Binding of Hoechst 33258 and 4',6-Diamidino-2-phenylindole to Self-Complementary Decadeoxynucleotides with Modified Exocyclic Base Substituents†

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ABSTRACT: Fluorescence titrations have been carried out to determine the association constants (K_a) for binding of the dyes Hoechst 33258 and DAPI to the self-complementary decamer d(CTGAATTCAG) and nine duplex derivatives with exocyclic substituent changes in the six central base pairs. Many K_a values are in the range (2–5) × 10^8 (duplex M)$^{-1}$ at 5.5 °C. Replacement of the leftmost adenine by 2-aminopurine in the sequence decreases K_a for Hoechst 33258 by a factor of 170. When the centermost adenine is replaced by 2-aminopurine, K_a for Hoechst 33258 and DAPI is too small to be evaluated. When the centermost adenine is replaced by purine, K_a for both dyes increases, but this very stable duplex–Hoechst 33258 complex is nonfluorescent. The measured affinities are compared to expectations derived from X-ray studies with dodecamer–dye complexes having an identical central binding sequence (Pjura et al., 1987; Teng et al., 1988; Larsen et al., 1989).

The dyes Hoechst 33258 and DAPI[1] are nonintercalating groove-binding DNA ligands that become brightly fluorescent when they bind to the minor groove of B-DNA, preferentially at 5 and 3 contiguous A·T base pairs (Zimmer & Wähnert, 1986). They are useful to specifically detect DNA and chromosomes in the light microscope (Latt & Wohleb, 1975; Stokke & Steen, 1986) and to quantify DNA in solution (Kapuscinski & Skoczylas, 1977). The complex of Hoechst 33258 with d(CGCGAATTCGCG) has been investigated by using X-ray diffraction by two groups (Pjura et al., 1987; Teng et al., 1988), and although the molecular constitution of the crystals is identical, they propose different binding contacts. Recently, Carrondo et al. (1989) have solved the structure of Hoechst 33258 bound to d(CGCGATATCGCG). The crystal structure of the DAPI–d(CGCGAATTCGCG) complex has been reported by Larsen et al. (1989).

We have recently found (Loontiens et al., 1990) that the association constants (K_a) for Hoechst 33258 binding to certain DNA sequences can be much higher than previously assumed: at 25 °C $K_a = 6 \times 10^8$ M^{-1} for the sites with the highest affinity in calf thymus DNA (an average of one independent site per 100 base pairs), and $K_a = 3.8 \times 10^8$ M^{-1} for binding to a contiguous 5 A·T base pair site in poly[d(A-T)] (accounting for excluded-site binding; McGhee & von Hippel, 1974). For binding to d(CCGGAATTCCGG) and d(CGCGAATTCGCG), with DNA concentrations expressed as a duplex, the K_a values are 3.2×10^8 M^{-1} and 4×10^8 M^{-1}. We have also shown that DAPI and Hoechst 33258 have comparable affinities for DNA (Loontiens et al., 1989b). Fluorescence lifetime measurements have shown that complexes of Hoechst 33258 (Kubota et al., 1986) and DAPI (Szabo et al., 1986) with repetitive- and heterogeneous-sequence DNA possess at least two decay processes (0.2–0.5 and 2.5–3.9 ns); the relative amplitudes depend upon the nucleic acid sequence and the ionic strength of the solution. For DAPI, these times are related to the two lifetimes observed in solution in the absence of DNA (Szabo et al., 1986; Barcellona & Gratton, 1990). The slower decay time of the DAPI–DNA complexes has been attributed to the bound DAPI molecules which are well removed from direct contact with the solvent molecules. DAPI complexes with poly[d(A-T)] and poly(A)·poly(T) at low dye/base pair ratios display only a single exponential decay (3.9 ns; Barcellona & Gratton, 1990), although complexes with poly[d(A-U)], at the same low dye/base pair ratios, have been reported to have two lifetimes (1 and 7 ns; Härd et al., 1990). The results of Barcellona and Gratton (1990) indicate that the multiple fluorescence decays observed with heterogeneous sequence DNA are a reflection of the variety of nucleotide binding sequences. When there is only a single type of binding sequence [e.g., poly[d(A-T)] and poly(A)·poly(T)], the observation of a single fluorescence lifetime at low dye to base pair ratios may correspond to a single DAPI–DNA complex, as we observe in the titrations with the well-defined short oligonucleotides reported here.

We report the affinities of Hoechst 33258 and DAPI for a series of self-complementary decadeoxynucleotides with exocyclic base-substituent changes in the six central base pairs of the parent compound d(CTGAATTCAG). The goal of these experiments was to better understand the solution structures of the tight complexes formed between these minor groove binding drugs and DNA; the details of the DNA binding surface were probed by perturbing the chemical and geometrical structure of the DNA molecule. Short oligomers were chosen to limit the extent of the binding site. Our approach minimally perturbs the DNA structure with small and well-defined chemical changes in the oligonucleotides in order to identify the important favorable and unfavorable interactions controlling the strong binding of the two dye molecules

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[1] Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.

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in solution. The K_a values for the parent compound are comparable to those we have measured with poly[d(A-T)] and poly(dA)·poly(dT) and with the highest affinity sites on calf thymus DNA. Some modifications in the parent compound increase K_a by a factor of 10. Remarkably, a very stable DNA duplex–Hoechst 33258 complex is nonfluorescent.

Materials and Methods

The buffer for all experiments was 50 mM Tris and 100 mM NaCl (pH 7.5). Hoechst 33258 was a gift from Dr. Loewe (Frankfurt), and DAPI was obtained from Serva. Working stock solutions of the dyes (140 nM and larger) were prepared daily by dilutions from a stock solution (1–5 mg of dye/mL of water) with 5 mM HCl in polystyrene tubes and vortex mixing. These working solutions were stable for at least 24 h. The dye concentrations were determined spectrophotometrically at pH 7.5 in polystyrene cuvettes (to which a neutral solution of a dye does not adsorb) using $\epsilon_{338} = 4.2 \times 10^4$ M^{-1} cm^{-1} for Hoechst 33258 (Latt & Wohlleb, 1975) and $\epsilon_{342} = 2.3 \times 10^4$ M^{-1} cm^{-1} for DAPI (Kapuscinski & Skoczylas, 1977).

