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NMR Studies of the Complex between the Decadeoxynucleotide d-(GCATTAATGC)$_2$ and a Minor-Groove-Binding Drug†

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ABSTRACT: Nearly all ^{1}H NMR lines of the complex formed between the bis(quaternary ammonium) heterocycle 4-[*p*-[*p*-(4-quinolylamino)benzamido]anilino]pyridine (**1**, also known as SN 6999) and the decadeoxyribonucleoside nonaphosphate d-(GCATTAATGC)$_2$ were sequentially assigned by using one- and two-dimensional NMR techniques. Intermolecular nuclear Overhauser effects between the ligand and the DNA show that the drug binds in the minor groove of the DNA, interacting with the central A-T base pairs. Over the temperature range from 277 to 313 K, the lifetime of the drug in the DNA binding sites is short relative to the NMR time scale, since fast exchange is observed for all but a few protons. A model for the binding of **1** to d-(GCATTAATGC)$_2$ is proposed, where the drug binds to two equivalent sites covering approximately five A-T base pairs, which assumes exchange of **1** between these two binding sites.

In the search of guidelines for the development of new drugs that are biologically active through binding to DNA, there has been a growing interest in the examination of drug–DNA complexes, both in the crystalline state and in solution. DNA–drug complexes may furthermore also serve as models for the study of gene expression and its control by interactions between DNA and proteins. Most NMR studies in this field so far focused on DNA complexes with various intercalating agents [e.g., Reid et al. (1983a), Assa-Munt et al. (1985), Patel et al. (1981), and Lown and Hanstock (1985)]. One minor-groove-binding drug was also studied in detail, i.e. netropsin [see Patel (1982) and Patel and Shapiro (1985) and references

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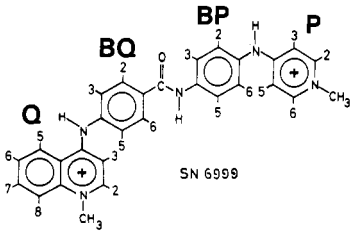

$$\begin{array}{l} C_{10}G_9T_8A_7A_6T_5T_4A_3C_2G_1\text{-d} \\ \quad I\ \ II\ \ III\ \ IV\ \ V\ \ V\ \ IV\ \ III\ \ II\ \ I \\ \text{d-}G_1C_2A_3T_4T_5A_6A_7T_8G_9C_{10} \end{array}$$

FIGURE 1: Structures of 4-[*p*-[*p*-(4-quinolylamino)benzamido]-anilino]pyridine (SN 6999), which is denoted by **1** in the text, and the decadeoxyribonucleoside nonaphosphate used in this study. In the latter the numeration of the base pairs with roman numerals reflects the twofold symmetry observed for the solution conformation of the free duplex. For the drug an unambiguous identification of individual protons includes the letter for the ring and the ring position, e.g. P2.

cited therein], as was the anthramycin–d-$(ATGCAT)_2$ adduct (Graves et al., 1985). This paper reports a study with the bis(quaternary ammonium) heterocycle 4-[*p*-[*p*-(4-quinolylamino)benzamido]anilino]pyridine[**1** also known as SN 6999 (Cain et al., 1969) (Figure 1)], for which previous studies [e.g., Braithwaite and Baguley (1980)] had indicated binding to DNA without intercalation. We have chosen to study the complex of **1** with the self-complementary decadeoxyribonucleoside nonaphosphate d-$(GCATTAATGC)_2$ to gain information on the structure of the complex and the kinetics of the binding process, using one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR).[1]

The binding of **1** to DNA and its biological activity have been studied extensively [(Braithwaite & Baguley, 1980; for a review see Baguley (1982)]. **1** binds on the outside of the DNA (it does not unwind closed circular superhelical PM2 DNA) and shows a pronounced selectivity for poly[d(AT)] relative to poly[d(GC)]. The binding constant to poly[d(AT)] in 0.2 M ionic strength buffer at 298 K is 2×10^6 M^{-1}, and there is an excluded site size of approximately five base pairs. **1** is a highly active experimental antitumor drug (Cain et al., 1969) in vitro and in vivo, and it also inhibits the production of bacteriophage (Robertson, 1978; Robertson & Baguley, 1982). Its 6-NH_2 derivative (NSC 176 319) has undergone preclinical and toxicological testing in preparation for clinical trials (Plowman & Adamson, 1978).

