Binding of 9-Aminoacridine to Bulged-Base DNA Oligomers from a Frame-Shift Hot Spot†

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ABSTRACT: Complexes of 9-aminoacridine and two derivatives with oligomers based on the sequence of a hot spot for frame-shift mutations, $^{5'}$dGATGGGGCAG, are investigated by proton NMR and equilibrium dialysis. Competition dialysis experiments show that the drug binds bulge-containing oligomers more strongly than regular duplexes of similar sequence and length, with one apparent strong site. A duplex containing an extra cytidine in a run of C's has the highest affinity for 9-aminoacridine among the sequences tested. An oligomer containing five consecutive G·C pairs shows cooperative drug binding, indicating that G tracts of this length may have an altered helical structure. Complexes of a regular 8-mer and a 9-mer containing a bulged guanosine are examined in detail by two-dimensional NMR techniques. 9-Aminoacridine preferentially binds at TpG sites in the 8-mer but binds primarily at the bulged guanosine in the G-bulge 9-mer. Drug–DNA NOE's in the 8-mer complex are compared with the crystal structure of 9-aminoacridine and 5-iodo-CpG [Sakore et al. (1979) *J. Mol. Biol. 135*, 763–785]. The NMR data suggest that the drug intercalates across the base pairs of both strands with the amino group projecting into the minor groove.

Frame-shift mutations are generally known to occur more frequently in runs of iterated base pairs in DNA (Drake, 1970; Okada et al., 1972) and are promoted by a number of simple DNA intercalators, such as proflavin and acridine orange (Lerman, 1963; Ames et al., 1973). Bulged or looped-out nucleotides have been proposed to be intermediates in frame-shift mutagenesis, presumably arising from slippage of one strand of the duplex relative to the other in homopolymeric sequences (Streisinger, 1966; Streisinger & Owen, 1985). Intercalation of a drug molecule at bulge sites would increase the lifetime of the mispaired state, thereby increasing the probability of a base addition or deletion (Streisinger et al., 1966). Nelson and Tinoco (1985) have shown that ethidium bromide has an increased affinity for an oligonucleotide containing an extrahelical cytidine compared to a normal duplex of the same sequence and will stack opposite an extrahelical nucleotide in a trinucleotide complex. Intercalation, however, appears to be necessary but not sufficient for the mutagenic activity of acridines, as some compounds bind DNA but are not mutagens (Drake, 1970).

Acridines are known to be potent frame-shift mutagens (Ames et al., 1973), and a variety of acridine derivatives have been tested for biological activity. The ionizability of the acridine ring is important, and in general, electron-withdrawing substituents reduce activity, while electron-donating substituents on the acridine ring increase activity (Ferguson et al., 1985; Müller & Crothers, 1975). Furthermore, the presence of a hydrogen bond donor at the 9-position appears to increase activity, and it has been postulated that the 9-amino group forms a hydrogen bond with the cytosine carbonyl at CpG sites (Young & Kallenbach, 1981). There is also evidence that biological activity may correlate with the lifetime of the drug on the DNA. Stably bound drugs can interfere with transcription (Crothers et al., 1985), and experiments performed by Denny and co-workers indicate that antitumor activity is related to the lifetime of the drug complexes (Rewcastle et al., 1986; Wakelin et al., 1987). 4-Carboxamide derivatives of 9-aminoacridine which permit an additional hydrogen bond contact to the neighboring base pair form complexes with dissociation constants up to several seconds and have greatly increased in vivo activity.

Complexes of 9-aminoacridine and ethidium bromide with dinucleotides and trinucleotides have been examined by proton NMR (Lee & Tinoco, 1978; Young & Kallenbach, 1981), and the stereochemistry of intercalation with several sequences was examined in detail. The order of preference for 9-aminoacridine binding is CpG·CpG > GpG·CpC > ApG·CpT, GpC·GpC (Young & Kallenbach, 1981). In a crystal structure of the 1:2 complex of 9-aminoacridine with 5-iodo-CpG by Sobell and co-workers, the drug is stacked with the base pairs in two geometries, where the amino group projects into the major and minor grooves, respectively (Sakore et al., 1977, 1979). More recently, the DNA complexes of some other intercalators, such as actinomycin and daunomycin, have been examined by proton NMR and X-ray crystallography (Patel et al., 1981a,b; Wang et al., 1987). In all cases, the aromatic ring system of the drug lies across the base pairs in such a way as to maximize the stacking interactions, and the backbone torsion angles are altered in order to accomodate the intercalated drug.

Although much work has been done in studying mechanisms of DNA damage and repair, frame-shift mutagenesis is not well understood on a molecular level. Various factors are undoubtedly important for mutagenesis, such as DNA conformation and stability, recognition by repair enzymes and polymerases, and interaction of mutagens with particular DNA sequences. By studying a series of small synthetic oligomers containing bulged bases and characterizing their complexes with intercalating drugs, we hope to begin to elucidate the role of DNA conformation and drug–DNA interactions in frame-shift mutagenesis.

We previously examined a series of synthetic oligomers containing bulged nucleotides based on a frame-shift "hot spot" sequence in the λ C_I gene (Skopek & Hutchinson, 1984). This particular sequence, containing a run of four G·C pairs, has

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Chart I

Perfect 8-mer		1	2	3	4	5	6	7	8	
C2G2	5′	dG	A	T	G	G	C	A	G	
		C	T	A	C	C	G	T	C	
		16	15	14	13	12	11	10	9	
		1	2	3	4	5		6	7	8	9
Isolated G-bulge	5′	dG	A	T	G	G	-	G	C	A	G
CG4G3		C	T	A	C	C	G	C	G	T	C
		19	18	17	16	15	14	13	12	11	10
Perfect 9-mer	5′	dG	A	T	G	G	G	C	A	G	
C3G3		C	T	A	C	C	C	G	T	C	
Perfect 10-mer	5′	dG	A	T	G	G	G	G	C	A	G
C4G4		C	T	A	C	C	C	C	G	T	C
Perfect 10-mer	5′	dG	A	T	G	G	G	G	G	C	A	G
C5G5		C	T	A	C	C	C	C	C	G	T	C
Migrating G-bulge	5′	dG	A	T	G	G	G	G	C	A	G
C3G4		C	T	A	C	-	C	C	G	T	C
Migrating C-bulge	5′	dG	A	T	G	-	G	G	C	A	G
C4G3		C	T	A	C	C	C	C	G	T	C
A-bulge 9-mer	5′	dG	A	T	G	G	G	-	C	A	G
CA4G3		C	T	A	C	C	C	A	G	T	C
G-bulge 8-mer	5′	dG	A	T	G	G	G	C	A	G	
C2G3		C	T	A	C	-	C	G	T	C	

a rate of frame shifting 60-fold higher than background when induced by an acridine derivative, ICR-191. Proton NMR experiments on these oligomers show that the position of an extra C in a C tract, or an extra G in a G tract, is delocalized across the C·G run and that guanines on the edge of the G tract are less likely to be unpaired than those internal to the G tract (Woodson & Crothers, 1987, 1988a). NOESY experiments on duplexes containing isolated bulged nucleotides indicate that these oligomers adopt a B conformation in solution and that extrahelical purines remain stacked in the helix (Woodson, 1987).

As a next step, we wished to test whether these oligonucleotides formed specific 1:1 complexes with 9-aminoacridine, and whether the drug displays a marked preference and altered sequence specificity for bulge-containing helices over regular duplexes. Sequences were chosen that contain bulged nucleotides at fixed and unfixed sites (see Chart I). The unpaired nucleotide is inserted in different flanking sequences in the A-bulge 9-mer and fixed G-bulged 9-mer, allowing comparison of drug binding at pyrimidine–purine and purine–purine steps. By comparing the affinity of 9-aminoacridine for a number of related oligonucleotides, we hope to gain a further understanding of what governs the selectivity of binding. The 9-aminoacridine complexes of the regular 8-mer, 5′dGATGGCAG·5′dCTGCCATC, the isolated G-bulge 9-mer, 5′dGATGGGCAG·5′dCTGCGCCATC, and the migrating C- and G-bulge 9-mers, 5′dGATGGGCAG·dCTGCCCCATC and 5′dGATGGGGCAG·dCTGCCCATC, are studied by one- and two-dimensional NMR. NOESY spectra of the complexes are compared to the spectra of the DNA alone, and at very long mixing times, specific drug–DNA NOE contacts are observed. The regular 8-mer and the isolated G-bulge 9-mer each appear to have a single strong binding site, whereas the drug appears to bind at a number of sites in sequences where the bulge is delocalized. We have also examined complexes of 4-carboxamide derivatives of 9-aminoacridine with the 8-mer and C-bulge 9-mer. Equilibrium dialysis experiments are a convenient method of measuring the relative binding constants of a series of oligomers. Competition dialysis between regular and bulge-containing sequences show that 9-aminoacridine has a higher affinity for bulged helices than normal oligomers of similar sequences and length.