The decadeoxynucleotides (Table I) were synthesized on an Applied Biosystems 381 DNA synthesizer and purified by HPLC on a reversed-phase column (McLaughlin & Piel, 1984), and their stock solutions (25–30 A_{260} units/mL in 10 mM Tris, 5 mM NaCl, and 2 mM EDTA, pH 7.0) were stored at −80 or −25 °C. DNA concentrations were determined at 260 nm and expressed as duplexes using a common value $\epsilon_{260} = 1.53 \times 10^5$ M^{-1} cm^{-1}; to avoid complications due to DNA-strand dissociation upon dilution, the A_{260} reading (between 0.2 and 0.25) was taken 1 min or less after diluting the stock solution (0 °C) into the buffer at 21 °C.

The fluorescence instrument has been constructed in the laboratory; high sensitivity and stability are achieved by chopping the light from a stabilized 150-W halogen–quartz lamp and employing lock-in phase detection (Ithaco Lock-In 397EO, Ithaca, NY) of the photomultiplier (EMI 9558QB) current. The lamp intensity was continuously monitored and electrically divided into the lock-in output. The fluorescence emission (excited at 365 nm, or sometimes 355 nm) was observed through a light filter that passes wavelengths longer than 450 nm (Schott KV450 cutoff filter). Before each titration, quartz cuvettes and glass stirrers were cleaned with Nochromix (Godax Laboratories, New York) and silanized (2% dimethyldichlorosilane in 1,1,1-trichloroethane, from BDH). To obtain a reproducible fluorescence signal, these precautions to prevent dye–surface adsorption were strictly necessary with DAPI. Cuvettes (1 × 1 × 4.5 cm) initially contained 2.270 mL of buffer and were not removed from the instrument holder (5.5 °C) during subsequent additions; solutions were mixed with motor-driven glass stirrers. A small volume (10–20 μL) of the dye solution was added, and the stable fluorescence intensity of the 1 nM dye in the cuvette was measured. The DNA duplex titrant (with 1–30 A_{260} units/mL, depending upon K_a) was then added in 0.11-μL portions from a 5-μL syringe kept at 0 °C. Throughout this paper, a T_m value refers to the melting temperature of a duplex (Table I) determined at 0.5–0.7 A_{260} units/mL (McLaughlin et al., 1987). To minimize the melting of duplexes with T_m < 31 °C, the corresponding titrations were carried out at 5.5 °C and limited to about 10 titrant additions over a period of 3 min or less. With all other duplexes, each digitized fluorescence reading was computer averaged for 30 s. The data were corrected by making a blank titration without dye. This correction was often negligible; it was always less than 5% of the maximal fluorescence for saturating concentrations of the parent duplex (**1**) or poly[d(A-T)]. The K_a values in Table I are defined for the association reaction

$$\text{decamer duplex} + \text{dye} \Leftrightarrow \text{decamer duplex–dye} \quad (1)$$

and correspond to affinities per duplex. The experimental increase in fluorescence, relative to the fluorescence of the input concentration of the free dye, was simulated by the full quadratic binding expression for reaction 1, taking into account that the concentration of the reaction components is diluted during a titration. The measured 5–85% fluorescence increase was interpreted as arising from the formation of a single fluorescent complex.

Results and Discussion

The Dodecamer Sequences Are Self-Complementary, and the Double-Stranded Duplexes Are Formed from Two Identical Single-Stranded Molecules. Each nucleotide modification in the single-stranded oligomers will appear twice in the double-stranded duplex. Thus a single modification in the nucleotide sequence can affect the dye–duplex interactions at two different positions of the dye molecule within the minor groove. The DNA duplexes free of dye have a dyad axis in the center of the duplex sequences and are symmetrical. When binding in the minor groove of the dodecamer duplex (see Figure 2), the dye molecule must choose one of two orientations along the floor of the minor groove, which are stereochemically identical. Once the dye is bound, the long axis of the duplex–dye complex acquires an orientation. For practical reasons we represent the sequence of the 5′→3′ strand only as the single strand in Table I, but in the discussion of the data the dual modification in the duplex is implicitely assumed.

In Order to Determine a K_a Value, It Is Necessary To Use Concentrations of Dye and DNA Low Enough So That the Complex Is Partially Dissociated. Since the duplexes are themselves double-stranded bimolecular complexes, they will dissociate into the single strands at sufficiently low concentrations. This limiting concentration range depends upon the stability of the duplex, and therefore upon the solution conditions, such as the ionic strength, pH, and temperature. The ionic strength of 0.1 M NaCl and the temperature of 5.5 °C were chosen as a precaution to stabilize the duplexes. The T_m and K_a values for binding of the parent decamer and its derivatives with Hoechst 33258 and DAPI at 5.5 °C are presented in Table I. The K_a values were determined by titrating a fixed dye concentration (1 nM) with an increasing DNA duplex concentration; the dye/duplex ratios were kept low to exclude binding phenomena other than the site-specific one (Loontiens et al., 1990).

Dilution of concentrated duplex solutions with 38 °C < T_m < 42 °C to $A_{260} \approx 0.001$ in a titration mixture at 5.5 °C did not cause melting problems; evidence for this comes from the steady fluorescence readings at any point in the titration and the good fit of the titration data to a binding equation, even at the start of the titration. With the lower melting duplexes (T_m 28–30 °C), slow melting upon dilution can occur at 5.5 °C, especially at the lower duplex concentrations. For instance, for 1 nM DAPI and compound **4** with $A_{260} = 0.001$, and for 1 nM DAPI or Hoechst 33258 and compound **5** with $A_{260} = 0.0005$, the dye–duplex fluorescence decreases to a constant value (comparable to the one for the free dye) over a period of 15–20 min. This is due to slow dissociation of the complex, not to adsorption of the dye to the cuvette walls. In the fluorescence titrations with the lower T_m duplexes, artifacts due to melting were avoided by carrying out the whole titration within 3 min or less. The reliability of this approach is illustrated by the following properties of the lowest T_m duplex

