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Unstable Hoogsteen base pairs adjacent to echinomycin binding sites within a DNA duplex

(¹H NMR/drug–DNA complexes/two-dimensional NMR/DNA oligonucleotides/bisintercalation)

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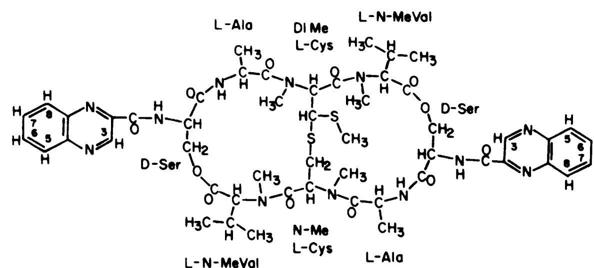
ABSTRACT **The bisintercalation complex present between the DNA octamer [d(ACGTACGT)]$_2$ and the cyclic octadepsipeptide antibiotic echinomycin has been studied by one- and two-dimensional proton NMR, and the results obtained have been compared with the crystal structures of related DNA–echinomycin complexes. Two echinomycins are found to bind cooperatively to each DNA duplex at the CpG steps, with the two quinoxaline rings of each echinomycin bisintercalating between the C·G and A·T base pairs. At low temperatures, the A·T base pairs on either side of the intercalation site adopt the Hoogsteen conformation, as observed in the crystal structures. However, as the temperature is raised, the Hoogsteen base pairs in the interior of the duplex are destabilized and are observed to be exchanging between the Hoogsteen base pair and either an open or a Watson–Crick base-paired state. The terminal A·T base pairs, which are not as constrained by the helix as the internal base pairs, remain stably Hoogsteen base-paired up to at least 45°C. The implications of these results for the biological role of Hoogsteen base pairs in echinomycin–DNA complexes *in vivo* are discussed.**

Echinomycin is a cyclic octadepsipeptide antibiotic with two quinoxaline rings (Structure 1) that bisintercalate into DNA (1, 2). The drug is a potent antitumor and antimicrobial agent (1, 2). Cleavage inhibition patterns ("footprinting") with DNases I and II (3) and methidiumpropyl-EDTA-Fe(II) [MPE-Fe(II)] (4) showed that the preferred binding sites are centered around a CpG step. The MPE-Fe(II) studies showed that the binding site is 4 base pairs and that the strongest binding sites are TCGT and ACGT. The DNase and MPE-Fe(II) studies also revealed that A·T base pairs adjacent to the binding site (and in some cases, runs of A·T base pairs distal to the binding sites) are more sensitive to cleavage by these reagents than is uncomplexed DNA, suggesting that these regions might have an altered conformation from B-DNA. Crystal structures of echinomycin and the related triostin A with the DNA hexamer [d(CGTACG)]$_2$ have been solved (5, 6). Two echinomycins are found to bind to each DNA duplex, with the echinomycin rings bracketing the CpG steps and the polypeptide arranged in the minor groove. A remarkable feature of these structures is that the A·T base pairs are Hoogsteen base-paired (7) with the adenines in the *syn* conformation.

More recently, Mendel and Dervan (8) demonstrated that echinomycin binding results in enhanced sensitivity to diethyl pyrocarbonate $(EtOOC)_2O$ of purines adjacent and distal to echinomycin-binding sites. These results were presented as being consistent with Hoogsteen base-pair formation as a result of drug binding in these longer DNA fragments. Waring and co-workers (9) have also investigated this enhanced sensitivity of $(EtOOC)_2O$ to purines in echi-



Structure 1. Structure of echinomycin including the numbering system for the quinoxaline rings.

nomycin-bound DNA, but they conclude that the evidence does not support the formation of Hoogsteen base pairs as the sole cause of enhanced sensitivity to $(EtOOC)_2O$ in the drug–DNA complexes.