Materials and Methods

The oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified by reverse-phase chromatography. NMR samples of oligonucleotide duplexes were prepared as described previously (Woodson & Crothers, 1987). Concentration of duplex was determined from the absorbance at 260 nm, with extinction coefficients calculated from values for di- and trinucleotides (Janik, 1971). The final concentration of duplex was between 2.0 and 4.0 mM, in 10 mM sodium phosphate, 83.9 mM NaCl, and 0.1 mM EDTA, pH 7.0. Aliquots of a solution containing 32.0 mM 9-aminoacridine hydrochloride (Aldrich) in D_2O were added to produce the desired ratios of drug:duplex. The concentration of 9-aminoacridine was determined by using an extinction coefficient of 9.646×10^3 M^{-1} in H_2O at 400 nm. *N*-[2-(Dimethylamino)ethyl]-6-methyl-9-aminoacridine-4-carboxamide dihydrochloride and *N*-[2-(dimethylamino)ethyl]-9-[[2-(dimethylamino)ethyl]amino]acridine-4-carboxamide trihydrochloride were generously provided by William Denny. Aliquots of a 9.44 and 17.9 mM solution, respectively, were added to the DNA sample. The sample was lyophilized and resuspended in 99.98% D_2O prior to acquisition of two-dimensional data sets.

Proton Magnetic Resonance. One-dimensional spectra were acquired on a WM-500 spectrometer between 288 and 303 K, with presaturation of the HOD resonance. Two-dimensional NMR spectra were recorded on the Yale 490 spectrometer at 298 or 303 K, with quadrature detection in both dimensions. 256 t_1 values with 2048 data points (Bruker) in t_2 were collected, resulting in 1024 points in both dimensions following transformation. A total of 128 transients were acquired per t_1 experiment. Phase-sensitive NOESY spectra (Macura & Ernst, 1980) were phase cycled according to States et al. (1982), with a 12% variation in the mixing time to suppress zero-quantum peaks. Mixing times of 500 and 600 ms were used to observe NOE's between drug and DNA protons. The solvent resonance was suppressed by presaturation during the relaxation delay where necessary. DQF-COSY (Rance et al., 1985) spectra were acquired on the WM-500 spectrometer in the usual fashion, with a relaxation delay of 1.3 s. The data were processed by using the software package for NMR processing written by Dennis Hare. FIDs were weighted in both dimensions by a skewed sine bell shifted 10–20°, and NOESY spectra were symmetrized about the diagonal.

Equilibrium Dialysis. Equilibrium dialysis experiments were carried out in 1-mL cells separated by M_r 1000 cutoff dialysis membrane (Spectropor) in a method similar to that of Chaires et al. (1982). Binding isotherms for C3G3 and C4G4 were determined by dialysis against free 9-aminoacridine (10–75 μM) at duplex concentrations of 5.0, 10.0, 20.0, and 30.0 μM. Total drug concentration in the chamber containing the DNA was determined from the absorbance at the isosbestic point, 427.8 nm. Concentration of free drug was determined from the absorbance at 400.0 nm of the dialysate. Extinction coefficients for 9-aminoacridine were 9.656×10^3 M^{-1} at 400.0 nm and 4.095×10^3 M^{-1} at 427.8 nm. The concentration of bound drug, C_B, is taken from the difference of total drug concentration and that of free drug, $C_B = C_T - C_f$. Binding constants for the 9-mer and 10-mer (C3G3 and C4G4) were determined from the best linear fit to r/C_f vs r, where r is the moles of bound 9-aminoacridine per mole of DNA base pairs (Scatchard, 1949).

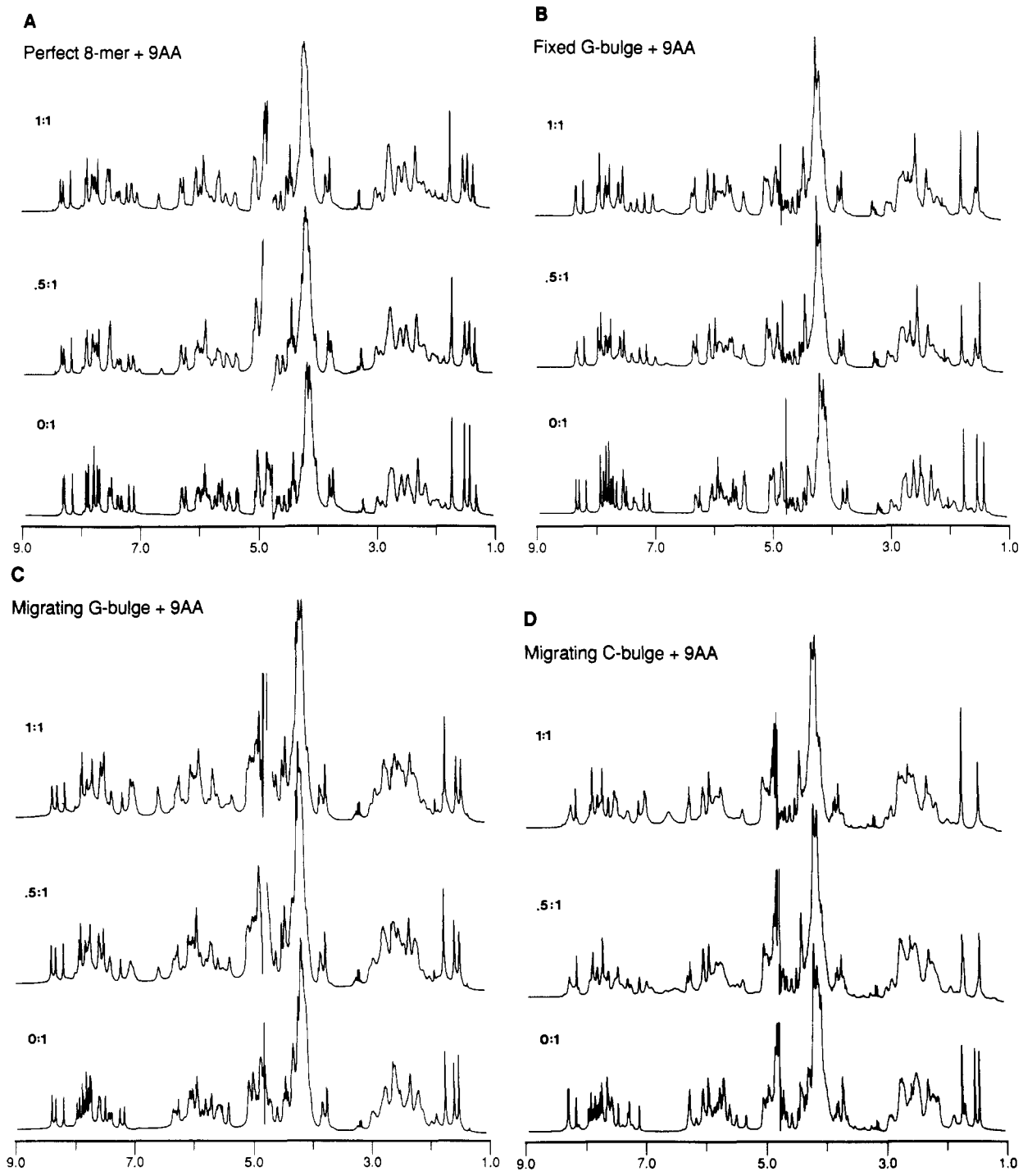

FIGURE 1: Proton NMR spectrum of the 9-aminoacridine–DNA complexes at 500 MHz. Spectra were taken in 10 mM phosphate and 100 mM Na^+, pH 7.0, at 298 K. The solvent resonance was presaturated during the relaxation delay. Increasing 9-aminoacridine was added to the sample until the ratio of drug to duplex was 1:1. The drug resonances appear at 7.5 and 6.7 ppm. Spectra of (A) perfect 8-mer, ^{5}'dGATGGCAG·dCTGCCATC, (B) fixed G-bulge 9-mer (CG4G3), ^{5}'dGATGGGCAG·dCTGCGCCATC, (C) migrating G-bulge 9-mer (C3G4), ^{5}'dGATGGGGCAG·dCTGCCCATC, and (D) migrating C-bulge 9-mer (C4G3), ^{5}'dGATGGGCAG·dCTGCCCCATC.