Table I: Association Constants of Hoechst 33258 and DAPI for Binding with the Parent d(CTGAATTCAG) and Its Derivatives at 5.5 °C

	duplex[a] (with position in sequence)											$10^{-8}K_a$[c] (M^{-1})	
compd no.	1	2	3	4	5	6	7	8	9	10	T_m[b] (°C)	Hoechst	DAPI
1	d(C	T	G	A	A	T	T	C	A	G)	40	5.1	2.9
2	d(C	T	G	$_+2^-$	A	T	T	C	A	G)	30	0.030	0.18
3	d(C	T	G	A	$_+2^-$	T	T	C	A	G)	30	<0.001	<0.001
4	d(C	T	G	A	P^-	T	T	C	A	G)	28	high;[d] no signal	24
5	d(C	T	G	P^-	A	T	T	C	A	G)	29	3.4	2.8
6	d(C	T	G	$I^<$	A	T	$M^>$	C	A	G)	42	3.7	1.4
7	d(C	T	I	A	A	T	T	C	A	G)	28	4.1	3.2
8	d(C	T	G	A	A	T	U°	C	A	G)	39	4.7	2.5
9	d(C	T	G	A	A	U°	T	C	A	G)	38	3.7	8.1
10	d(C	T	$D^>$	A	A	T	T	$U^<$	A	G)	39	13	9.1

[a] P = purine; 2 = 2-aminopurine; D = 2,6-diaminopurine; I = inosine; M = 5-methylcytosine. Whether, with respect to the parent sequence **1**, a substituent has been changed in the minor groove, or in the major groove, is indicated by a symbol subscripted to the left, or superscripted to the right, of the base printed in boldface. Deletion or addition of an NH_2 group is indicated by − or +; thus $_+2^-$ means that in 2-aminopurine an NH_2 has been added in the minor groove and an NH_2 group has been removed from the major groove, as compared to the original adenine base. With P^-, there is only a single hydrogen bond due to the deletion of an amino group from the major groove. The right-superscripted symbol °, as in U°, is for deletion of a CH_3 group. Substitution of NH_2 by keto O is indicated as superscript < and substitution of keto O by NH_2 as superscript >; this results in a donor–acceptor interchange of the hydrogen bond near the major groove; due to the additional NH–N interchange, the direction of the hydrogen bond in the center of the base pair is also changed. [b] Determined at 0.5–0.7A_{260} units/mL in 100 mM NaCl, 10 mM $MgCl_2$, and 10 mM Tris (pH 7.5) (McLaughlin et al., 1987). [c] DNA concentration expressed as duplex and corresponding to site affinities; when expressed as base pairs, the K_a values are smaller by a factor of 10 (see Table II). [d] See Table III.

4: (a) the data fit a theoretical titration curve (see Figure 1A, top curve), and (b) this compound has the highest affinity for DAPI and also for Hoechst 33258; appreciable melting of the duplex would lower the apparent affinity of the dyes for the DNA. As the titration proceeds, the duplex concentration increases and any tendency of the DNA to dissociate into single strands diminishes.

Titration of DAPI and Hoechst 33258 with the Parent Decamer d(CTGAATTCAG) (1). The data are presented in Figure 1. The fluorescence of the DNA–Hoechst 33258 complex is about 30% more intense than that of the DNA–DAPI complex, except when A is substituted by P. For **1** and several of the modified duplexes in Table I, K_a for Hoechst 33258 is in the range (3–5) × 10^8 M^{-1}, which is only slightly but consistently larger than K_a = (2–3) × 10^8 M^{-1} for the binding of DAPI to the same compounds. Table II shows that these K_a values (with DNA concentrations expressed as base pairs and as sites) for the two dyes are very close to those we have obtained (Loontiens et al., 1989b, 1990) under identical conditions with d(CCGGAATTCCGG), poly[d(A-T)], and poly(dA)·poly(dT) (unpublished data) and, with Hoechst 33258, also with calf thymus DNA. For Hoechst 33258 we have measured the affinity of the low-abundance, high-affinity sites on calf thymus DNA to be K_a = 6 × 10^8 (site $M)^{-1}$ (Loontiens et al., 1990); these sites occur with a frequency of about 1 in 100 base pairs. With calf thymus DNA and Hoechst 33258 we have also observed a wide range of affinities of sequence-specific sites with K_a values ranging from ≈1 × 10^9 to 1 × 10^6 M^{-1}; which sites are observed depends upon the concentration of the dye and the ratio of the dye to base pairs. Photofootprinting experiments at relatively high concentrations of binding species have provided evidence of a variety of sequences providing specific binding sites for Hoechst 33258 and DAPI (Jeppsen & Nielsen, 1989); Manzini et al. (1983) and Kubista et al. (1987) have also previously reported more than one binding mode with calf thymus DNA. Our K_a values are higher than those earlier reported in the literature which have often been considerably underestimated. Many of the determinations and discussions of the affinity constants for these two dyes with DNA in the earlier literature must be interpreted with caution, because the concentration of the binding species are far too high to render a determination of K_a. Our titrations at low occupancy and low concentrations reported here are not related to the weaker or unspecific binding phenomena involving dye–dye interactions and binding to backbone phosphate which have been suggested for DAPI (Manzini et al., 1983; Kubista et al., 1987) and Hoechst 33258 (Steiner & Sternberg, 1979; Loontiens et al., 1990). With Hoechst 33258 these weaker complexes seem to involve five minor groove and two phosphate-mediated complexes (Loontiens et al., 1990). In contrast, our Job plots with DAPI and poly[d(A-T)] at 15–30 μM show only two equivalence points: one for 1 DAPI per 3 A·T pairs, without evidence for binding of a DAPI dimer, and one for 1 DAPI per phosphate (Loontiens et al., 1989a).