We have investigated complex formation between echinomycin and the DNA octamer [d(ACGTACGT)]$_2$ using solution NMR methods to compare the solution structure to the crystal structure and specifically to answer the question of whether Hoogsteen base pairs are formed adjacent to echinomycin-binding sites in solution. Previous NMR results on echinomycin–DNA complexes focused on DNA tetramers with single drug-binding sites and, thus, do not address the question of Hoogsteen base-pair formation in the interior of a DNA duplex (10). Our results indicate that two echinomycin molecules bind cooperatively to the DNA by bisintercalation and that at low temperatures both the terminal and the interior A·T base pairs are Hoogsteen base-paired in the drug–DNA complex in solution. However, as the temperature is raised, the internal A·T Hoogsteen base pairs become increasingly less stable and are exchanging between Hoogsteen and open or Watson–Crick base-paired conformations. In contrast, the base pairs on the ends of the duplex remain in the Hoogsteen conformation up to at least 45°C.

MATERIALS AND METHODS

Sample Preparation. The DNA octamer d(A[1]C[2]G[3]T[4]A[5]C[6]G[7]T[8]) was synthesized and purified as described (11). The DNA was converted from the ammonium to the sodium form by passage over a Bio-Rad AG 50W-X4 column. The NMR samples contained 2 mM DNA duplex and 50 mM NaCl (pH 6.5; meter reading, no added buffer) in 400 μl of 99.996% D_2O. Spectra in H_2O were obtained on the same sample after redrying in the NMR tube under a stream of N_2(g) and redissolving in 90% H_2O/10% D_2O.

Echinomycin was a gift from the National Cancer Institute. A saturated echinomycin–DNA complex of two drugs per

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Abbreviations: NOESY, nuclear Overhauser effect spectroscopy; τ_m, mixing time.
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DNA octamer was formed by adding 2 equivalents of echinomycin in methanol to the DNA sample in the NMR tube. The aqueous solution of methanol was evaporated over a period of about 12 hr by a stream of $N_2(g)$, allowing the drug to partition from the methanol into the DNA. The dried sample was then redissolved in D_2O and redried and redissolved as necessary for D_2O or H_2O experiments. Partially saturated drug–DNA complexes were prepared similarly.

The DNA octamer was deuterated at the H8(1,3,5,7) positions, which we shall refer to as A(1,5)H8 and G(3,7)H8, by heating a sample of the DNA at 60°C for 48 hr (12), and then the complex was prepared as described above.

NMR Spectroscopy. All NMR experiments were done at 500 MHz on a General Electric GN500 spectrometer. Chemical shifts were obtained by reference to the chemical shift of water, which had been previously calibrated relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DDS). Nuclear Overhauser effect spectroscopy (NOESY) spectra in D_2O were obtained in the pure absorption mode by following the method of States *et al.* (13) and using the standard pulse sequence (14) with presaturation of the residual HDO peak during the 2-sec recycle delay. Spectra were acquired with 1024 complex points in t_2 and 232–332 t_1 values. Phase-sensitive NOESY spectra of samples in H_2O were obtained by replacing the last 90° pulse with a 1–$\bar{1}$ spin-echo pulse sequence (15) and phase cycling appropriately to suppress the large water resonance. Two-dimensional NMR spectra were processed with the Fortran program FTNMR (Hare Research, Woodinville, WA).

RESULTS

Echinomycin–DNA Complex Formation. Spectra of the aromatic region of $[d(ACGTACGT)]_2$ as a function of drug concentration at 30°C are given in Fig. 1. As increasing amounts of drug were added to the DNA, intensities of the resonances for the free DNA decreased, and a new set of resonances appeared from the DNA in the complex plus resonances from the echinomycin quinoxaline rings. This indicates that the drug is in slow exchange with the DNA on the NMR time scale. Only one new set of resonances appeared, which corresponded exactly with resonances of the fully saturated complex at all DNA:drug ratios studied. This is consistent with positive cooperative binding—i.e., either no drugs or else two drugs bound per duplex.