The DNA fragment d-(GCATTAATGC) was designed for this study with a central core of AT base pairs as potential drug binding sites and two GC base pairs at each end to diminish fraying of the duplex. As demonstrated with different systems, NMR studies of DNA–drug interactions allow determination of base pair and sequence specificity, mode of binding, kinetics of the binding process, and, for short DNA fragments of known sequence, delineation of close contacts between protons of the drug and the DNA molecule (Patel, 1982; Feigon et al., 1982, 1984; Brown et al., 1984; Graves et al., 1985; Leupin et al., 1985; Patel & Shapiro, 1985; Reid et al., 1983a). Previously, 2D NMR was used to obtain sequence-specific assignments of the ^1H resonance in the free DNA duplex (Chazin et al., 1986). In this paper these assignments provide the starting points for determination of structural changes in the DNA introduced by the binding of the drug and for investigations of static and dynamic aspects of the intermolecular interactions in the DNA–drug complexes.

Materials and Methods

The decadeoxynucleoside nonaphosphate d-$(GCATTAATGC)_2$ was synthesized by the phosphotriester method in liquid phase as described in earlier papers (Chazin et al., 1986; Denny et al., 1982). The oligonucleotide was used as the sodium salt. The synthesis of the drug (Figure 1) is described by Cain et al. (1969). It was used as the dibromide salt. Because the free drug **1** is only poorly soluble in aqueous solution of medium ionic strength, NMR spectra of the free drug were recorded in unbuffered ^2H_2O solution (p^{2}H 6.9, c 3.0×10^{-3} M, T = 301 K). For the complex, all NMR experiments were performed on a single sample containing a 1:1 ratio of d-$(GCATTAATGC)_2$ and **1**. The H_2O and ^2H_2O solutions were buffered at pH 7.0 with 50 mM phosphate buffer and also contained 100 mM sodium chloride and 0.1% (w/v) sodium azide. For ^2H_2O spectra, the labile protons of the DNA fragment and the drug were replaced by ^2H by dissolving the complex in 98% ^2H_2O, allowing the solution to stand at room temperature for 1 h, and lyophilizing. The procedure was repeated twice, and then the final solution was made in "100%" ^2H_2O.

^1H NMR spectra were recorded on Bruker WM-500 and AM-360 spectrometers, both equipped with Aspect 2000 computers, except for the 2QF-COSY and NOESY experiments on the free drug and the 1D NOE experiments on the complex at 283 K, which were recorded on the WM-500 spectrometer equipped with an Aspect 3000 computer. Experimental schemes used in these studies to acquire phase-sensitive 2D NMR spectra with quadrature detection in both dimensions have been previously described (Chazin et al., 1986; Marion & Wüthrich, 1983). The experimental conditions for the 2D experiments in ^2H_2O solution at 301 K were matched to those used in the studies of the free d-$(GCATTAATGC)_2$ duplex (Chazin et al., 1986), except that 512 (rather than 740) experiments were acquired for the 2-quantum spectrum. In addition, 2QF-COSY and NOESY spectra (τ_m = 300 ms) were acquired at 283 K. NOESY spectra in ^2H_2O solution at 301 K were run with 150- and 300-ms mixing times. NOESY spectra at 301 K in H_2O solution with the solvent suppressed by continuous irradiation were acquired with mixing times of 200 and 300 ms, on 450 experiments with t_1 values from 3 μs to 30 ms, with each free induction decay consisting of 4096 data points. These data were processed in a manner similar to that used for the ^2H_2O spectra.

NOESY experiments in H_2O solution at 301 K with a $1\text{–}\bar{3}\text{–}3\text{–}\bar{1}$ observation pulse (Hore, 1983) were run with mixing times of 300 ms (Santos et al., 1984). The excitation maximum was set to 11.5 ppm, midway between the exchangeable drug resonances and the exchangeable DNA resonances. A total of 256 experiments was acquired with t_1 values from 3 μs to 13 ms over a period of 45 h, with each free induction decay consisting of 4096 data points. Prior to Fourier transformation of these data sets, the time domain data were filtered either with a shifted sine bell or with exponential window functions and expanded to 8192 × 4096 data points by zero filling. The resulting digital resolution was 2.4 Hz/point in ω_2 and 5.0 Hz/point in ω_1. 1D-truncated driven NOE experiments (TOE) in H_2O solution with $1\text{–}\bar{3}\text{–}3\text{–}\bar{1}$ observation pulses (Hore, 1983) were acquired at 301 K with 300- and 600-ms saturation periods and a recycle time of 2.7

[1] Abbreviations: NMR, nuclear magnetic resonance; fid, free induction decay; 1D and 2D, one and two dimensional; COSY, 2D correlated spectroscopy; 2QF, two quantum filtered; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; TOE, truncated-driven NOE experiment; CD, circular dichroism; d_i(X;Y), intraresidue distance between protons X and Y; d_s(X;Y), sequential distance between protons X and Y; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt.