Relative binding constants of bulge-containing sequences were determined by competition dialysis against the regular oligomers in a similar fashion. Concentrations of the duplexes were generally 70 μM with drug concentrations between 20 and 80 μM. Solutions were allowed to equilibrate 24 h at room temperature before the absorbance was measured. (In trials up to 48 h, no change in absorbance was seen after 24 h.) The concentration of free drug on both sides of the membrane at equilibrium was calculated from the binding isotherm previously measured for the regular duplex. Moles of drug bound per mole of duplex (ρ) can then be calculated for the bulged duplex from the difference in the total drug concentration on the side containing the test sequence and the free drug concentration, and the best line fit to a plot of ρ/C_f vs ρ for the bulge-containing molecule.

RESULTS

One-Dimensional Proton NMR Spectra. The proton spectra of the regular 8-mer and bulge-containing duplexes with 9-aminoacridine are shown in Figure 1. The sequences are diagrammed in Chart I. The ratio of 9-aminoacridine to duplex was increased from 0:1 to 1:1 drug:DNA for each sequence. As the concentration of the drug is increased, several general changes in the proton spectra can be observed. The

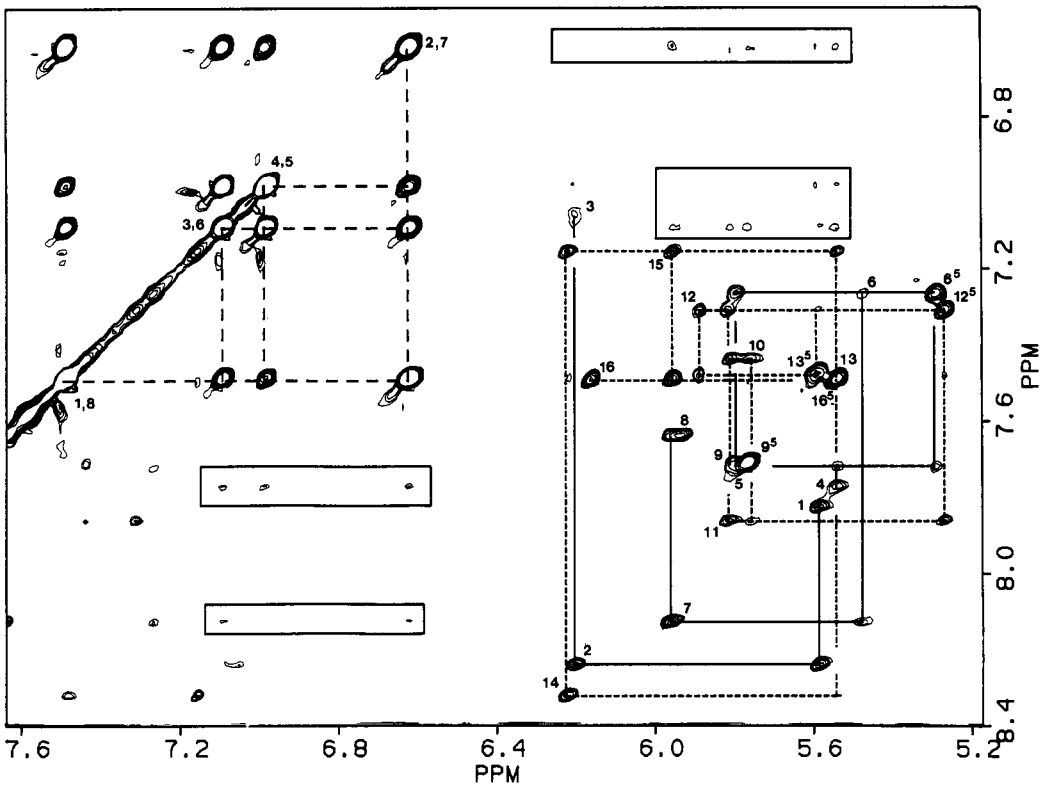

FIGURE 2: Region of the phase-sensitive NOESY spectrum of the 2:1 complex of 9-aminoacridine with the perfect 8-mer showing the aromatic resonances of the drug and the H1′–H6,H8 DNA cross-peaks. The spectrum was acquired at 490 MHz with a mixing time of 600 ms, at 298 K. Solid lines, connectivities along the top strand of the DNA; short dashed lines, connectivities along the bottom strand; long dashed lines, connectivities between drug resonances. The intraresidue H1′–H6,H8 cross-peaks are labeled by residue number, and "5" indicates cytosine H5–H6 cross-peaks. Drug–DNA NOE's are outlined in the boxes.

line widths of all the proton resonances increase, but some resonances broaden more than others and undergo a noticeable change in chemical shift. For instance, in Figure 1A, the resonances of A2 H8 and A14 H8 in the perfect 8-mer broaden while that of A7 H8 does not. Likewise, T3 and T15 methyl resonances broaden much more than the T10 methyl resonance. In general, the line broadening increases with added drug to about 0.75:1 drug:DNA and then decreases again slightly at 1:1 drug:DNA.

A closer examination of the one-dimensional spectra demonstrates a remarkable variability from sequence to sequence in the nonexchangeable proton spectra of the 9-aminoacridine complexes. The spectrum of the isolated G-bulge 9-mer is shown in Figure 1B. Again, the resonances assigned to the left side of the sequence undergo a change in chemical shift, in addition to a number of other resonances in the aromatic region. The spectrum of the migrating G-bulge 9-mer is shown in Figure 1C. In this sequence the adenine H8 and thymine methyl resonance do not broaden as much as in the 8-mer, but the G H8 and C H6 resonances do appear to broaden considerably. The degree of line broadening in the C-bulge 9-mer (Figure 1D) is quite dramatic and may indicate slower exchange or several competing sites of intercalation.

The line broadening in these spectra is most likely caused by chemical exchange between uncomplexed and one or more complexed forms of the DNA. Similarly, the line widths of the drug proton resonances are also quite broad and differ surprisingly in both chemical shift and line width from sequence to sequence. The 9-amino and base imino proton resonances of all the acridine–oligonucleotide complexes studied were extremely broad and were unresolved even at 5 °C. Consequently, the imino proton data were not very useful and are not shown here. The lifetime of 9-aminoacridine on the DNA is about 10 ms (Wakelin et al., 1987), which corresponds to intermediate exchange at 500 MHz. The line widths of the nonexchangeable proton resonances sharpen considerably as the temperature is increased from 15 to 35 °C, implying that we are moving toward the fast-exchange limit for these complexes. This has the unfortunate effect of making these drug–DNA complexes very difficult to study by NMR. For this reason, we are only able to draw general conclusions about the binding of 9-aminoacridine to these sequences. We also present data on derivatives of 9-aminoacridine which form longer lived complexes (Wakelin et al., 1987) but did not observe specific drug–DNA NOE's in these complexes.

The DNA resonances of the 9-aminoacridine complexes were assigned in the usual manner from sequential NOESY connectivities, as outlined in Figures 2 and 3 (Weiss et al., 1984). Assignment of weak resonances could often be achieved by cross-checking ribose connectivities. The drug resonances in the complex were assigned from double quantum filtered (DQF) COSY spectra (data not shown). The COSY connectivities permitted an order of assignment of the four nonexchangeable proton resonances, and the correct orientation was obtained from the assignments of Reuben et al. (1978). Because of the rapid chemical exchange in these complexes, it was necessary to use very long mixing times (500–600 ms) in the NOESY experiments to see any drug–DNA contacts. At these long mixing times, there is undoubtedly substantial spin diffusion and the intensities of the cross-peaks no longer reliably reflect the interproton distances.