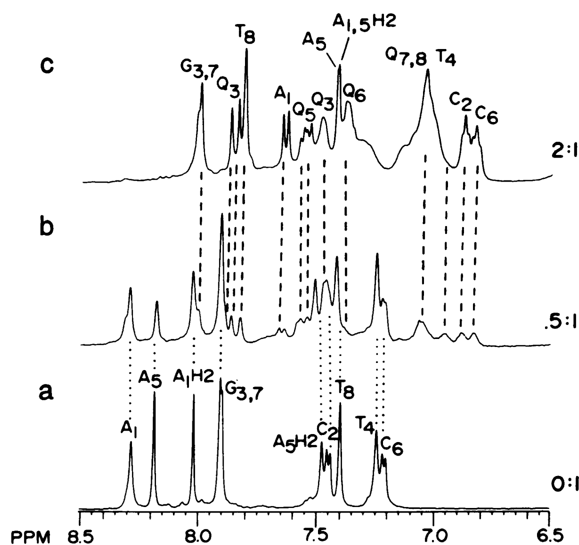

FIG. 1. ^1H NMR spectra of the aromatic proton resonances as a function of drug concentration at 20°C. (*a*) Free $[d(ACGTACGT)]_2$. (*b*) Ratio of 0.5:1 of echinomycin to $[d(ACGTACGT)]_2$. (*c*) Ratio of 2:1 of echinomycin to $[d(ACGTACGT)]_2$. Dotted lines connect resonance positions of the free DNA in *a*, and dashed lines connect resonance positions of the DNA in the 2:1 complex in *c*. Assignments of some of the DNA resonances are indicated on the figure. Data were line-broadened by 1 Hz prior to Fourier transformation.

Assignments of the Nonexchangeable DNA Resonances in the Complex. Assignments of some of the aromatic resonances are indicated on the spectra in Fig. 1. The assignments of the DNA base, sugar H1′, and quinoxaline ring protons at 20°C are summarized in Table 1. These assignments, as well as more complete assignments of the DNA sugar and drug resonances and the drug–DNA contacts, will be discussed in detail elsewhere. However, several points about the assignment strategy are relevant here. We anticipated that regardless of base-pairing type, intercalative binding of the echinomycin would disrupt the usual 5′ base–H1′–3′ base connectivities used to sequentially assign DNA oligonucleotides that adopt an A-DNA- or B-DNA-like structure (16–18), thus making the assignments much more difficult, and this was indeed the case. Therefore, we assigned the DNA base protons by using a combination of methods including standard analysis of the NOESY, correlated spectroscopy (COSY; ref. 19), double quantum-filtered (DQF)-COSY (20), and homonuclear Hartmann–Hahn (HOHAHA; ref. 21) spectra of the complex and by chemical exchange of the G(3,7)H8 and A(1,5)H8 protons with D_2O (12). The chemical exchange experiments (not shown) allowed us to unambiguously distinguish between the H8 and H2 resonances of the adenine bases, which was crucial in the identification of Hoogsteen versus Watson–Crick base pairing (discussed below). We were also able to unambiguously distinguish between the internal and terminal A·T base-pair resonances by comparison of the NOESY spectra of the complex with those observed for a similar complex with the hexamer $[d(CGTACG)]_2$ (spectra not shown).

Imino Proton Spectra of the Complex as a Function of Temperature. Spectra of the imino proton resonances of the free DNA and the DNA in the complex are shown in Fig. 2. At 1°C, four imino resonances were observed corresponding to the terminal A(1)·T(8) (13.3 ppm) and interior T(4)·A(5) (13.56 ppm) base pairs and the C(2)·G(7) and G(3)·C(6) base pairs (22). In the complex, all of the imino proton resonances were upfield-shifted by at least 1 ppm. These large upfield shifts are a characteristic effect of intercalative binding (23). Some very small imino resonances were observed at the chemical shifts of the free DNA because of a small fraction of uncomplexed DNA. The terminal A·T imino resonance appeared downfield of the internal A·T imino resonance. As the temperature was raised, the A·T imino resonances of the drug–DNA complex rapidly began to broaden and disappear. This indicates that these were exchanging much more rapidly

Table 1. Assignments of base, H1′, and drug aromatic proton resonances in the 2:1 echinomycin–$[d(ACGTACGT)]_2$ complex at 20°C.