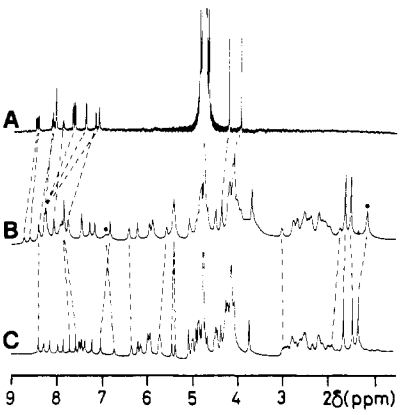

FIGURE 2: Comparison of the 500-MHz ^1H NMR spectra of the nonexchangeable protons of the drug SN 6999 (Figure 1) (A), d-(GCATTAATGC)$_2$ (C), and their 1:1 complex (B) in ^2H_2O at 301 K. The solutions for (B) and (C) were 2.8 mM in duplex with 100 mM NaCl, 50 mM phosphate buffer, and p^{2}H 7.0. Lines connect those resonances in the spectrum of the complex with the corresponding resonances in the free drug and the free duplex, for which individual assignments were obtained both in the individual components and in the aggregate. Stars indicate exchange-broadened resonances in the complex.

s and at 283 K with a 1-s saturation period and a recycle time of 3.2 s. In the experiments run with the Aspect 2000 computer and analog phase shifters it was observed that the H_2O suppression could be significantly improved by using only a single set of phases in the $1\text{-}\bar{3}\text{-}3\text{-}\bar{1}$ composite pulse and no CYCLOPS (Hoult & Richard, 1975).

RESULTS

General Characterization of the Binding of Drug 1 to the DNA. In Figure 2 the 500-MHz NMR spectra of the nonlabile protons of d-(GCATTAATGC)$_2$ and **1** are compared with the spectrum of their 1:1 complex. The increased line width of the well-separated drug resonances at the low-field end of spectrum B provide clear evidence for binding of **1** to the DNA. In spite of this and other evidence for binding of the asymmetric drug molecule **1** the molecular symmetry manifested, e.g. by the appearance of only three methyl resonances in the spectrum of the uncomplexed DNA, appears to be retained in the complexed. Comparison of parts B and C of Figure 2 further shows that in the complex the methyl resonance at highest field is clearly broader than both the corresponding line in the free DNA and the other two methyl lines in the complex.

The appearance of the highest field methyl resonance varies pronouncedly with temperature (Figure 3). At 305 K a single, relatively narrow line is observed, which broadens at lower temperature and eventually splits into two lines of equal intensity at temperatures below ca. 290 K, indicating two-site exchange. Coalescence is observed at 289 K. From the standard formula for two-site exchange with equal population of the sites (Sutherland, 1971)

$$k_{coal} = (\pi/2^{1/2})\Delta\nu = 2.22\Delta\nu \qquad (1)$$

where $\Delta\nu = 43$ s^{-1} is the difference between the chemical shifts of the exchanging spins, the rate of exchange of **1** between different binding sites is estimated to be ≥ 96 s^{-1} at 289 K. The apparent retention of the twofold symmetry of the free DNA in the complex could thus arise from exchange of the drug between two binding sites that is fast enough on the NMR time scale to result in chemical shift averaging.

Figure 4 shows the ^1H NMR spectra of the imino protons in the base pairs of d-(CGATTAATGC)$_2$ in the free DNA and in the complex with **1**. Clearly, complex formation is

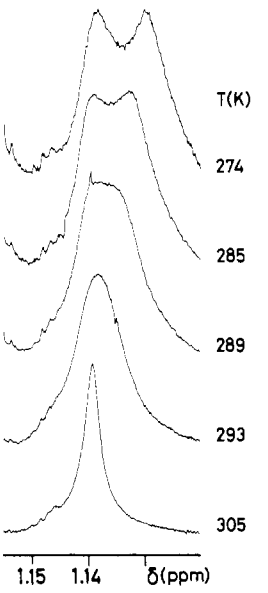

FIGURE 3: Temperature dependence of the highest field methyl resonance in the 500-MHz ^1H NMR spectrum of the 1:1 complex of d-(GCATTAATGC)$_2$ with **1**; same solution as for Figure 2B. Different expansions along the vertical axis were used at the different temperatures, so that the resonance intensities cannot be directly compared. (This resonance was subsequently assigned to T_8.)