Table II: Comparison of K_a Values at 5.5 °C for Hoechst 33258 and DAPI Binding with the Parent Decamer Duplex and Other DNAs[a]

	$10^{-8}K_a$			
	Hoechst 33258		DAPI	
	M^{-1} (bp)	M^{-1} (sites)	M^{-1} (bp)	M^{-1} (sites)
d(CTGAATTCAG)	0.51	5.1	0.29	2.9
d(CCGGAATTCCGG)	0.6	7.2	0.43	5.2
poly[d(A-T)]	0.29	2.9	0.58	2.9
poly(dA)·poly(dT)	0.085	0.85	0.27	1.3
calf thymus DNA[b]	0.07	6	*c*	

[a] K_a values, determined by titrating a 1 nM dye solution with an excess of DNA, are expressed in base pairs (bp) and in sites; the latter correspond to the molarity of the decamer and dodecamer duplexes. The data are taken from Table I, from Loontiens et al. (1989b, 1990), and from our unpublished data. For the two A-T polymers, the effect of statistically excluded binding (McGhee & von Hippel, 1974; Zasedatelev et al., 1971) of the dye covering n base pairs, n = 5 or 6 for Hoechst 33258 (Loontiens et al., 1990) and n = 3 for DAPI (Kapuscinski & Szer, 1979; Loontiens et al., 1989a), is not apparent at the low DNA occupancy. Therefore, K_a is given also with the concentration of the A-T polymers expressed as the number of base pairs N according to the independent-site model with $N = 2n - 1$ (Schellman, 1974; Macgregor et al., 1985). Thus, N = 10 for Hoechst 33258 and N = 5 for DAPI binding to poly[d(A-T)] and poly(dA)·poly(dT). For Hoechst 33258 binding to calf thymus DNA, K_a is for the sites with the highest affinity that occur once per N = 80 base pairs (Loontiens et al., 1990). The small K_a values for Hoechst binding to poly(dA)·poly(dT) are due to an endothermic reaction ($\Delta H°$ = +8.6 kcal mol^{-1}) that contrasts with the exothermic binding to poly[d(A-T)] and d(CCGGAATTCCGG) with $\Delta H°$ = −4 to −7 kcal mol^{-1} (unpublished results). [b] Determined at 25 °C. [c] Estimated for the high-affinity mode by Manzini et al. (1983) and Kubista et al. (1987) as (1–2) × 10^7 M^{-1} (in bp) at 25 °C and 100–10 mM NaCl.

Base Substitutions Outside the Central AATT Region of the Duplex or Anywhere in the Major Groove Do Not Sig-

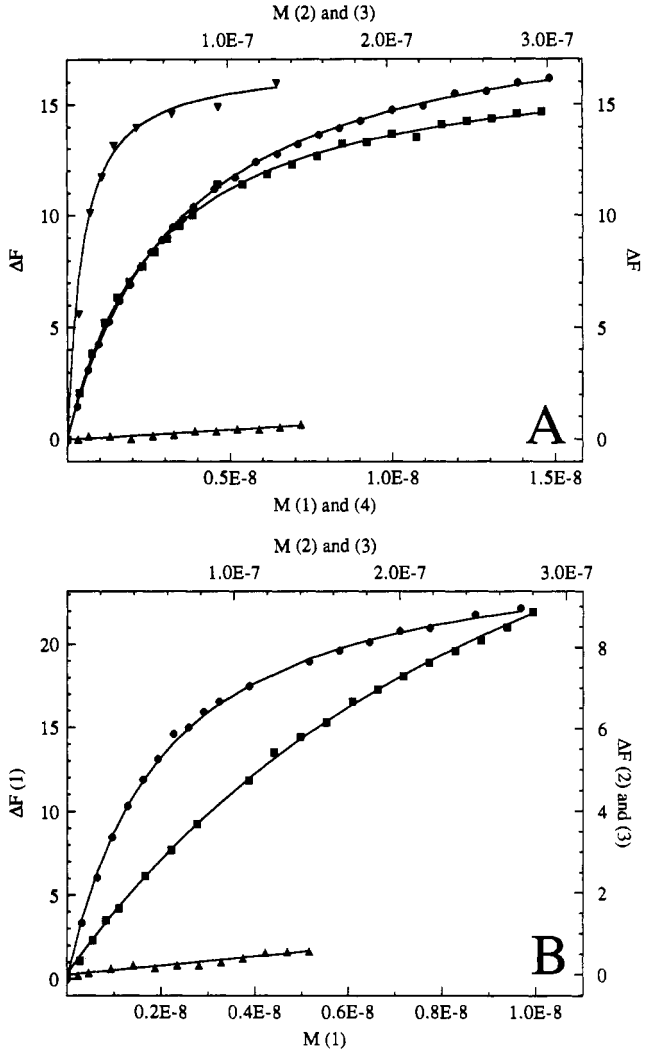

FIGURE 1: Fluorescence titrations of DAPI (A) and Hoechst 33258 (B) with the decamer duplexes **1**–**4**. To 2.287 mL of the dye solution (1.0 nM, 5.5 °C, pH 7.5) was added a decamer duplex solution (between 6.6 and 164 μM; 0 °C) in successive 0.11-μL aliquots. The fluorescence increase, ΔF, is expressed relative to the fluorescence of each free dye, and the data have been corrected for a corresponding blank titration without dye. In (A) and (B), a given sequence is represented by the same symbol: **1** (●), **2** (■), **3** (▲), and **4** (▼). The small increase in dye fluorescence observed with **3** is 4 times larger than the blank for this duplex. With **4** and DAPI, the titration was done in 2.5 min; compound **4** did not increase the fluorescence of Hoechst 33258. The curves are simulations of the experimental data using the K_a values in Table I.

nificantly Decrease the Binding Constants of the Dyes. This is illustrated in Table I and Figures 3 and 4. For instance, removing the 2-amino group from the minor groove at position 3 (G to I substitution, **7** of Table I) hardly changes the affinity of the dyes for DNA. Replacing G_3 by 2,6-diaminopurine and C_8 by U as in **10**, which reverses the direction of two out of three hydrogen bonds, actually increases the affinity for both dyes. At this stage a detailed explanation for the increased K_a values with **10** cannot be given. Also, many nucleotide substituent changes located in the major groove (Table I), whether inside or outside of the AATT region, do not disturb the binding free energy upon formation of the duplex–dye complex (see Table I and Figure 4). Some modifications in the major groove of the central AATT sequence, such as replacement of A_4 in **1** by purine as in **5**, or replacements of A_4 by inosine together with T_7 by 5-methylcytosine as in **6** with a reversal in direction of the two hydrogen bonds, or replacement of T_7 alone by U as in **8**, hardly change K_a for