	Chemical shifts, ppm relative to DDS				
	Base proton		Sugar		
Base	H8/H6	H2/H5/CH_3	H1′	Quinoxaline	Ring H
A(1)	7.64, 7.62	7.37	6.00	Q(3)	7.77, 7.45
C(2)	6.86, 6.84	5.42	5.86	Q(5)	7.55, 7.50
G(3)	7.98		5.78	Q(6)	7.39, 7.25
T(4)	6.97	1.83	5.54*	Q(7)	7.02, 7.08
A(5)	7.38	7.37	5.83	Q(8)	7.02, 7.00
C(6)	6.82, 6.80	5.40	5.79		
G(7)	7.98		5.95		
T(8)	7.77	1.95	6.33		

DDS, 2,2-dimethyl-2-silapentane-5-sulfonate.
*Tentative assignment.

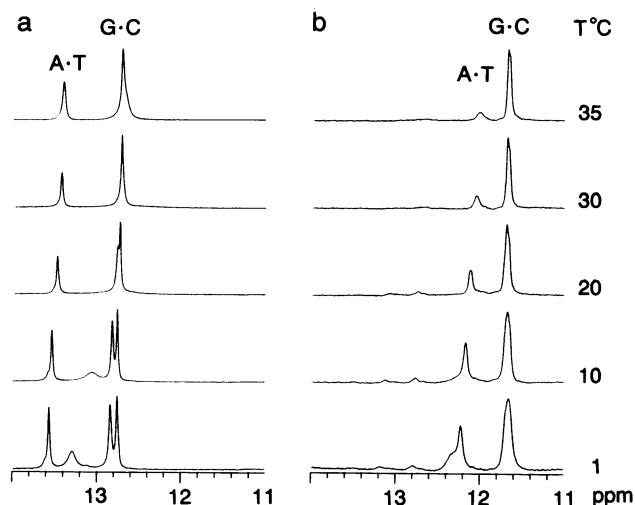

FIG. 2. Spectra of the imino resonances as a function of temperature. (*a*) Free $[d(ACGTACGT)]_2$. (*b*) Echinomycin–$[d(ACGTACGT)]_2$ 2:1 complex. Spectra were acquired by using the 1–$\bar{1}$ spin-echo pulse sequence (90°_x–t–90°_{-x}–Δ–90°_ϕ–2t–$90^\circ_{-\phi}$) (15) to suppress the water resonance. The water resonance was centered at the carrier, and the delays t = 60 μsec and Δ = 50 μsec were used to give a null at water and a maximum at the imino region; 128 acquisitions were collected with a sweep width of 10,000 Hz. Spectra were line-broadened by 1 Hz. No baseline correction was applied to the spectra.

with water than the A·T iminos in the uncomplexed DNA, which were much sharper by comparison.

NOESY Spectra of the Complex in Water. A portion of the NOESY spectrum of the 2:1 echinomycin–$[d(ACGTACGT)]_2$ complex in water at 1°C is shown in Fig. 3*a*. A strong cross peak was observed between the internal T(4)·A(5) imino protons and the base proton resonance identified as A(5)H8. Strong cross peaks are normally observed between A·T imino and adenosine H2 protons in Watson–Crick base-paired DNA. In contrast, for Hoogsteen base pairs, a strong cross peak is expected from the imino protons to the adenosine H8 (Fig. 3*b*), and this is what we observed. We noted that the A(5)H8 resonance partially overlapped with one of the adenosine H2 resonances at 20°C, and this overlap was worse at 1°C. However, the strong cross peak from the imino proton resonance to the A(5)H8 was unambiguously identified, since this cross peak was reduced in intensity in the NOESY spectra taken on the deuterated DNA–echinomycin complex in H_2O at 1°C (not shown). The residual cross peak intensity was due to overlap with an imino–Q(3) quinoxaline cross peak. We were unable to observe cross peaks from the imino resonances of the terminal A·T base pairs to the aromatic protons because of rapid exchange of these imino protons with water even at 1°C (observed as an exchange cross peak with the water in the NOESY spectrum). If the adenine bases in the Hoogsteen base pairs adopt the *syn* conformation as observed in the crystal structure (5), we also would expect a strong cross peak between the adenosine H8 resonances and their sugar H1′ resonances. We observed these strong cross peaks for both the terminal and the interior A·T base pairs in the NOESY spectra in water at 1°C and also in the corresponding NOESY spectra in D_2O (see Fig. 4*a*).