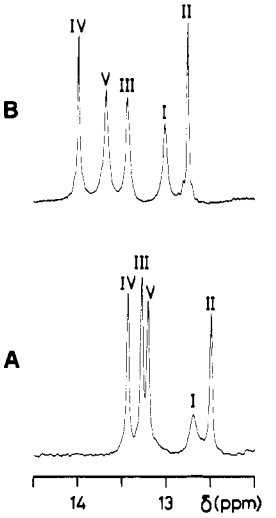

FIGURE 4: 500-MHz ^1H NMR spectra of the imino protons of d-(GCATTAATGC)$_2$ in the free DNA duplex (A) and in the 1:1 complex with **1** (B). The temperature was 288 K; otherwise, the solution conditions were identical with those given in Figure 2. The spectra were obtained with the $1\text{-}\bar{3}\text{-}3\text{-}\bar{1}$ selective excitation pulse sequence as described under Materials and Methods. Assignments are indicated above each resonance (see Figure 1 for the numeration used).

manifested in the sizable chemical shift differences in this spectral region. Furthermore, the labile protons also manifest the apparent retention of the twofold symmetry of the DNA duplex in the complex with **1**, since only five resonance lines are observed. A unique feature of this complex is that the exchange of the imino protons of the DNA fragment and of the amino and amide protons of the drug with the solvent are significantly slowed down, so that observation of nearly all labile protons is possible while saturating the solvent resonance. In the spectra of the free drug or the free DNA, no exchangeable protons can be observed under these experimental conditions. To our knowledge, this is the first example of an observation of exchangeable protons in a DNA fragment by using this convenient method of solvent suppression.

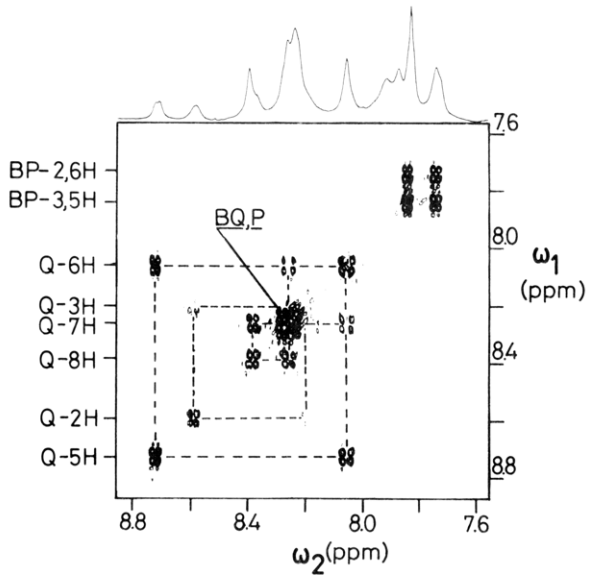

FIGURE 5: Identification of the aromatic spin systems of **1** in a phase-sensitive 2QF-COSY spectrum of the 1:1 complex of d-$(GCATTAATGC)_2$ and **1** at 500 MHz, T = 301 K. The corresponding region in the 1D NMR spectrum is shown at the top. Experimental details are given in Materials and Methods. Proton resonances within each spin system are connected by broken lines and are identified on the left. The resonances of BQ and P are all overlapped near 8.2 ppm (see Table I). For the notation see Figure 1.

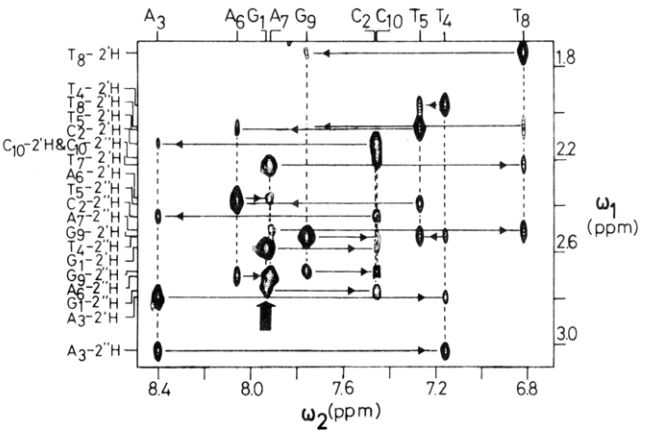

FIGURE 6: Absorption mode NOESY spectrum at 500 MHz of the 1:1 complex of d-$(GCATTAATGC)_2$ and **1**, T = 301 K. The spectral region is shown that was used for obtaining sequential resonance assignments of the DNA in the complex. The spectrum was recorded with a 150-ms mixing time. Further experimental details are given under Materials and Methods. Sequence-specific assignments are indicated at the top for the base protons 6H or 8H and near the cross peaks for 2'H and 2''H. The thick arrow indicates the start of the assignment pathway from G_1 to C_{10}.

Resonance Assignments. Sequence-specific proton resonance assignments for the drug–DNA complex were obtained by using 2QF-COSY, 2-quantum spectroscopy, RELAYED-COSY, and NOESY in a manner similar to the assignment of the free DNA duplex d-$(GCATTAATGC)_2$ (Chazin et al., 1986). Compared to the work with the free DNA, which was recently presented in detail (Chazin et al., 1986), the following are some important novel aspects of the NMR experiments with the complex.