Complex of the Regular 8-mer. The region of the NOESY spectrum of the 2:1 complex of the regular 8-mer with 9-aminoacridine containing the aromatic–H1′ cross-peaks is shown in Figure 2. The resonances were assigned as indicated, and their chemical shifts are listed in Table I. The most striking change in the spectrum is the reduced intensity of the

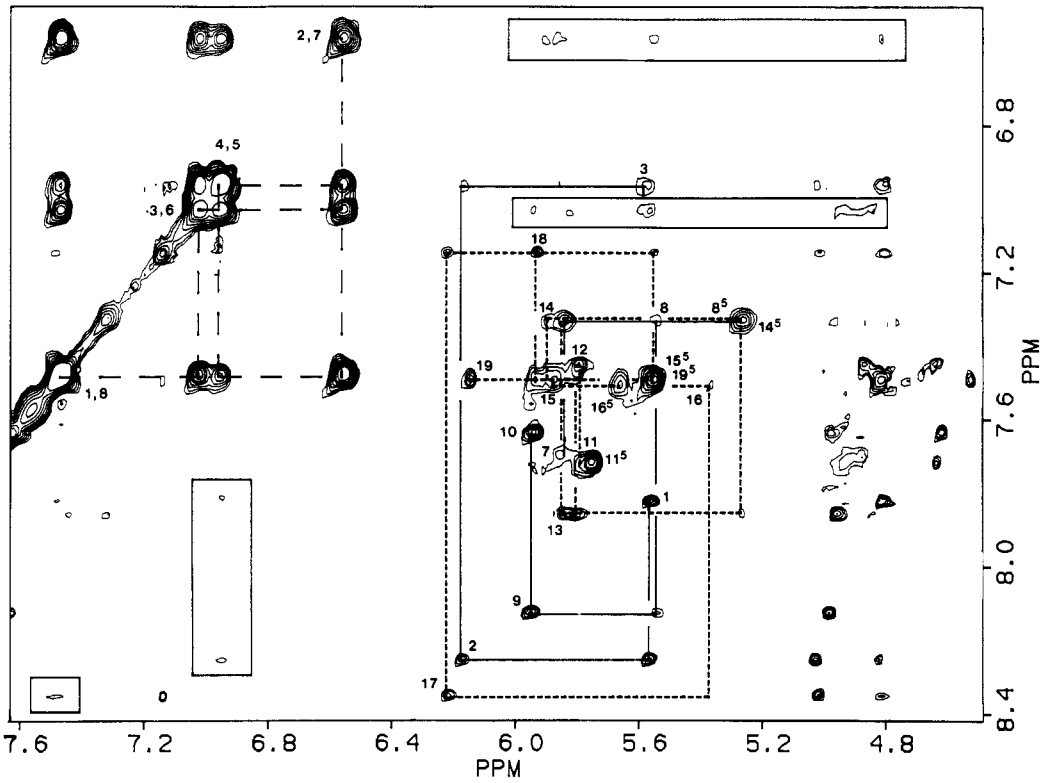

FIGURE 3: Phase-sensitive NOESY spectrum of the 2:1 complex of 9-aminoacridine with the G-bulge 9-mer. The spectrum was acquired with a mixing time of 600 ms. The DNA connectivities are outlined by the solid and dashed lines as in Figure 2, and the drug resonances are connected by long dashes. Intraresidual H1′–base proton cross-peaks are labeled by residue number.

Table I: Chemical Shifts of Nonexchangeable Protons in 2:1 9-Aminoacridines–Perfect 8-mer Complex (ppm)[a]

residue	H8	H6	H5	H1′	H2′	H2″	H3′	CH_3
G1	7.82			5.58	2.50	2.69	4.83	
A2	8.23			6.20	2.68	2.88	5.03	
T3		7.06		5.76	2.39	2.25	4.84	1.36
G4	7.77			5.54	2.65	2.65	4.97	
G5	7.71			5.80	2.54	2.65	4.95	
C6		7.26	5.29	5.48	1.92	2.23	4.79	
A7	8.12			5.96	2.68	2.82	5.00	
G8	7.63			5.93	2.46	2.29	4.63	
C9		7.71	5.76	5.81	2.07	2.50	4.65	
T10		7.43		5.76	2.19	2.47	4.88	1.64
G11	7.86			5.82	2.63	2.66	4.97	
C12		7.31	5.27	5.89	2.05	2.40	4.81	
C13		7.48	5.59	5.54	2.25	2.39	4.83	
A14	8.31			6.22	2.72	2.91	5.01	
T15		7.15		5.95	2.02	2.43	4.82	1.42
C16		7.49	5.54	6.16	2.25	2.25	4.54	
9-Aminoacridine								
$H_{1,8}$	7.52	$H_{2,7}$	6.64	$H_{3,6}$	7.12	$H_{4,5}$	7.00	

[a] Measured relative to TSP at 298 K in 10 mM phosphate, 100 mM Na^+, and 0.1 mM EDTA, pH 7.0.

NOESY cross-peaks at T3 resonances, which have intensities comparable to those of the other residues in the uncomplexed oligomer. Furthermore, the expected cross-peaks between T3 sugar proton resonances and G4 H8, as well as between C13 sugar protons and A14 H8, are missing or are very weak. There are few changes in the intraribose cross-peaks, although the intensities of the G4 and A14 cross-peaks are difficult to evaluate, since the H2′ and H2″ resonances are poorly resolved. Interestingly, the cross-peaks between the H1′ and H2′,2″ resonances of T3 and C13 are missing in the magnitude COSY spectrum (see Supplementary Material) as well as in the NOESY spectrum. The other ribose COSY connectivities appear normal. Comparison of chemical shifts in the complexed and uncomplexed oligomers (Woodson, 1987) shows that the largest changes in chemical shift occur at the sugar resonances of T3 and G4, and of A14 and C13.

The drug–DNA NOE contacts are outlined by boxes in Figure 2. The only cross-peak present in the spectrum of the 1:1 complex (data not shown) is between G4 H8 and H2,7 on the acridine ring. In the 2:1 complex, this cross-peak increases in intensity, and other cross-peaks to the H2′,H2″ resonances from the H2,7, H3,6, and H4,5 drug protons appear. One also sees connectivities to T3 H2′,2″ and methyl resonances. In addition, there are weaker NOE's to A7 H8 and H1′, T10 H1′ and methyl, and G11 H1′ protons, although some of these connectivities are ambiguous due to chemical shift overlap. Taken together, the chemical shift and NOE data indicate T3-G4 as the preferred site of intercalation, and T10-G11 as a secondary site. This is quite reasonable in light of the known preference of 9-aminoacridine for CpG steps (Young &

Table II: Chemical Shifts of 2:1 9-Aminoacridine–Isolated G-Bulge 9-mer (CG4G3) Complex (ppm)[a]

residue	H8	H6	H5	H1′	H2′	H2″	H3′	CH_3
G1	7.83			5.63	2.54	2.72	4.84	
A2	8.22			6.23	2.70	2.91	5.03	
T3		7.07		5.75	1.97	2.37	4.86	1.38
G4	7.72			5.64	2.60	2.68	4.95	
G5	7.72			5.64	2.58	2.68	4.95	
C7		7.28	5.33	5.33	1.91	2.19	4.78	
A8	8.12			5.95	2.69	2.82	5.00	
G9	7.64			5.92	2.47	2.29	4.63	
C10		7.70	5.77	5.82	2.04	2.49	4.66	
T11		7.45		5.62	2.19	2.42	4.87	1.64
G12	7.86			5.80	2.62	2.62	5.05	
C13		7.28	5.33	5.80	2.22	2.49	4.78	
G14	7.86			5.34	2.63	2.63	5.05	
C15		7.46	5.49	5.77	2.02	2.49	4.98	
C16		7.47	5.77	5.49	2.26	2.44	4.83	
A17	8.27			6.19	2.66	2.88	4.98	
T18		7.19		5.97	2.03	2.44	4.84	1.45
C19		7.47	5.49	6.17	2.25	2.25	4.55	
9-Aminoacridine								
$H_{1,8}$	7.45	$H_{2,7}$	6.47	$H_{3,6}$	6.93	$H_{4,5}$	6.93	

[a] As in Table I.

Kallenbach, 1981). The sequence of the 8-mer does not contain CpG, but apparently TpG is an acceptable alternative.