NOESY Spectra of the Complex as a Function of Temperature. A series of NOESY spectra of the complex in D_2O were taken at temperatures ranging from 1°C to 45°C. Stacked plots of NOESY spectra of the echinomycin–DNA complex at 1, 20, 30, and 45°C are shown in Fig. 4. The region of cross peaks between the aromatic resonances and the DNA sugar, H5, thymidine CH_3, and echinomycin peptide resonances is shown. Cross peaks between adenosine H8–H1′, cytidine H6–H5, and thymidine H6–CH_3 are labeled. At 1°C (Fig. 4*a*), a strong cross peak was observed from each adenosine H8 to its own H1′ resonance. The only other cross peaks of comparable size in this region (6.5–5.2 ppm) were between the two cytidine H6–H5 resonances, which overlap to form one peak, and from the quinoxaline drug resonances. All of the other DNA nucleotides showed small cross peaks from the base H6 or H8 to their own H1′ and much larger cross peaks to the H2′ and H2″ protons, as expected for nucleotides with bases in the *anti* conformation normally adopted in B-DNA helices (24). As noted above, sequential connectivities were not observed here. Comparison of the integrated intensity of the adenosine H8–H1′ cross peaks to those of cytidine H6–H5 (assuming two cross peaks of equal intensity) gave interproton distances in adenosine from H8 to H1′ of about 2.6–2.7 Å. This distance is in the range expected if the adenine bases adopt the *syn* conformation as observed in the Hoogsteen base pairs in the crystal structure and is considerably less than the 3.7–3.9 Å expected for the *anti* conformation (25).