The identification of the ^1H spin systems in the individual nucleotides of the DNA and the different aromatic rings in **1** (Figure 1) was complicated by the fact that there was overlap between resonances with similar chemical shifts. In this respect 2QF-COSY proved to be a powerful experiment for the identification of the proton spin systems of **1**. Because the DNA singlet lines in the region from 7.5 to 8.5 ppm were suppressed by the 2QF (Piantini et al., 1982; Rance et al., 1984; Shaka & Freeman, 1983), the drug spectrum could be investigated without interference from the presence of the DNA. Figure 5 illustrates the identification of the drug spin systems in this spectral region. It further shows that there is particularly severe overlap of drug resonances near 8.25 ppm, which subsequently also made differentiation of certain NOESY cross peaks difficult. As a consequence of the rather broad lines, not all of the deoxyribose proton systems 1'H–2'CH_2–3'H–4'H could be completely identified with the use of scalar couplings. The remaining assignments were made on the basis of intraresidue NOEs observed at short mixing times.

The spin systems of the nonlabile DNA protons were sequentially assigned by the usual methods (Broido et al., 1984; Clore & Gronenborn, 1985; Feigon et al., 1982, 1983; Frechet et al., 1983; Haasnoot et al., 1983; Hare et al., 1983; Hosur et al., 1985; Reid et al., 1983b; Sanderson et al., 1983; Scheek et al., 1983, 1984; Weiss et al., 1984), relying on observation of NOESY cross peaks arising from short proton–proton distances between adjacent residues. Figure 6 shows that very similar NOESY cross peak patterns connecting the base protons with the 2'-methylene protons of the deoxyribose are observed for the DNA in the complex with the drug **1** as for the free DNA (Chazin et al., 1986). Since the NOESY spectrum of Figure 6 was recorded with a long mixing time, spin diffusion was sufficiently efficient to cause the appearance of four cross peaks of comparable intensity for each base proton. These correspond to the connectivities $d_i(6,8;2')$, $d_i(6,8;2'')$, $d_s(2';6,8)$, and $d_s(2'';6,8)$ (Chazin et al., 1986). Following the arrows in Figure 6, a continuous assignment pathway extends from G_1 to C_{10}, where there are in general two parallel sequential connectivities represented by the horizontal lines.

Sequential resonance assignments for the labile DNA protons were made by both 1D TOE difference spectroscopy in H_2O, using a $1\text{–}\bar{3}\text{–}3\text{–}\bar{1}$ observation pulse, and NOESY recorded with presaturation of the solvent resonance or with a $1\text{–}\bar{3}\text{–}3\text{–}\bar{1}$ selective observation pulse. The nonexchangeable A2H resonances could also be sequentially assigned in these spectra, on the basis of large interstrand NOEs with the thymidine N3H of the same Watson–Crick base pairs. The two labile amino protons and the amide proton of **1** (Figure 1) were sequentially assigned by the occurrence of large intramolecular NOEs to nonexchanging resonances.

The results of the NMR assignments for the drug–DNA complex are summarized in Table I, which lists the chemical shifts of the assigned resonances.

*Intermolecular Contacts in the Complex of DNA with **1**.* On the basis of the sequence-specific resonance assignments, the intermolecular contacts between d-$(GCATTAATGC)_2$ and **1** were delineated by chemical shift measurements and by NOESY.

In Figure 7 the drug-induced changes of the chemical shifts of specific protons of the DNA duplex are plotted vs. the sequence. These graphs demonstrate that the nucleotides A_3 to T_8 are clearly influenced by the binding of **1**. Nearly all resonances of the nonexchangeable DNA protons are shifted upfield upon binding of **1**, an exception being the adenine 2-protons (not shown in Figure 7). In contrast, the exchangeable protons and A2H are shifted toward lower field. Overall, the chemical shift measurements thus indicate that the drug **1** binds predominantly to the segment d-$(A_3T_4T_5A_6A_7T_8)_2$ in the DNA duplex and that qualitatively different effects of drug binding are observed for the deoxyribose protons and the base protons 6 and 8 on the one hand

Table I: ^{1}H NMR Chemical Shifts of the 1:1 DNA–Drug Complex (0.05 M PO_4^{2-}, 0.1 M NaCl, pH 7.0, T = 301 K)[a]

	chemical shifts of d-(GCATTAATGC)$_2$[b] (ppm)											
residue	N1H	2H	N3H[c]	5H	5CH$_3$	6H	8H	1′H	2′H	2″H	3′H	4′H
G_1	*12.95*						7.94	5.96	2.59	2.77	4.86	4.23
C_2				5.44		7.46		5.58	2.13	2.45	4.90	4.21
A_3		*7.82*					8.40	6.40	2.79	3.02	5.10	4.49
T_4			*13.94*		1.51	7.16		5.89	1.97	2.53	4.83	
T_5			*13.64*		1.63	7.27		*5.39*	2.07	2.39	*4.72*	
A_6		*6.91*					*8.07*	*5.43*	*2.37*	*2.70*	*4.84*	*4.03*
A_7		*7.85*					*7.93*	*5.41*	*2.22*	*2.51*	*4.83*	
T_8			*13.39*		*1.14*	*6.82*		*5.05*	*1.74*	*2.06*	*4.70*	
G_9	*12.76*						7.76	5.88	2.54	2.68	4.95	*4.18*
C_{10}				5.43		7.45		6.22	2.21	2.22	4.53	4.08