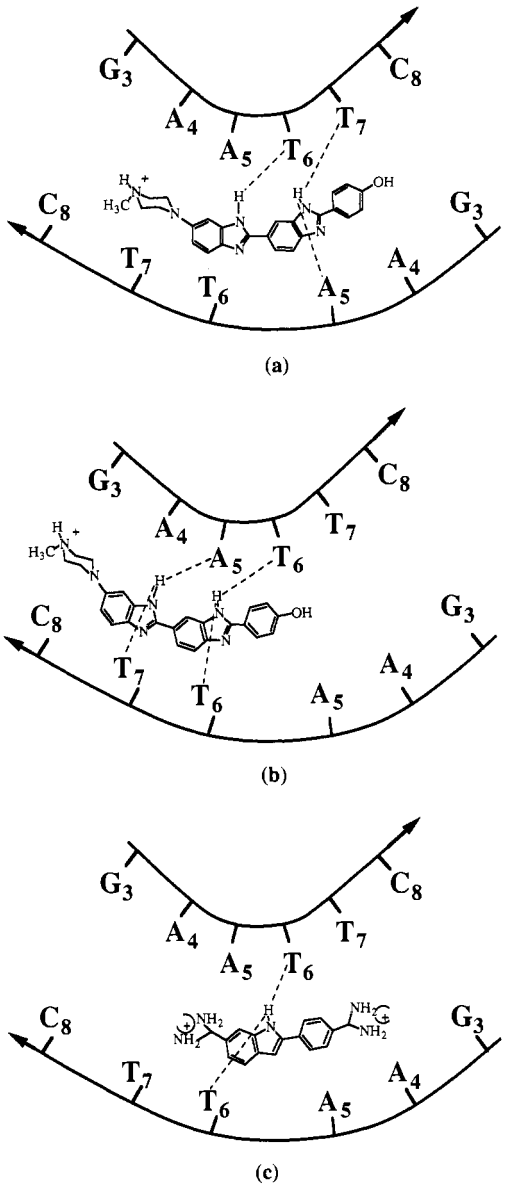

FIGURE 2: Schematic views of the GAATTC center sequence of the parent oligonucleotide showing the relative placement of the Hoechst 33258 molecule (a and b) and DAPI (c) within the minor groove according to the Teng et al. (1988) model (a), the Pjura et al. (1987) model (b), and the Larsen et al. (1989) model (c). The numbering of the nucleotides in the self-complementary strands is according to the sequence designation given in Table I. The top sequence runs 5′–3′ from left to right, and the bottom sequence is 5′–3′ from right to left. The dye is shown schematically in the minor groove; the actual three-dimensional configuration of the dye–DNA complex has the dye rotated 90°, such that the top edge of the molecule with the designated hydrogen bonds (dashed lines) are pointing into the minor groove. The proposed hydrogen bonds, shown as dashed lines, are between the benzimidazole nitrogens to the 3′-nitrogen of adenine or to the 2′-oxygen of thymine.

Hoechst 33258 or DAPI. The modification **6** decreases the K_a for DAPI by a factor of 2. The replacement of T_6 by U in **9** noticeably increases K_a for DAPI only; the reason for this is not clear but may result from increased steric freedom in the major groove due to the deleted methyl group. Such a local change in the major groove may locally affect the minor groove dimensions, which may have a more pronounced effect upon the smaller DAPI molecule than the more extended Hoechst 33258 molecule. The fact that many of the above derivatives of the parent sequence do not significantly affect the binding constant can reflect possible compensating structural adaptations of the dye–DNA complex to produce a tight binding

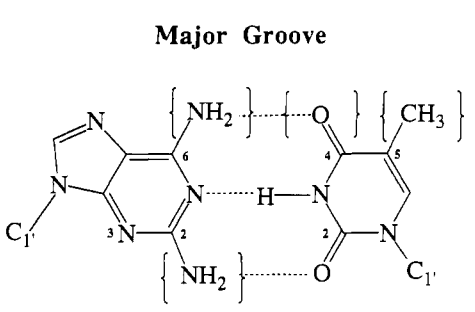

FIGURE 3: Molecular model of a base pair showing the minor and major groove sides; the brackets indicate which chemical groups are modified in the oligonucleotide derivatives of Table I. The actual nucleoside bases shown are 2,6-diaminopurine and thymine. Hydrogen bonds between the bases have been indicated with dotted lines. The atomic numbering of the positions which are discussed in the text are given in the figure.

Table III: Dissociation of the DAPI–d(CTGAPTTCAG) Complex by Hoechst 33258

[Hoechst 33258] (nM)	ΔF
0	11.4
1.95	5.8
4.15	3.3
6.1	1.3

[a] To a mixture (5.5 °C) of DAPI (0.97 nM) and Hoechst 33258, with concentratinos as given, a small volume of a 7.7 μM solution of **4** (0 °C) was added to give a duplex concentration (1.0 nM) equimolar with DAPI. The increase in fluorescence was measured within 1 min. ΔF is the increase in fluorescence relative to the fluorescence of 0.97 nM DAPI alone (2.1 for a nM solution), corrected for the fluorescence of Hoechst 33258 by itself (2.3 for a nanomolar solution). In the absence of Hoechst 33258, ΔF corresponds to almost 70% complex formation with ΔF_{max} = 16.8 at infinite duplex concentration (see Figure 2a). In the absence of DAPI no increase in Hoechst 33258 fluorescence could be observed at this low concentration.

interaction. The insensitivity of K_a toward changes in the major groove nucleotide substituents is a direct indication that the tight binding of these dyes takes place solely in the minor groove.