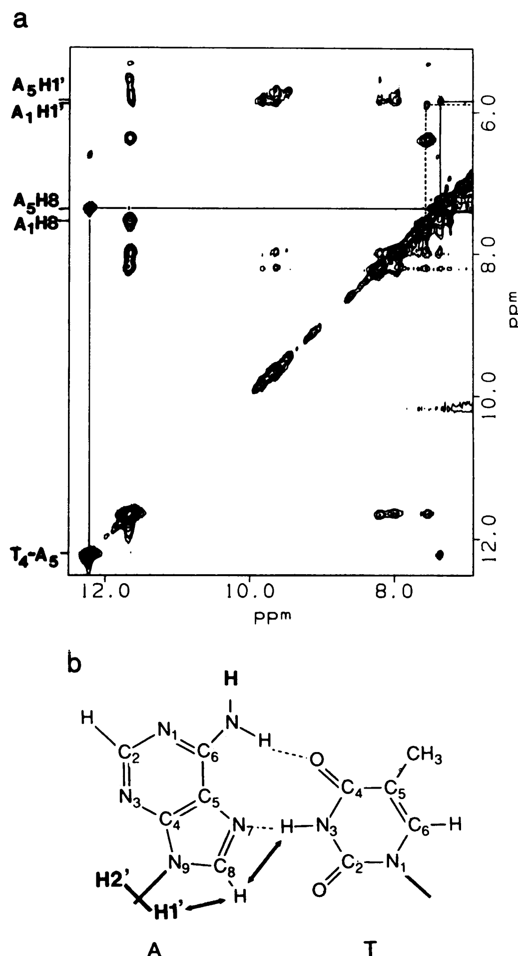

FIG. 3. (*a*) NOESY spectrum of the 2:1 echinomycin–$[d(ACGTACGT)]_2$ complex in 90% H_2O/10% D_2O at 1°C with mixing time (τ_m) = 50 msec. An expanded region showing the imino and aromatic resonances and cross peaks is shown. The solid line shows the imino to H8 to H1′ cross peaks for Ado-5. The dotted line shows the H8 to H1′ cross peaks for Ado-1. Data were processed with a skewed sine bell squared (skew = 1.1) phase-shifted by 80°; 232 points were apodized in each dimension. The t_1 dimension was zero-filled to 2048 points prior to Fourier transformation. (*b*) Structure of a Hoogsteen A·T base pair. Arrows indicate the short interproton distances, which should give rise to large NOEs. Subscripted numbers refer to atom numbering (except for methyl).