	chemical shifts of **1**[b] (ppm)			chemical shifts of **1**[b] (ppm)			chemical shifts of **1**[b] (ppm)	
proton	free drug **1**	**1** bound to DNA	proton	free drug **1**	**1** bound to DNA	proton	free drug **1**	**1** bound to DNA
Q-2H	8.41	*8.59*	Q-CH$_3$	4.22	*4.36*	P-3,5H	7.08	*8.28*
Q-3H	7.16	*8.19*	BQ-2,6H	8.03	*8.26*	P-CH$_3$	3.96	*4.10*
Q-5H	8.46	*8.72*	BQ-3,5H	7.61	not assigned	amide NH	not obsd	9.58[c]
Q-6H	7.89	*8.06*	BP-2,6H	7.37	*7.74*	amino NH betw PB and P	not obsd	10.13[c]
Q-7H	8.12	*8.25*	BP-3,5H	7.65	*7.84*	amino NH betw Q and BQ	not obsd	10.42[c]
Q-8H	8.08	*8.38*	P-2,6H	8.03	*8.23*			

[a] Italicized resonances exhibit a drug-induced chemical shift difference of >|0.1| ppm relative to the resonance position in the free DNA. [b] Indirectly referenced to DSS, using the H^{2}HO resonance previously calibrated in stock buffer solution. [c] Measured in H_2O.

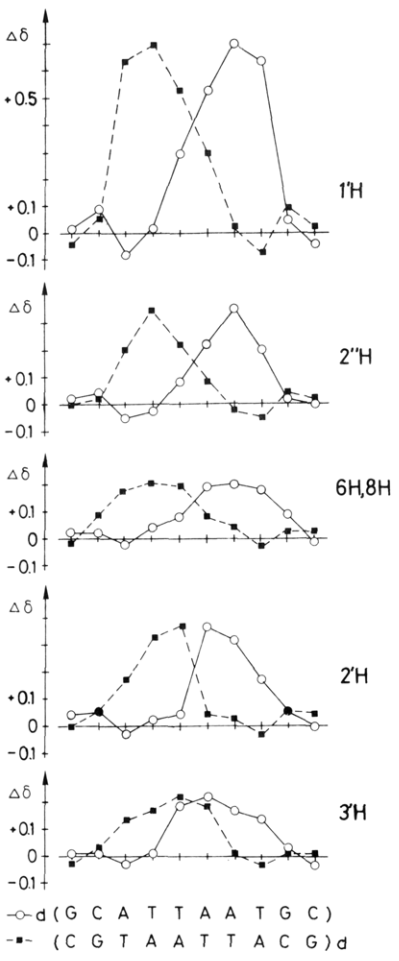

FIGURE 7: Graphical presentation of the drug-induced chemical shift changes for selected nonlabile protons in the DNA sequence. Positive values indicate that the resonances in the complex are at higher field than in the free DNA duplex. The + and − strands of the DNA are shown at the bottom and are identified with the symbols ○ and ■, respectively. (The actual experimental observation was the prevalence of a single set of NMR lines corresponding to the palindromic symmetry shown in Figure 1; see text.)

and for the imino protons and A2H in the Watson–Crick base pairs on the other hand. This agrees quite well with the

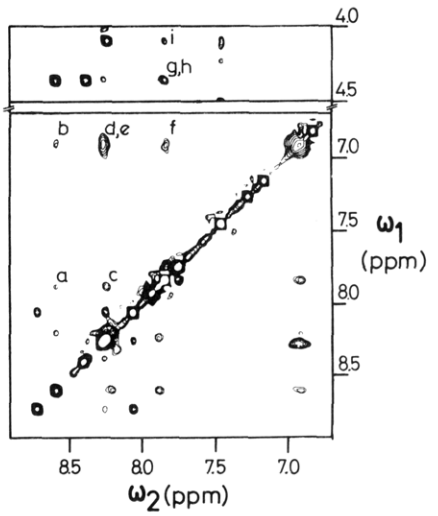

FIGURE 8: Two regions of a 500-MHz absorption mode NOESY spectrum of the 1:1 complex of **1** and d-(GCATTAATGC)$_2$ in ^2H_2O at 301 K containing intermolecular cross peaks. The mixing time was 150 ms. Other experimental details are given under Materials and Methods. Intermolecular NOESY cross peaks are indicated by lower case letters: (a) Q-2H/$A_7$2H; (b) Q-2H/$A_6$2H; (c) P-2,6H/$A_7$2H; (d) P-3,5H/$A_6$2H; (e) Q-3H/$A_6$2H; (f) BP-3,5H/$A_6$2H; (g) Q-CH$_3$/$A_7$2H; (h) Q-CH$_3$/$A_3$2H; (i) P-CH$_3$/$A_7$2H.

