach a maximum value, and then on the basis of the difference in quantum yield for the duplex vs triplex minor groove complex, the signal should drop off as triplex formation occurs.

Under stopped flow conditions, we examined complex formation as a function of fluorescence emission for the triplexes illustrated in Table 2. All of the complexes exhibited two emission vs time processes. The first process was completed with a half-life of between 0.5 and 1.3 s, and this process could be fit to a second-order exponential equation (see Figure 5a). This process is consistent with an initial bimolecular interaction between the target and the conjugate in which the tethered Hoechst dye binds the A-T rich minor groove (prior to triplex formation). The second process involved a reduction in the magnitude of the fluorescence quantum yield over a period of at least 1 h and could be fit to a first-order exponential process (Figure 5b, pH 6.4). We interpret this slower second process as reflecting the third strand binding to the DNA duplex to form the triplex. To further confirm this interpretation of the binding order, we repeated the stopped flow analyses at neutral and basic pH values. The first process, illustrated in Figure 5a, remained largely the same, although the intensity of the fluorescence signal decreased with increasing pH as expected (see Figure 4). However, the second process appeared to disappear at higher pH values (compare signals at pH values 7.0 and 8.0 with that of pH 6.4, Figure 5b). This result is consistent with a much slower rate of triplex formation at higher pH values owing to the necessity of protonation of the M^+-G-C base triplets. The observed reduction in quantum yield as the triplex is formed (Figure 5b, pH 6.4) suggests that there is a change in the nature of the minor groove structure as the DNA duplex is converted to a DNA triplex. The decrease in quantum yield with this transformation could be the result of the Hoechst ligand not fitting as tightly into the triplex minor groove as it does in the duplex. A "looser" fitting complex may permit access of water to the dye excited state and result in more of the noted nonradiative relaxation processes. These stopped flow experiments are not conclusive, but we believe highly suggestive that, after equilibrium is reached, the complex is composed of (i) a third strand bound in the major groove and (ii) the tethered Hoechst ligand bound simultaneously in the minor groove.

Conclusions

Tethering the Hoechst 33258 fluorophore to the 5′-terminus of the pyrimidine third strand of parallel-stranded DNA triplexes results in enhanced stability that is dependent upon both the pH of the solution and the sequence of the minor groove. The presence of M^+-G-C base triplets interferes with the enhanced stabilization of the triplex structure by the tethered ligand. The Hoechst ligand is very effective in stabilizing G-T-A base triplets, in part because the $(AATT)_2$ minor groove may offer the ligand a preferred binding site. Binding to the triplex results in an enhanced quantum yield for the emission spectra, the magnitude of which is dependent upon the sequence effects. Stopped flow experiments have provided some insight into the nature of the process; rapid ligand binding to the A-T rich minor groove of the duplex target is then followed by a slower process, triplex binding, which can be monitored by a second process involving a decrease in quantum yield.

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