The Largest Perturbation of the Binding Free Energy for Both Dyes Is Found for the Base Substituents in the Minor Groove within the Central AATT Region. This was expected from the X-ray diffraction studies (Pjura et al., 1987; Teng et al., 1988; Larsen et al., 1989). In addition, the site extension and nucleotide specificities have been proposed from studies by the footprinting technique (Martin & Holmes, 1983; Harshman & Dervan, 1985; Jorgenson et al., 1988; Portugal & Waring, 1988; Jeppesen & Nielsen, 1989). Our data are a direct physicochemical corroboration of these earlier studies. Interesting results were obtained with **2**–**4** (Table I); data for the parent sequence **1** and the derivatives **2** and **3** are presented in Figure 1, and those for **4** are shown in Figure 1 and Table III. The replacement of A_4 or A_5 by 2-aminopurine in the parent decamer, as in **2** and **3**, drastically decreases the affinity for both Hoechst 33258 and DAPI. The structural change in the purine base is 2-fold: (a) deletion of the 6-amino group, in the major groove, and (b) introduction of the 2-amino group in the minor groove; the latter leads to hydrogen bonding with thymine, with a local similarity in the minor groove to a dG·dC or a dD·dU base pairing (McLaughlin et al., 1987).

When 2-Aminopurine Replaces A_4 As in **2**, *the Binding of Hoechst 33258 Is Affected More Than That of DAPI.* For this replacement the K_a for Hoechst 33258 drops by a factor of 170 ($-\Delta\Delta G$ = 2.8 kcal). Teng et al. (1988) have suggested that the 2-amino group of guanine in the minor groove at G_4

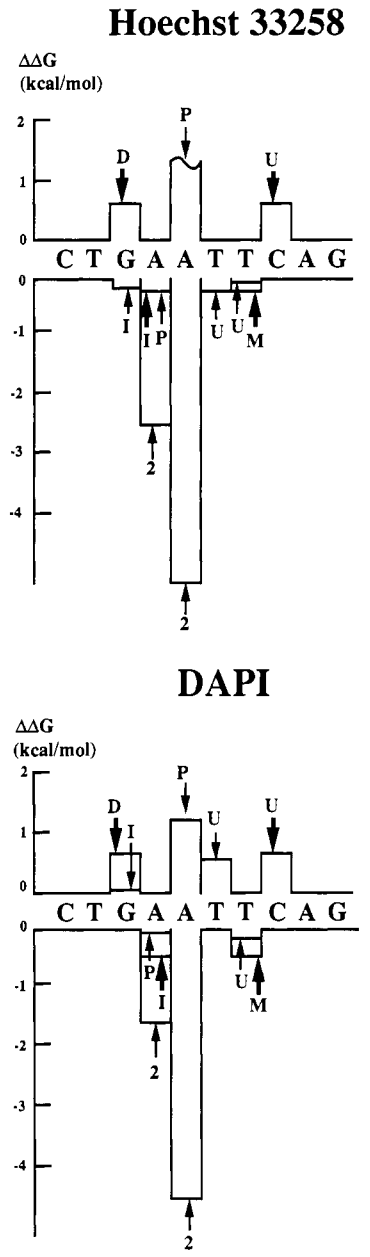

FIGURE 4: Schematic representation of the changes in free energy of binding ($\Delta\Delta G$, in kcal mol^{-1}) for the dyes Hoechst 33258 and DAPI with the duplexes from Table I. The base changes are given above (increased binding) and below (decreased binding) the parent sequence **1**. Bold arrows indicate simultaneous changes with other positions (see Table I).

in the dodecamer d(CGCGAATTCGCG) sterically prevents Hoechst 33258 from binding to this position on the dodecamer and that the dye binds to the sequence AATT (Figure 2a). One would expect a similar steric hindrance from the 2-amino group of 2-aminopurine in decamer **2**; this 2-amino group would repel the dye. In addition to this steric effect, a base-pair hydrogen bond can now form between this 2-amino group of 2-aminopurine and O2 of the opposite thymine T_7 in the complementary strand (see Figure 2); thus, any hydrogen bonding between a benzimidazole nitrogen and the O2 of this thymine is probably either abolished or weakened due to hydrogen-bonding competition. The shortest hydrogen bond from the dye, as proposed in both X-ray studies (Pjura et al., 1987; Teng et al., 1988), involves a bifurcated hydrogen bond including one of these thymine O2 (Figure 2), and this interaction would be directly affected in both Hoechst 33258 binding configurations shown in Figure 2 when A_4 is replaced

by 2-aminopurine. The steric hindrance and new base pairing caused by the 2-amino group of 2-aminopurine at position 4 would only disturb the positioning of the distal piperazine and phenyl groups in the model of Teng et al. (Figure 2a), whereas in the model of Pjura et al. (Figure 2b) this substitution would drastically affect the correct positioning of the central bisbenzimidazole of Hoechst 33258. The decrease in the binding free energy due to the substitution of 2-aminopurine for adenine at position 4 in the sequence **2** is only 2.8 kcal. The presence of the bulky 2-amino group in the minor groove at position 4 of the sequence would not allow the proper placement of the benzimidazole at this position for the binding configuration of Figure 2b, which is needed to promote the hydrogen bonding, the van der Waals interactions, and the interaction between the π electron clouds of the Hoechst 33258 bezimidazole rings and the O'_4 atoms of the deoxyribose rings. All these interactions have been suggested to be responsible for the binding interaction. Such a drastic effect would not be expected for the configuration of Figure 2a. This result could indicate that the binding of Hoechst 33258 to the AATT sequence (Figure 2a) of **1** is more likely than binding to the ATTC sequence (Figure 2b). Otherwise, a $\Delta\Delta G$ larger than 2.8 kcal would be expected.

For DAPI, replacing A_4 by 2-aminopurine has a similar but less drastic effect; the K_a for binding of DAPI to **2** is only a factor of 16 smaller ($-\Delta\Delta G$ = 1.5 kcal) than the K_a for binding to **1**. This quantitative difference between the two dyes is consistent with recent X-ray diffraction data indicating that DAPI binds at the central AATT sequence of d-(CGCGAATTCGCG) covering three A·T pairs (Larsen et al., 1989). When DAPI binds to **2**, the configuration shown in Figure 2c will have the charged amidino ends nudged away from the minor groove by the extra 2-amino group of the 2-aminopurines at position 4, but the hydrogen-bonding and other interactions with the dye are still possible at positions 5 and 6 of the decamer. DAPI binding with **2** is probably mostly impaired by two effects: (1) the steric repulsion of the phenyl group due to the 2-amino group and (2) the decrease of the electrostatic attraction at both amidino ends since the negative electrostatic potential in the minor grooves is decreased by inclusion of the electropositive 2-amino group.