excluded-site size of five base pairs for the binding of **1** to DNA derived from optical studies (Braithwaite & Baguley, 1980). Considering that the overall length of **1** is approximately 18.5 Å, there is also the indication that the entire drug molecule is in close contact with the DNA.

More direct identification of the intermolecular contacts was obtained from observation of NOEs between protons located in d-(GCATTAATGC)$_2$ and in **1**, respectively. Figure 8 contains some NOESY cross peaks manifesting drug–DNA NOEs between nonlabile protons in the complex. Drug–DNA NOEs were also observed between exchangeable drug protons and nonexchangeable DNA protons, and vice versa. A complete list of all assigned NOEs is presented in Table II. Additional intermolecular NOEs were observed between the drug protons around 8.25 ppm and several ribose 4′,5′- and 5″-protons of the DNA. These cross peaks could not be individually assigned because of the limited spectral resolution

Table II: Intermolecular NOEs in the 1:1 Complex of **1** with d-(GCATTAATGC)$_2$ Observed with NOESY Using a Mixing Time of 150 ms

drug proton	DNA proton
P-CH_3	$T_4$1′H; $A_6$1′H; $A_7$2′H; $A_7$2H
Q-CH_3	$A_3$2H; $A_3$1′H; $A_3$2″H; $T_4$1′H; $T_5$1′H; $A_6$3′H; $A_7$2H; $T_8$1′H; $G_9$4′H
Q-2H	$A_6$2H; $A_7$2H; T_4N3H
Q-3H	$A_6$2H
Q-7H	$A_6$4′H
P-2,6H	T_5N3H; $A_7$2H; $T_5$1′H; $A_6$1′H; $A_7$1′H; T_4N3H
P-3,5H	$A_6$2H; $T_5$1′H; $A_6$1′H; $A_7$1′H
BP-2,6H	$A_7$1′H; $A_6$1′H; $A_6$2H
BP-3,5H	$T_4$1′H; $T_5$1′H; $A_6$1′H; $A_7$1′H
amide NH	$T_5$1′H; $A_6$1′H; $A_7$2H

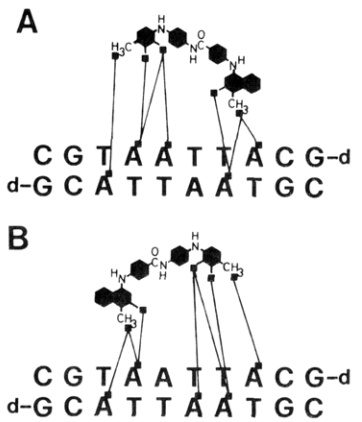

FIGURE 9: Alignment of **1** relative to d-(GCATTAATGC)$_2$ (A) based on NOESY observation of short intermolecular ^{1}H–^{1}H contacts at 301 K. Only a small selection from the NOEs listed in Table II is shown. Filled squares represent protons involved in intermolecular NOEs. Note that the two strands in the DNA duplex are no longer equivalent in the complex formed with **1**. Therefore, a second, identical structure B can be drawn.

in this spectral region. However, with regard to the following discussions it is of interest that these ribose 4′,5′- and 5″-protons are more readily accessible to the drug in the minor groove of the DNA duplex. All intermolecular NOESY cross peaks were thus observed for DNA protons in the minor groove. This provides direct evidence that **1** binds to the DNA decamer in the minor groove. The intermolecular NOE contacts were used to align the drug along the DNA duplex, as is shown in Figure 9. This scheme is also compatible with the qualitative conclusions from Figure 7. However, since a 1:1 complex is formed, the twofold symmetry of the ^{1}H NMR spectrum of the DNA duplex could not be preserved in a single kinetically stable complex of the form shown in Figure 9. This apparent discrepancy could be resolved with the data presented in the following section.