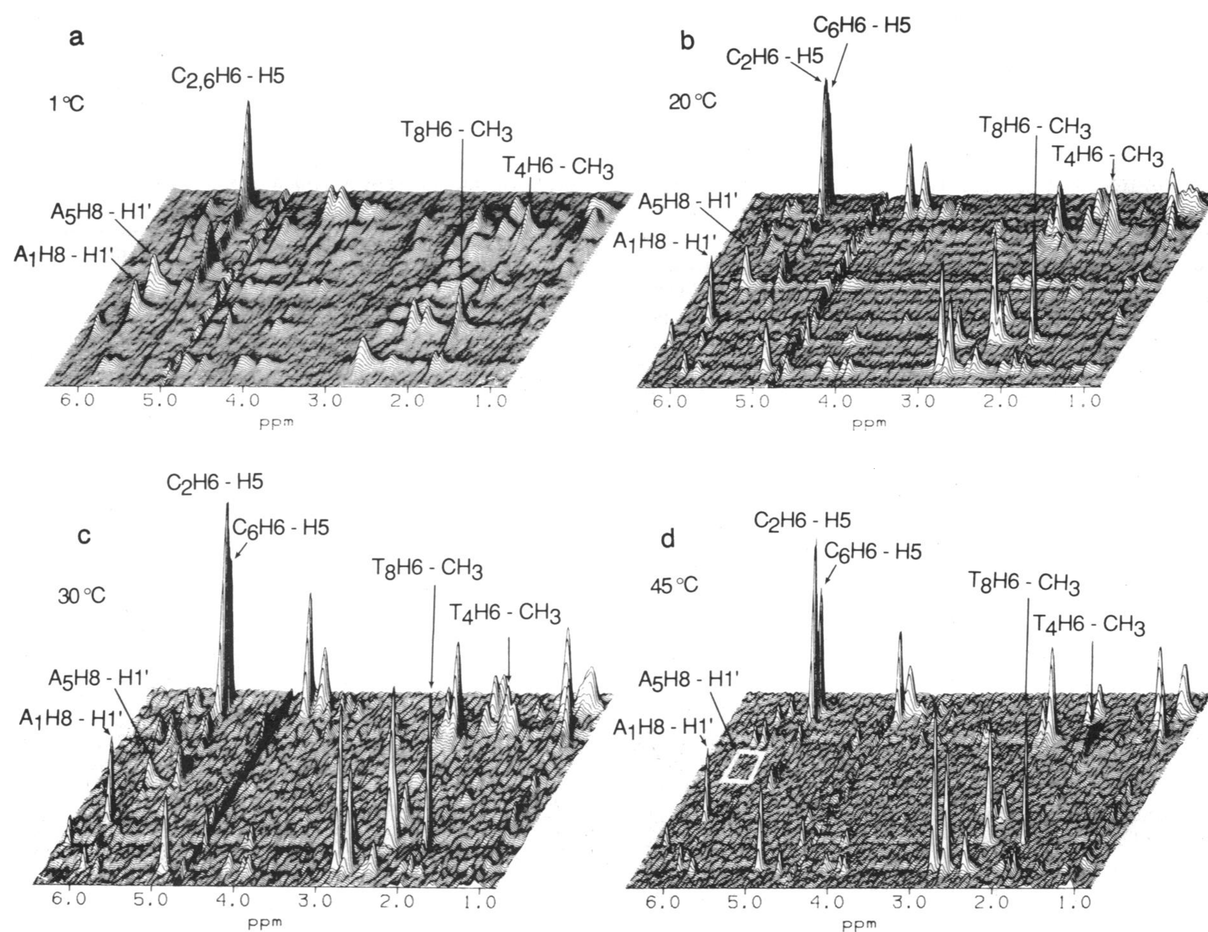

FIG. 4. Stacked plots of NOESY spectra of the 2:1 echinomycin–$[d(ACGTACGT)]_2$ complex in D_2O as a function of temperature: 1°C, τ_m = 50 msec (*a*); 20°C, τ_m = 100 msec (*b*); 30°C, τ_m = 150 msec (*c*); and 45°C, τ_m = 180 msec (*d*). The region of cross peaks between the aromatic and the DNA sugar, H5, CH_3, and drug peptide proton resonances is shown; nucleotide positions are subscripted. The spectrum was processed with a skewed sine bell squared (skew = 1.1) and phase-shifted by 80°. The t_1 time domain was zero-filled to 1024 points prior to Fourier transform. The boxed region in *d* shows the absence of the A(5)H8–A(5)H1′ cross peak.

As the temperature was raised, the A(5)H8–A(5)H1′ cross peak began to broaden extensively and completely disappeared by 45°C (boxed region in Fig. 4*d*). In contrast the A(1)H8–A(1)H1′ cross peak remained sharp, and the intensity of this cross peak relative to the cytidine H5–H6 cross peak remained essentially constant throughout the temperature range studied. The T(4)H6–T(4)CH_3 cross peak also broadened and disappeared as the temperature was raised.

DISCUSSION

Binding Mode of Echinomycin to the DNA Octamer. Our studies indicate that echinomycin binds cooperatively to both of the strong binding sites on the DNA octamer $[d(ACGT-ACGT)]_2$. This is consistent with the footprinting studies of Waring and coworkers (3, 9), which showed an all-or-nothing effect as the drug concentration was increased. A doubling of some but not all of the DNA resonances is observed in the drug–DNA complex, which indicates that binding of the drug breaks the symmetry of the DNA duplex. The A(1)H8 resonance is split into two resonances and the C(2,6)H6 resonances appear as overlapping doublets (Fig. 1). Intercalative binding of the antibiotic via the quinoxaline rings is confirmed by the large upfield shifts observed in the imino proton spectra of the DNA upon drug binding. NOESY cross peaks observed between the quinoxaline and the DNA base resonances indicated that the quinoxaline rings intercalate on either side of the CpG steps as expected; the detailed DNA–drug contacts will be presented elsewhere.

Hoogsteen Base Pairs in the A·T Base Pairs Flanking the Intercalation Site. Cross peaks observed in the NOESY spectra of the complex in D_2O and H_2O confirm that both the terminal and the interior A·T base pairs in the 2:1 echinomycin–DNA complex adopt the Hoogsteen conformation at 1°C (Figs. 3*a* and 4*a*). Analysis of the NOESY spectra in D_2O as a function of temperature indicates that while the 1°C structure appears to correspond to those observed in the crystal, the conformation begins to change at higher temperatures. As the temperature is raised, resonances and cross peaks arising from resonances in the interior A·T base pairs are observed to broaden and disappear. We interpret this to indicate that these base pairs are in intermediate exchange between the Hoogsteen and an unpaired state (or possibly between the Hoogsteen and a Watson–Crick base-paired state). The broadening and upfield shifts observed for the A·T imino resonances of the complex as a function of temperature (Fig. 2) are consistent with the behavior normally seen in base pairs exchanging with water. Therefore, we favor the former explanation. In contrast to the interior Hoogsteen A·T base pairs, the resonances from the Hoogsteen base pairs on the ends of the duplex remain sharp up to at least 45°C. This indicates that the exchange broadening of the DNA resonances for the interior Hoogsteen base pairs is not due to changes in kinetics or mode of drug binding because, if that were the case, one would expect exchange broadening for the terminal base pairs also.