Complex of the Isolated G-Bulge 9-mer. The region of the NOESY spectrum of the complex of the isolated G-bulge 9-mer with 9-aminoacridine containing cross-peaks between aromatic and H1′ sugar resonances is shown in Figure 3. In this sequence, the cross-peaks relating to T3 protons are present and more or less unaffected by the presence of the drug, but those of G5 and G6 have become very weak. The chemical shifts of the 2:1 complex are listed in Table II. Comparison to those of the oligomer alone (Woodson & Crothers, 1988b) indicates that the largest changes are no longer at T3 and G4 as for the 8-mer, but at the sugar resonances of G5, G6, G14, and G15. The H1′ resonance of the bulged G, G14, is shifted upfield by 0.5 ppm. NOE contacts from the $H_{3,6}$ and $H_{4,5}$ drug protons (which both fall at 6.93 ppm) are observed to C15 H1′, H5 (strong), G14 H1′ (medium), and G6, G14 and/or G12 H8 (weak), which all overlap at 7.85 ppm. It appears that, in the bulge-containing duplex, the drug no longer binds preferentially at TpG but instead binds at the bulge site and seems to interact with G4-C15.

Complex with Migrating G-Bulge 9-mer and C-Bulge 9-mer. The NOESY spectra of the complexes of the migrating G-bulge 9-mer, 5′dGATGGGGCAG·dCTGCCCATC, and migrating C-bulge 9-mer, 5′dGATGGGCAG·dCTGCCCCATC, were also acquired (data not shown). These spectra could not be completely assigned, because all of the cross-peaks corresponding to the guanosines in the G tract of the G-bulge oligomer were missing, even at a mixing time of 500 ms. Similarly, those of the cytidines in the C tract of the C-bulge duplex were also absent. This is presumably due to increased motion or conformational averaging in this region of the molecule. All of the connectivities are present in the spectra of the uncomplexed oligomers (Woodson, 1987), which we have shown to be undergoing rapid exchange among bulge conformers. One would expect drug binding in this region to slow isomerization of the bulged site, since the lifetime of the 9-aminoacridine complex is longer than that of a bulge conformer. Intercalation of a drug molecule will also change the flexibility and conformation of the DNA helix, as evidenced by the disappearance of normal NOE contacts at T3 in the regular 8-mer and at G5 in the isolated G-bulge oligomer. There are a number of NOE contacts from $H_{2,7}$, $H_{3,6}$, and $H_{4,5}$ on the drug to T3 H1′ and methyl protons, T18 methyl, H8 and H3′, and C16 H2′, as well as to sugar resonances on T12 and A9 and A9 H8 in the G-bulge 9-mer, and a number of weak contacts to the DNA in the C-bulge 9-mer. This spread in the drug–DNA NOE contacts seem to suggest that there is no strongly preferred binding site in these sequences, and the drug most likely intercalates at a number of positions along the G·C run.

Complexes of 9-Aminoacridine-4-carboxamides. 4-Carboxamide derivatives of 9-aminoacridine (Rewcastle et al., 1986) have been shown to form long-lived DNA complexes with dissociation time constants greater than 1 s. The lifetime of the complexes has been associated with their increased in vivo antitumor activity (Wakelin et al., 1987). The parent compound, *N*-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide, is proposed to intercalate from the minor groove, allowing the protonated terminal amino group on the side chain to form a hydrogen bond with the O2 oxygen of an adjacent cytosine. We wished to examine the complexes of these compounds with bulge-containing oligomers in the hope that more stable complexes would allow us to precisely determine the conformation of the bound drug.

The spectrum of the complex of *N*-[2-(dimethylamino)-ethyl]-6-methyl-9-aminoacridine-4-carboxamide with the perfect 8-mer is shown in Figure 4A, with ratios of 0:1, 0.5:1, and 1:1 drug:duplex at 298 K. As in the case of 9-aminoacridine, all of the DNA resonances broaden with the addition of drug, indicating intermediate exchange between different complexes. This was somewhat surprising, as the longest dissociation time constant for this molecule is on the order of 1 s. The resonances of all the complexes examined with these acridine derivatives have broadened lines, which sharpen at higher temperatures. This is independent of concentration and is more likely to be due to exchange than aggregation. A number of DNA resonances appear to split with addition of drug, such as the methyl and adenine H8 resonances. The resonances of the drug protons appear between 6.5 and 7.0 ppm, at 3.8 ppm (methylene), and overlapping with the H2″ sugar resonances at 3.0 ppm (methyl) and were assigned by one-dimensional NOE experiments on the free drug and NOESY cross-peaks of the complex. The drug resonances are themselves very broad, including the terminal methyl protons on the side chain, suggesting that the drug is binding at a number of different sites and that the terminal amino group is making contact with the DNA.

Table III: Chemical Shifts of the 1:1 Complex of the 9-Aminoacridine-4-carboxamide Derivative with the Migrating C Bulge (C4G3) (ppm)[a]

residue	H8	H6	H5	H1′	H2′	H2″	H3′	CH_3
G1	7.84			5.62	2.47	2.65	4.82	
A2	8.28			6.22	2.72	2.85	5.04	
T3		7.05		5.70	1.81	2.14	4.82	1.41
G4	7.68			5.83	2.53	2.53	4.96	
G5	7.74			5.82	2.62	2.62	4.95	
G6	7.73			5.82	2.64	2.64	4.93	
C7		7.34	5.32	5.54	1.90	2.27	4.80	
A8	8.16			6.01	2.69	2.85	5.01	
G9	7.71			6.02	2.47	2.31	4.64	
C10		7.79	5.90	5.90	2.13	2.54	4.68	
T11		7.47		5.81	2.20	2.50	4.90	1.68
G12	7.90			5.89	2.68	2.71	5.00	
C13		7.38	5.34	5.94	2.15	2.41	4.82	
C14		7.54	5.69	5.90	2.18	2.38	4.84	
C15		7.53	5.63	5.89	2.17	2.35	4.84	
C16		7.50	5.67	5.62	2.14	2.33	4.84	
A17	8.37			6.26	2.79	2.92	5.03	
T18		7.21		6.01	2.07	2.44	4.83	1.52
C19		7.61	5.76	6.23	2.27	2.27	4.56	

Drug Resonances							
acridine ring				9-amino		4-carboxamide	
H_1	7.68	H_8	7.76	H_α	4.17	H_α	3.27
H_2	6.71	H_7	6.38	H_β	3.70	H_β	3.18
H_3	7.00	H_6	7.23	H_γ	3.00	H_γ	2.73

[a] As in Table I, at 303 K.

The spectrum of *N*-[2-(dimethylamino)ethyl]-9-[[2-(dimethylamino)ethyl]amino]acridine-4-carboxamide complexed to the migrating C bulge at 298 K is shown in Figure 4B. This compound contains two aliphatic chains which have the possibility of forming hydrogen bonds with adjacent bases; the apparent lifetime of this drug on the DNA is 10 s (William Denny, personal communication). The resonances of this particular complex are narrower than the others, and moreover, the broadening of the DNA resonances seems to be somewhat more selective. For instance, the H6 and methyl resonances of T3 are both broadened substantially, while the other methyl resonances are not so severely affected. The DNA resonances of the 1:1 complex were assigned from NOESY connectivities (data not shown), and their chemical shifts are listed in Table III. Although there were very few drug–DNA NOE's except to the side-chain resonances, the NOE contacts between T3 and G4 were entirely disrupted, and those between C16 and A17 were much weaker than in the DNA spectrum. This suggests that the drug is intercalating preferentially at T3-G4, which is also the preferred binding site of 9-aminoacridine in the regular 8-mer. This may stabilize the oligomer in a conformation where C16 is bulged out of the helix, and the other cytosines pair with the guanines in the C·G run. It is also interesting to note that the methyl resonance assigned to the 4-carboxamide chain (2.75 ppm) is very sharp, suggesting that it is tumbling more freely in solution than the complex, while that assigned to the 9-amino chain is extremely broad (3.0 ppm) and makes a number of NOE contacts to the DNA resonances.