When 2-Aminopurine Replaces A_5 As in **3**, *the Binding of Hoechst 33258 or DAPI Is Virtually Abolished.* The titrations show a very small and monotonically increasing fluorescence (see Figure 1). The unmeasurably low affinity of both dyes for **3** is in line with all the X-ray diffraction data (Pjura et al., 1987; Teng et al., 1988; Larsen et al., 1989) and with the idea that at the minor groove binding site, a 2-amino group, as in 2-aminopurine or guanine, prevents binding of A-T preferential dyes and drugs. According to the Teng et al. (1988) structure, the bisbenzimidazole of the Hoechst 33258 dye is directly juxtaposed and snuggly fits into the central A_5T_6 part of duplex **1** in Figure 2a, but according to the Pjura et al (1987) model (Figure 2b), the piperazinic benzimidazole is displaced into the A_4 position, which would retain the van der Waals, hydrogen-bonding, and orbital overlap interactions with decamer **3** at this position. Since the binding of Hoechst 33258 to **3** is too weak to determine the K_a, this may be interpreted to be more in accord with the Teng et al. (1988) binding configuration in solution to **1** than with that of Pjura et al. (1987). However, all binding configurations which require the central AT (i.e., A_5 and T_6) sequence to be intact would be drastically affected by the addition of the 2-amino group within the minor groove at these two base pairs. Decamer **3** has been shown to adopt the B-DNA conformation below 20 °C with a disturbance to the 5′ side of the 2-aminopurine, probably due to increased local mobility involving stacking–unstacking reactions and local motions on a 40–400-ps time scale (Nordlund et al., 1989).

Decamer **4** *Offers a Peculiar and Interesting Case with Regard to Binding of Hoechst 33258 and DAPI.* Here A_5 is replaced by purine, resulting in a single base-pairing hydrogen bond; this destabilizes the helix and decreases the T_m value (Table I). This modification increases K_a of DAPI for **4** to $K_a = 2.4 \times 10^9\ M^{-1}$, or by a factor of 10 ($\Delta\Delta G$ = 1.2 kcal) with respect to **1**, and hardly affects the fluorescence of the DAPI complex. However, with Hoechst 33258 and **4** no fluorescence increase could be observed. Despite this total absence of Hoechst 33258 fluorescence increase upon binding to **4**, inhibition of DAPI binding by Hoechst 33258 shows that the K_a for Hoechst 33258 binding to **4** is comparable to or higher than that for DAPI. This conclusion follows from the decrease in fluorescence of the DAPI–duplex **4** complex caused by very low concentrations of Hoechst 33258 (see Table III). This large increase in K_a of DAPI and Hoechst 33258 for duplex **4** as compared to **1** results from two singly hydrogen-bonded dP·dT base pairings in the center of duplex **4** that might alter the local helix parameters and optimize the minor groove binding contacts for both dyes. It is known from NMR studies on self-complementary duplexes, such as d(CGPAATTTCG) and **5**, that a dP·dT base pair retains Watson and Crick base pairing and that the duplex retains the characteristics of B-DNA (Ikuta et al., 1987; Clore et al., 1988); however, the dP·dT base pair increases the imino proton exchange rate and introduces small structural changes such as bending toward the major groove in the central part of duplex **5**. Our dye-binding data with **4** show that the presence of the two central dP·dT base pairs, flanked by two A·T pairs in the duplex, increases the binding of the two dyes. Perhaps the hydrogen-bonding interaction between the benzimidazole NH of the dye and the O2 of the complementary thymine increases due to better positioning of hydrogen-bonding partners within the minor groove. Further studies are required to define the factors that prevent Hoechst 33258 from becoming fluorescent with this high-affinity derivative **4** of the series. One possibility might be a change in the 32–36° angle between the two benzimidazoles found in the crystalline d-(CGCGAATTCGCG)–Hoechst 33258 complex (Pjura et al., 1987; Teng et al., 1988) accompanied by an increased rotational flexibility about this bond allowed by the loosening of constraints in the minor groove. Such rotational movements around bonds connecting conjugated dye systems are known to quench fluorescence (Förster, 1951). It has been known that the replacement of T by 5-BrU in DNA affects Hoechst 33258 binding in two ways: (a) an increase in K_{assoc} which we have shown kinetically by comparison of poly[d(A-T)] and poly[d(A-5-BrU)] (Regenfuss et al., 1989); (b) a reduction in fluorescence quantum yield (Latt & Wohlleb, 1975) usually attributed to an increase in intersystem crossing due to the electron-dense bromine atom. In addition, there is an indication of a photochemical reaction of the dye Hoechst 33258 itself (Härd et al., 1990). However, the present finding with Hoechst 33258 and duplex **4** of a very stable complex with almost zero quantum yield suggests an alternative origin of the decrease in Hoechst 33258 fluorescence with 5-BrU-DNA; perhaps the dye is allowed enough freedom in the minor groove to increase the rate of internal conversion from the excited to the ground electronic state. The structure of Figure 2a places the bisbenzimidazole directly in the central AT region, and both of the base pairs at these positions are involved in the

A to P substitution of **4**. On the other hand, the model of Figure 2b places the bisbenzimidazole over the AA region, where one of the adenines is not changed to purine. Considering the unexpectedly large increase in the K_a for Hoechst 33258 and **4**, this could indicate that the bound configuration in solution is more similar to the model of Figure 2a than to that of Figure 2b.