The temperature study of the imino proton resonances of the complex in H_2O showed that Hoogsteen A·T base pairs

in the center of the helix exchange unusually rapidly with water compared to the free DNA (Fig. 2). This may be indicative, but does not prove (26), that the lifetime of the Hoogsteen A·T base pairs in the center of the helix is much shorter than that of the Watson–Crick A·T base pairs in the free DNA. While further experiments need to be done to obtain accurate lifetimes for these base pairs, exchange broadening as a function of temperature observed for the imino protons of the interior A·T Hoogsteen base pairs is consistent with the results obtained in the D_2O spectra that indicate that the Hoogsteen A·T base pairs in the center of the duplex are kinetically less stable than Watson–Crick base pairs, and that these base pairs are also kinetically less stable than the Hoogsteen A·T base pairs at the ends of the duplex. Formation of stable Hoogsteen base pairs at the ends of the DNA tetramer d(ACGT) complexed to echinomycin at 24°C has also been reported by Gao and Patel (10), consistent with the results reported here.

The possibility of Hoogsteen A·T base-pair formation has been known for many years; in fact, early crystal structures of adenosine and thymidine nucleotides indicated that their bases adopted the Hoogsteen conformation (7). Thus, it is clear that in the absence of helical constraints, adenosine and thymidine will readily form Hoogsteen base pairs. In the echinomycin–DNA complex reported here, we observe that stable Hoogsteen A·T base pairs are formed at the ends of the DNA helix. In contrast, Hoogsteen A·T base pairs in the center of the duplex, which are constrained by the helix, are much less stable. By 20°C, resonances from these base pairs already exhibit significant line broadening. Therefore, we conclude that for echinomycin–DNA complexes *in vivo*, the relevant conformation of A·T base pairs flanking echinomycin-binding sites may be an open rather than a Hoogsteen base-paired state. We note that the majority of the echinomycin-binding sites in the footprinting experiments were separated by <8 base pairs.

Relevance to Footprinting Studies. The structural basis for the increased sensitivity to footprinting reagents of A+T-rich regions adjacent to echinomycin-binding sites is far from clear. We have shown that the base pairs at the center of the helix in the complex are less stable than those at the center of the helix in free DNA. This destabilization of the base pairs may be partially responsible for the increased sensitivity of the regions adjacent to echinomycin-binding sites. Local unwinding due to intercalation of the drug can account for the increased sensitivity of the A+T-rich regions to DNase I. This enzyme is used as a probe of the DNA groove (27). The reactivity to $(EtCOOH_2)O$ is more problematic. One problem is that it is not clear whether $(EtCOOH_2)O$ is reacting at N7, N1, or N3. The reagent also has been used to identify regions of Z-DNA, where it reacts at N7 (28), a position that should be protected if Hoogsteen base pairs are formed. Mendel and Dervan (8) propose that in the echinomycin–DNA complexes, $(EtCOOH_2)O$ is reacting at N1 or N3 or both. If the base pairs open, all three nitrogens of adenine would be available for reaction with $(EtCOOH_2)O$. Thus, destabilized or open base pairs near echinomycin-binding sites are equally consistent with the footprinting results as Hoogsteen base pairs could be.

Summary. The NMR results we have obtained on the 2:1 complex of echinomycin with $[d(ACGTACGT)]_2$ are consistent at low temperatures with the crystal structures determined by Rich and coworkers (5, 6, 29). However, we have presented evidence that the Hoogsteen base pairs observed in the crystal structure and at low temperatures in solution may not be the relevant structures for echinomycin binding within a DNA duplex under physiological conditions. More complete details of the structure of the echinomycin–DNA complex will be presented elsewhere.

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