Equilibrium Dialysis of 9-Aminoacridine Complexes. The relative binding affinities of bulge-containing and regular duplexes for 9-aminoacridine were measured by competition dialysis experiments. The binding isotherm of the regular 9-mer and 10-mer (Chart I) were measured by dialysis against free aminoacridine solution, as described under Materials and Methods. A Scatchard plot of r/C_f vs r for the 9-mer and 10-mer, where r is the moles of drug bound per mole of base pairs and C_f is the free drug concentration, is shown in Figure 5A. The data for each oligomer were fit to a straight line,

Table IV: Results of Competition Dialysis Experiments[a]

duplex	K_a'[b]	$K_a'B_{app}'$[b]	B_{app}
perfect 9-mer	5.88×10^4	2.78×10^4	4.3
perfect 10-mer	8.15×10^4	2.81×10^4	3.4
duplex	**K_a[c]**	**K_aB_{app}[c]**	**B_{app}**
C-bulge 9-mer	1.26×10^6	1.55×10^6	1.2
G-bulge 9-mer	5.31×10^5	8.09×10^5	1.5
A-bulge 9-mer	2.93×10^5	4.59×10^5	1.6
migrating G bulge	7.38×10^4	2.35×10^5	
G-bulge 8-mer	2.13×10^5	3.02×10^5	1.4

[a] Measured in 10 mM Phosphate, pH 7.0, and 100 mM Na^+, at room temperature. [b] In M^{-1} (base pairs). [c] In M^{-1} (duplex).

with the results listed in Table IV. No attempt was made to disentangle the influence of neighbor exclusion and sequence-specific binding on the shape of the binding isotherm, since both of these effects yield nonlinear binding isotherms (Crothers, 1968). It should be recognized that the apparent binding site size B_{app} reflects both neighbor exclusion and the tendency of a single strong binding site to dominate the binding equilibrium at the low degrees of binding that we studied.

The relative affinity of 9-aminoacridine for the bulge-containing duplexes was determined by competition dialysis against a regular duplex, a procedure that is intrinsically more reproducible than measurement of absolute binding equilibria. The free drug concentration was calculated from the previously determined Scatchard plot for the regular duplex. The ratio of moles of drug bound per mole of duplex (ρ) for several bulge-containing duplexes to that of the regular 9-mer is plotted vs ρ of the 9-mer in Figure 5C, and the results of Scatchard plots for the test sequences are listed in Table IV. The sequences of the bulge-containing duplexes are diagrammed in Chart I.

In general, the bulged sequences bind the drug more strongly than the regular duplexes, at values of ρ less than 1. Furthermore, the apparent number of binding sites in the bulged sequences is 1.5, compared to 4.3 in the perfect 9-mer and 3.4 in the perfect 10-mer, indicating dominance of a single strong site in the bulged duplexes. The migrating C-bulge 9-mer

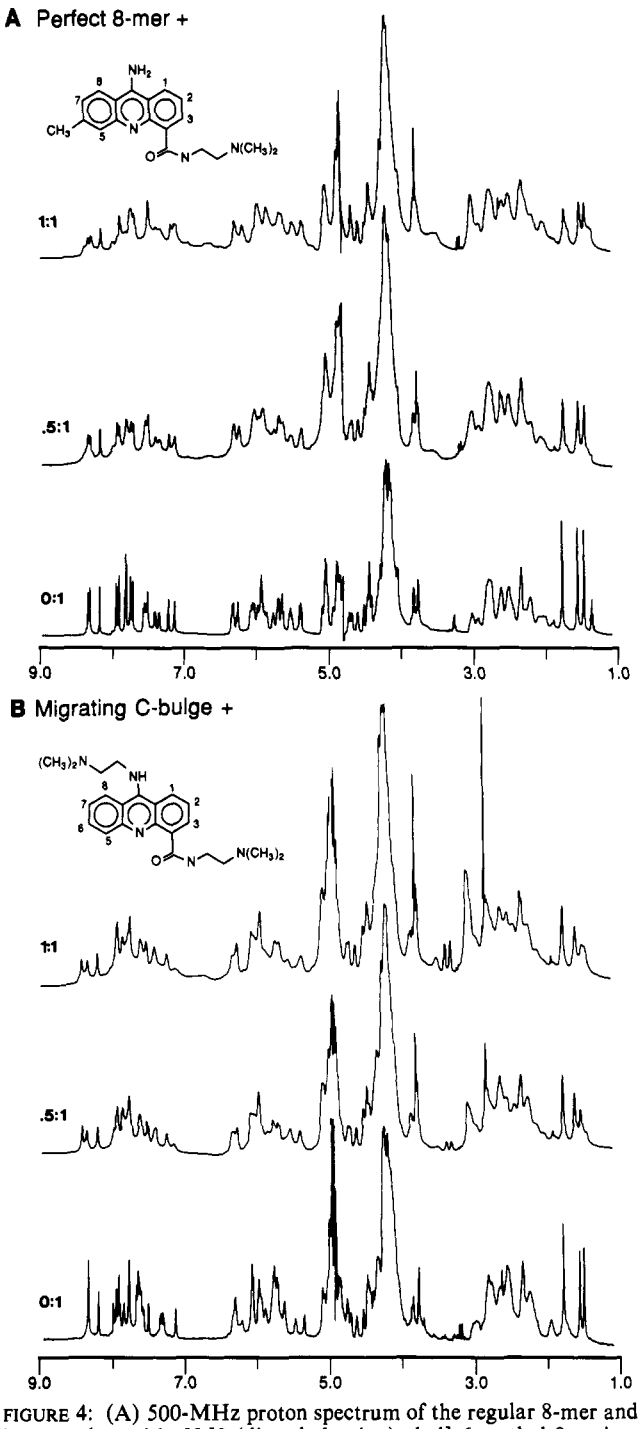

FIGURE 4: (A) 500-MHz proton spectrum of the regular 8-mer and its complex with *N*-[2-(dimethylamino)ethyl]-6-methyl-9-aminoacridine-4-carboxamide at 298 K. Aliquots of drug were added until the ratio of drug to duplex was 1:1. Drug resonances are visible at 6.5–7.5 ppm and at 3.0–3.8 ppm. (B) Spectrum of the migrating C-bulge 9-mer and *N*-[2-(dimethylamino)ethyl]-9-[[2-dimethylamino)ethyl]amino]acridine-4-carboxamide at 298 K. Drug resonances are visible at 6.5–7.5 ppm, at 3.2–4.2 ppm (methylene), and at 3.00 and 2.73 ppm (methyl).

(C4G3) has by far the strongest affinity for 9-aminoacridine, and it is interesting that only this sequence forms a well-defined complex with the acridine derivatives. Duplexes containing an isolated extrahelical purine in the pyrimidine-rich strand (CG4G3 and CA4G3) also preferentially bind the drug. The G-bulge 8-mer also behaves similarly to the other bulge-containing sequences, but the overall affinity is lower than that of the 9-mer, most likely because it has fewer base pairs. The migrating G-bulge 9-mer displays cooperative binding, where drug affinity increases with added drug up to 1 drug molecule per duplex.

Duplexes containing runs of five G·C pairs also show cooperative binding. A Scatchard plot of ρ/C_f vs ρ is shown in Figure 5B for the regular 11-mer (C5G5), which was measured by competition against the regular 10-mer (C4G4). Binding isotherms for the 9-mer and 10-mer are also drawn for comparison. The 9-mer and 10-mer show no cooperative behavior, and the 10-mer has a slightly greater affinity for the drug than the 9-mer, as expected. The 11-mer shows strong cooperativity where there is less than 1 drug bound per duplex. At low values of ρ, it binds more weakly than the 10-mer, and at $\rho = 1$, it has a greater affinity for the drug, as expected. These results suggest that runs of G·C pairs greater than four may adopt some altered helical conformation that does not bind drug as readily. Binding of the first acridine could revert the helix to a more usual conformation, raising the binding constant for subsequent drug molecules.

DISCUSSION

Preferential Binding to Bulge-Containing Oligomers. An important feature of the bulged-base model for frame-shift mutagenesis is the stabilization of looped-out bases by binding of mutagens. Nelson and Tinoco (1985) found that ethidium bromide preferentially binds a duplex containing an extrahelical cytidine. Competition dialysis experiments show that 9-aminoacridine preferentially binds molecules that contain an extrahelical base, with apparently one strong site. The results of the dialysis experiments also agree with estimates of the relative stabilization of these sequences upon binding of 9-aminoacridine obtained from optical melting curves (Tao Pan, S. A. Woodson, and D. M. Crothers, unpublished results). The data were fit to a two-state model for samples with drug:DNA duplex ratios between 0:1 and 2:1. In all cases, the T_m of bulge-containing oligomers increased more with addition of drug than that of the regular duplex, and most of the additional stabilization was gained at 1:1 drug:duplex.

In both experiments, the relative affinities and stabilization are the same for the sequences studied. The large difference between the migrating C-bulge 9-mer and the migrating G-bulge 9-mer is of particular interest. In the first case, there is an extra cytosine in the C tract and, in the latter, an extra guanine in the G-tract. If the drug stabilizes the bulge by intercalating between the bases of the shorter strand, then the differences between these sequences could be rationalized by the fact that the drug can stack with purines in the C-bulge duplex but must stack with pyrimidines in the G-bulge molecule. This does not explain, however, the difference between the isolated G-bulge molecule and the migrating C-bulge molecule. As we discuss below, the NMR data on the G-bulge complex suggest that the drug stacks with the bases of both strands.