In contrast, by replacing A_4 by purine as in **5**, the K_a values for both Hocchst 33258 and DAPI do not increase as with **4** but remain practically unchanged compared to **1**. This again indicates the primary role of the central two A·T base pairs for creating a high-affinity site. Therefore, the small structural changes observed by Clore et al. (1988) due to the dP·dT base pairing as in **5** have only a minor effect on the binding of the two dyes unless the substitution occurs in the center of the binding region, as in **4**. Again, for reasons similar to those given above, this result seems more in accord with the model of Figure 2a than with that of Figure 2b.

The above self-complementary oligonucleotide sequences have been used in other studies, and a comparison of results seems appropriate here. Repeats of these and other oligonucleotides have been used to study the effect of exocyclic base substituent changes on the degree of DNA curvature (Diekmann & McLaughlin, 1988); the curvature is noticeable with the parent compound **1** in Table I, and this property can be either enhanced or diminished by the nature of the substituent changes. However, with the sequences common to both studies there is no correlation between Hoechst 33258 and DAPI binding on one hand and DNA curvature on the other; this conclusion follows from a comparison of our Table I with Table I in Diekmann and McLaughlin (1988). The sequences in Table I have also been used to probe the recognition site of the *Eco*RI restriction endonuclease and its activity (McLaughlin et al., 1987), and again there is no apparent correlation between K_m, k_{cat}, or k_{cat}/K_m and our dye binding affinities.

Conclusions

This is the first quantitative solution binding study of Hoechst 33258 and DAPI to well-defined DNA oligomers which have strong, sequence-specific binding sites on the DNA duplexes. By employing base derivatives with minor structural changes in defined positions of a self-complementary oligonucleotide sequence, we are able to probe the details of the binding surface of the DNA. The affinity of both Hoechst 33258 and DAPI to DNA sequences can be very high, and these results substantiate our earlier conclusions, using large natural-sequence DNA, that particular specific complexes formed between these dyes and DNA are much stronger than generally recognized. Natural DNA sequences contain many types of high-affinity binding sites for these dyes with a large spectrum of association constants, and the most stable dye–DNA complexes show K_a values that are comparable to the one for the parent compound **1** in this study (see Table II). A minor groove amino group at the 2-position of purines in a binding sequence of double-stranded DNA drastically interferes with the binding of these dyes; this is a direct physicochemical illustration that these dyes bind within the minor groove of DNA in solution and shows the stringent steric constraints required for tight binding in the minor groove.

Our finding that the highest affinity DNA duplex–Hoechst 33258 complex can be nonfluorescent (when the central adenine is replaced by purine) emphasizes that precautions must be taken when evaluating Hoechst 33258 as a fluorescent reporter dye for A-T-specific minor groove binding in DNA and chromosomes. Obviously, the existence of such very stable, nonfluorescent complexes means that the intensity of Hoechst 33258 fluorescence cannot be uncritically used as a measure of DNA concentration. One must, of course, bear in mind that the presence of a purine, rather than adenine, is not a usual occurrence in DNA; however, this result points out that the quantum yield may be a function of the exact DNA sequence. The largest changes in the free energy of binding are found when the structure of the decamer within the central AATT region is changed; however, as shown by decamer **10**, changes adjacent to this sequence can also have significant effects. Our fluorescence titrations with derivatives of the parent decamer, d(CTGAATTCAG), indicate that Hoechst 33258 may bind with a higher affinity to the sequence AATT than to ATTC; thus, in solution, the position of Hoechst 33258 proposed by Teng et al. (1988) is in complete accordance with our results. However, a definite choice between the two models of Teng et al. (1988) and Pjura et al. (1987) cannot be made. The variance in the association constants upon minor changes in the decamer structure indicate that the dye can adjust its conformation to adapt to the DNA structure.

Added in Proof

A recent ^{1}H NMR study on the Hoechst 33258–d(CTTTTGCAAAAG) interaction by Searle and Embrey (1990) is consistent with our proposed positioning of this dye in Figure 2a.

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Preparation and Characterization of *N*-(1-Pyrenyl)iodoacetamide-Labeled *Escherichia coli* RNA Polymerase

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ABSTRACT: *N*-(1-Pyrenyl)iodoacetamide has been used to introduce fluorescent probes into *Escherichia coli* RNA polymerase. After an incubation time of 15 min, approximately 2 pyrene equiv was introduced per enzyme molecule. There was no further increase in modification after more extended periods of incubation. Neither calf thymus DNA nor nucleotides protected the holoenzyme from modification. Thus, the sites of modification do not appear to involve the binding sites for polynucleotides or the ribonucleoside triphosphates. From the isolation and analysis of the individual subunits, it was found that σ contained approximately 1 pyrene equiv, β contained 0.6, β' contained 0.6, and α less than 0.1. Spectral and Stern–Volmer analyses indicate that the covalently attached pyrene molecules are in comparable apolar microenvironments. On the basis of CD analyses, the introduction of pyrene molecules into RNA polymerase alters its secondary structure. This alteration in secondary structure manifests itself by a reduction in overall enzymatic activity. Transcript analysis of the products obtained by using a linearized plasmid containing the A1 promoter and the T_e terminator of bacteriophage T7 indicates that the pyrenyl derivative is capable of producing full-length transcripts and that it has an efficiency of chain termination comparable to the native enzyme. Analysis of τ plots for the interaction of the pyrenyl derivative and the native enzyme, respectively, with the A1 promoter yielded comparable values for the isomerization constant in the conversion of the closed complex to an open one. Comparable values were also obtained for the association constant. The rate of chain elongation for the pyrenyl derivative, however, is approximately 54% of that observed for the native enzyme. Thus, the decrease in overall transcriptional activity observed with the pyrenyl derivative is not due to a decrease in the efficiency of initiation or premature termination, but rather to a decrease in the rate of chain elongation.

The reaction catalyzed by *Escherichia coli* RNA polymerase has been divided formally into four major steps, each of which consists of several substeps (Chamberlin, 1970; McClure, 1985). RNA polymerase recognizes and binds to promoter sites, initiates transcription at these sites, catalyzes the process of chain elongation, and terminates transcription at discrete sites. It has been postulated that many of these steps are accompanied by conformational changes in the enzyme. However, precise experimental documentation of these structural alterations is limited. A biphasic change in the

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