Site-Specific Binding at the Bulged Nucleotide. Although the short lifetime of the acridine–DNA complexes unfortunately prevents one from making a detailed analysis of its complexes with these sequences by NMR, it is possible to draw several general conclusions about intercalation of acridines into bulge-containing duplexes. Intermolecular NOE's and the strong effect on the resonances of T3 clearly identify T3-G4 as the preferred binding site in the perfect 8-mer. This is also supported by the reduction of NOE contacts between T3 and G4, and between C13 and A14. The loss of the expected cross-peak between T3 H1′ and H6 could be due to a change in the glycosidic torsion angle caused by intercalation of the acridine ring. Binding at TpG is consistent with previous work by Young and Kallenbach (1981), where they found CpG to

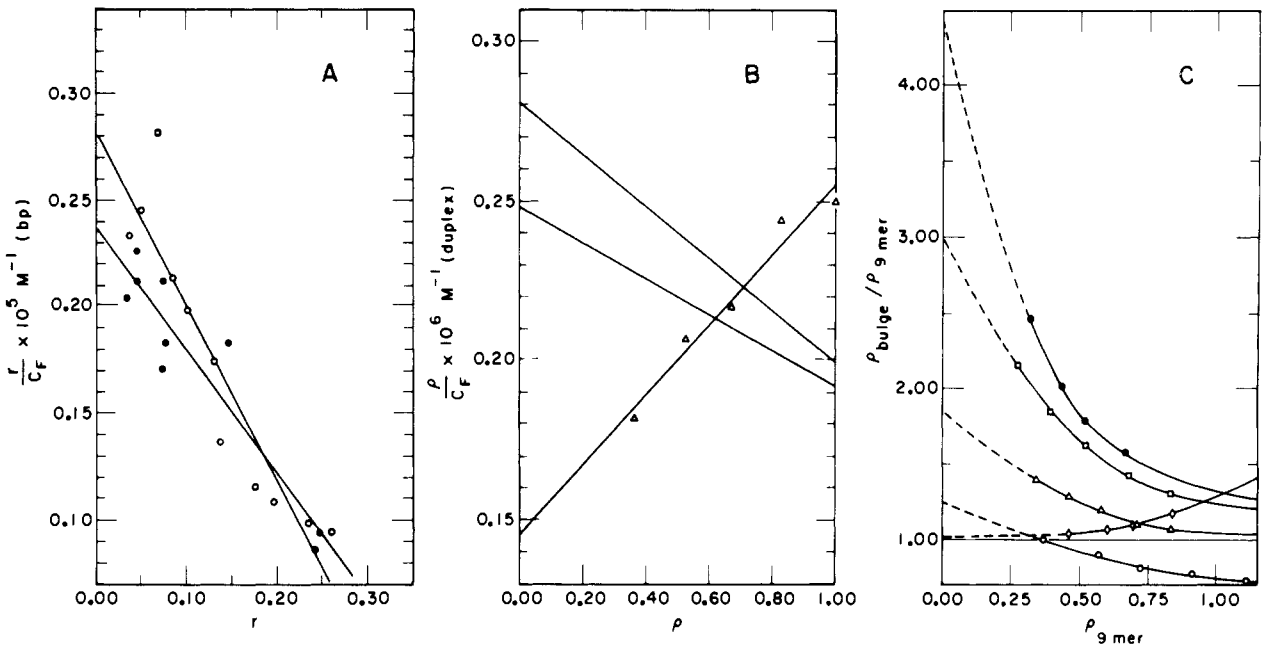

FIGURE 5: Binding of 9-aminoacridine to regular and bulge-containing duplexes from equilibrium dialysis experiments. (A) r/C_f vs r for the perfect 9-mer (●) and perfect 10-mer (○), where r is moles of drug bound per moles of base pairs of C_f is the free drug concentration. Solutions containing 5–30 μM duplex in 10 mM phosphate, pH 7.0, and 100 mM Na^+ were dialyzed against free 9-aminoacridine solution. (B) Comparison of regular 9-mer, 10-mer, and 11-mer, containing 3, 4, and 5 consecutive G·C base pairs, respectively. ρ/C_f is plotted vs ρ, where ρ is the moles of drug bound per mole of duplex. Curves for the 9-mer and 10-mer were calculated from the Scatchard plots in (A); ρ(11-mer) (△) was measured by competition dialysis against the regular 10-mer. (C) Relative binding affinities of bulge-containing duplexes. The ratio of drug bound to a bulge-containing duplex, ρ_{bulge}, to drug bound to the regular 9-mer, $\rho_{9\text{-}mer}$, is plotted vs $\rho_{9\text{-}mer}$, where ρ is the moles of drug bound per moles of duplex. The concentration of drug bound to the bulge-containing duplexes was determined by competition dialysis against the perfect 9-mer. The best fits to the data points are extrapolated to $\rho = 0$, and the horizontal line at 1.0 represents the ratio of $\rho_{9\text{-}mer}$ to itself. (●) C-bulge 9-mer, C4G3; (□) fixed G-bulge 9-mer, CG4G3; (△) A-bulge 9-mer, CA4G3; (◇) migrating G-bulge 9-mer, C3G4; (○) G-bulge 8-mer, C2G3.

bind 9-aminoacridine better than GpG. The NOESY spectrum of the complex of the isolated G-bulge duplex (CG4G3) does not show any of the perturbations at T3-G4 seen in the 8-mer, but rather the NOE's and chemical shift changes indicate preferential binding of the drug at the extrahelical guanosine, G14. Although there remains the possibility that this is due to the creation of a CpG rather than the presence of a bulged nucleotide, the equilibrium dialysis experiments show that this sequence contains an unusually strong binding site for 9-aminoacridine. We conclude that insertion of an extra nucleotide alters the binding specificity of 9-aminoacridine in this sequence and that the drug binds preferentially in the region of the bulge.

The low intensity of the cross-peaks in the NOESY spectra collected on complexes with C3G4 and C4G3 makes it difficult to identify the drug binding sites. In fact, the diffusion of weak drug–DNA NOE's across the sequence, and the loss of DNA–DNA cross-peaks at more than one or two residues, itself strongly suggests that binding of 9-aminoacridine is delocalized in sequences where the position of the bulge is delocalized. We have previously estimated the lifetime of the bulge conformers to be a millisecond or shorter (Woodson & Crothers, 1987, 1988), and the lifetime of 9-aminoacridine complexes is roughly 10 ms (Wakelin et al., 1987). Binding of 9-aminoacridine should slow the rate of isomerization of the bulge site in homopolymeric sequences but may not be sufficient to resolve the complexes on the NMR time scale. We therefore hoped that formation of longer lived complexes would hold the duplex in one conformation.

Binding of 9-Aminoacridine-4-carboxamide to the C-Bulge Oligomer. The complex of the C-bulge 9-mer with the 4-carboxamide derivative (see Figure 4B) does appear to be more stable than that of 9-aminoacridine. Although the resonances are still broad, nearly all of the expected connectivities among the DNA protons are present, whereas in the NOESY spectrum of the 9-aminoacridine complex with the same sequence, all of the cross-peaks corresponding to the resonances of the C tract were absent. The spectrum of the C-bulge 9-mer complex had the narrowest resonances of all the oligomers studied with this compound. This particular sequence also binds 9-aminoacridine most strongly. Although we were unable to observe specific NOE's between the acridine and base protons, the interruption of sequential connectivities at T3-G4 and C16-A17 suggests that the drug is intercalating at this site, which is also the preferred site in the regular 8-mer. The duplex could then be stabilized in a conformation where C16 remains unpaired and the rest of the cytosines remain in the helix. Other evidence that the bulge is "trapped" at the 5′ end of the G·C run comes from the chemical shifts of C13 H6 and G6 H8. The chemical shift of C13 H6 in the uncomplexed C-bulge duplex is very close to those of the other cytosines in the C tract, C14–C16, whereas in the normal duplex, the chemical shift of C13 is closer to that of C7, which is also part of a GpC dinucleotide. This was thought to be due to averaging of the bulge site across the C tract. In the drug complex, the chemical shift of C13 H6 (and also of G6 H8) now reverts back to its position in the normal duplex, near to that of C7.

The question remains of why the proton resonances of such a stable drug complex still appear to be exchange broadened and why we are unable to observe NOE contacts between the acridine ring protons and the DNA bases. The dissociation time constant for this molecule as measured by stopped-flow phase partitioning is on the order of 10 s. This technique would measure complete dissociation from the DNA. It possible that the drug is held to the DNA by the hydrogen bonds formed by the amino groups of the side chains but that there is considerable motion or slippage of the acridine ring itself, which results in broadening of the proton resonances. The aromatic

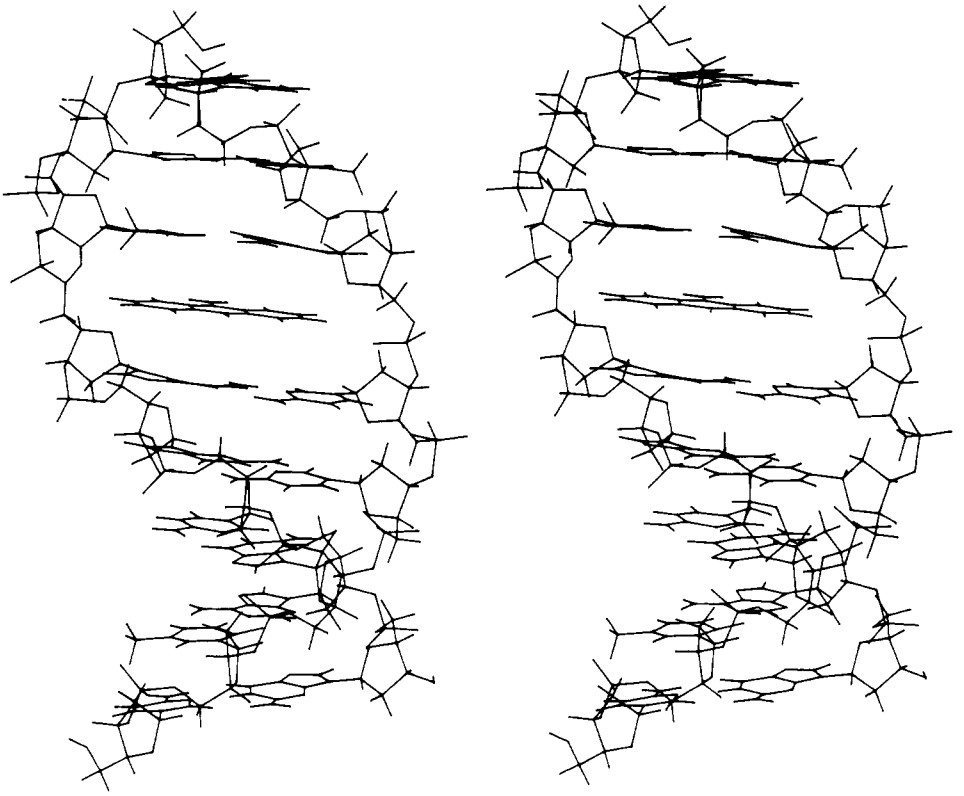

FIGURE 6: A model of 9-aminoacridine intercalated into the 8-mer between base pairs T3·A14 and G4·C13. The diagram was constructed by inserting 9-aminoacridine into regular B-DNA coordinates (Arnott & Hukins, 1972) for the 8-mer by using FRODO on an Evans & Sutherland PS390. The drug was oriented relative to the base pairs following the crystal structure of Sakore et al. (1977), with the 9-amino group facing the minor groove. The bond distances and angles of the sugar backbone were then regularized, resulting in an unwinding angle of 20°.

ring protons are shifted upfield approximately 0.7 ppm in the complex compared to the free drug, which indicates that the drug really is intercalated, and the broadening of the side-chain resonances suggests that they are also bound to the DNA. It is interesting to note that the only drug–DNA NOE's observed for this complex are to the side-chain proton resonances.

Stereochemistry of 9-Aminoacridine Complexes. The drug–DNA NOE contacts in the spectrum of the perfect 8-mer were compared with the crystal structure of 9-aminoacridine bound to 5-iodo-CpG from Sobell and co-workers (Sakore et al., 1977, 1979). In the crystal, the drug stacks with the dinucleotide in two orientations, with the 9-amino group projecting into the minor or major groove. We attempted to see whether the NOE contacts at T3-G4 would fit either of these models. The models can be distinguished from each other by recognizing that, in the one case, the $H_{1,8}$ protons of the acridine ring will not be in a position to make NOE contacts to the protons of the flanking base pairs, whereas the strongest NOE's to the base protons should arise from $H_{3,6}$ and $H_{4,5}$, and to the sugar protons from $H_{2,7}$ and $H_{3,6}$. On the other hand, if the amino group is placed in the major groove, now H1 and H8 should make good NOE contacts to the DNA, but not H4 and H5. There are almost no NOE's to $H_{1,8}$ in the spectrum of the 8-mer (see Figure 2), and many of the strongest contacts are to $H_{3,6}$ and $H_{4,5}$. Furthermore, the $H_{2,7}$ resonance has a number of cross-peaks to sugar proton resonances. This fits the intercalated conformation in the CpG crystal where the amino group is in the minor groove quite well and discriminates against the intermolecular orientation with the amino group facing the major groove. A model of 9-aminoacridine intercalated into the 8-mer at T3-G4 is shown in Figure 6. The diagram was constructed by inserting the drug into regular B-DNA coordinates (Arnott & Hukins, 1972) for the sequence of the 8-mer following the conformation of the crystal structure with the amino group facing the minor groove. The bond distances and angles along the backbone were then regularized, resulting in a helix unwinding of 20°.

The complex of the G-bulge 9-mer, CG4G3, is somewhat more difficult to model. Although the DNA proton resonances of the guanosines opposite the bulged guanosine, G5 and G6, are clearly affected by the presence of the drug, all the strong NOE contacts observed are to C15 and G14. Furthermore, there is a very large change in the chemical shift of G14 H1′. The stereochemistry proposed for the GpG·CpC complex by Young and Kallenbach (1981) shows the drug stacked with the purines on one strand. In this arrangement, all the strong NOE's would be to the purine strand, and there would be almost no NOE's to the pyrimidines, which is not what is observed. This implies that the drug is stacked with, or near to, the extrahelical guanine. It is conceivable that the guanine is now unstacked from the helix, but this would not explain the effect on the G5 resonances. Alternatively, the drug could be stacking across C15·G5 and also with G14.

CONCLUSIONS

Although the data presented here are limited by the rapid exchange between drug complexes, these experiments confirm our expectation that intercalating drugs will specifically bind and stabilize bulge-containing helices. Furthermore, the specific nature of the bulged base and certainly the local sequence are important in determining the nature of the drug–DNA complex. The binding affinities and stereochemistry, and most likely the lifetimes of the complexes of 9-aminoacridine, appear to vary substantially from one sequence to the next. For both homopolymeric runs of bases and mixed sequences, these findings will be important for understanding how mutagens interact with specific sequences, and how they determine the frequencies of mutation at specific sites.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Region of the magnitude COSY spectrum of the 2:1 complex of 9-aminoacridine with the perfect 8-mer (Figure A), phase-sensitive NOESY spectrum of the 1:1 complex of the C-bulge 9-mer and *N*-[2-(dimethylamino)ethyl]-9-[[2-(dimethylamino)ethyl]amino]acridine-4-carboxamide at 490 MHz (Figure B), and region of the NOESY spectrum of the 4-carboxamide complex of the C-bulge 9-mer showing cross-peaks between aromatic and H2′/H2″ and thymine methyl resonances (Figure C) (3 pages). Ordering information is given on any current masthead page.

Registry No. C2G2, 116436-77-2; CG4G3, 116436-82-9; C3G3, 116436-78-3; C4G4, 116436-83-0; C5G5, 116466-09-2; C3G4, 116436-81-8; C4G3, 116436-79-4; CA4G3, 116436-80-7; C2G3, 116502-60-4; 9-aminoacridine, 90-45-9; *N*-[2-(dimethylamino)-ethyl]-6-methyl-9-aminoacridine, 22089-42-5; *N*-[2-(dimethylamino)ethyl]-9-[[2-(dimethylamino)ethyl]amino]acridine-4-carboxamide, 91549-78-9.

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