Dynamic Properties of the DNA–Drug Complex. To gain further insight into the dynamic process observed in Figure 3, NOESY and COSY spectra were recorded at 283 K in order to search for additional evidence of chemical exchange among the many resonances that cannot be resolved in 1D ^{1}H NMR spectra. Assignments at 283 K were made by correlation with the data obtained at 301 K. One example of chemical exchange apparent in Figure 10 is that seen in the resonance of 2H of A_6. This proton exhibits a single broad diagonal peak at 301 K, but at 284 K it is split into two peaks separated by 80 Hz (the average chemical shift is 6.86 ppm). The NOESY cross peaks observed between these two proton resonances are indicative of a chemical exchange process. (Additional evidence for this interpretation of Figure 10 is that the two resonance lines attributed to $A_6$2H exhibit an identical

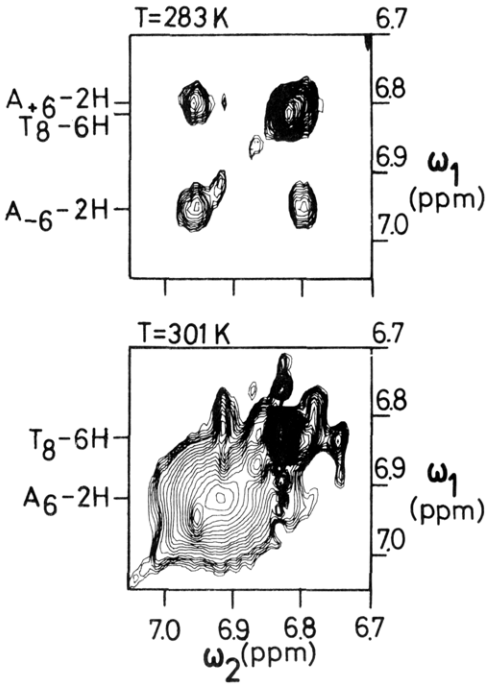

FIGURE 10: Comparison of corresponding regions in 500-MHz absorption mode NOESY spectra of the 1:1 complex of d-(GCATTAATGC)$_2$ and **1** at 283 and 301 K. The mixing time was 150 ms in both cases. Other experimental details are given under Materials and Methods. Sequence-specific assignments are given on the left.

set of NOESY cross peaks, including cross peaks to the drug resonances P-2,6H.) From examination of the 1D ^{1}H NMR spectra of the complex at different temperatures, the coalescence temperature for this resonance was estimated to be $T \approx 299$ K. Using eq 1, one finds an exchange rate of k_{coal} (299 K) ≥ 178 s^{-1}.

From eq 2 (Günther, 1980), an estimate of the activation energy for the observed exchange process at the coalescence temperature T_{coal} can be made.

$$\Delta G^{\ddagger} = 19.14 T_{coal}[9.97 + \log (T_{coal}/\Delta\nu)] \text{ (J mol}^{-1}) \quad (2)$$

This equation is based on transition-state theory. From the coalescence involving resonances of A_{+6}2H and A_{-6}2H (T_{coal} = 299 K), a $\Delta G^{\ddagger}$ of 60.3 ± 5 kJ mol^{-1} is calculated, whereas for the coalescence involving resonances of $T_{+4}5CH_3$ and $T_{-4}5CH_3$ (T_{coal} = 289 K) $\Delta G^{\ddagger}$ is 59.7 ± 5 kJ mol^{-1}. The close coincidence of the $\Delta G^{\ddagger}$ values suggests that the two observed coalescence phenomena are due to the same exchange process. The nature of this exchange process can be further characterized on the basis of the symmetry properties of the ^{1}H NMR spectrum of the complex, as is described in the Discussion.

Discussion

The preceding sections described observations on the chemical shifts, on intermolecular ^{1}H–^{1}H NOEs, and on the temperature dependence of certain NMR line shapes in the complex formed between **1** and the DNA duplex d-(GCATTAATGC)$_2$. In the following we examine to what extent the structural properties of this complex can be characterized by the combination of these different observations with the sequence-specific NMR assignments.

In recent reports Feigon et al. (1984) and Leupin et al. (1985) studied the influence of over 90 different drugs on the chemical shifts of the imino protons in short natural DNA fragments of random sequence. These studies led to the empirical rule that drugs binding in the minor groove induce a downfield shift for the imino protons of the Watson–Crick base pair of the DNA, whereas intercalating drugs induce an upfield

shift. Feigon et al. (1984) followed also the titration of DNA by **1**, which resulted in downfield-shifted A-T imino proton resonances. These observations with random DNA are now corroborated by the results of the present study with d-$(GCATTAATGC)_2$, which shows drug-induced downfield shifts of 0.12–0.49 ppm for the different imino protons. The ^1H chemical shifts thus not only indicate that **1** interacts primarily with the central A-T base pairs in d-(GCATTAATGC)$_2$ (Figure 7) but in addition provide evidence, based on the previously established empirical criterion (Feigon et al., 1984), for a classification of **1** as a minor-groove-binding drug. This classification received support also from ^{31}P NMR measurements (data not shown), which showed that the ^{31}P resonances in the spectrum of the complex are shifted toward higher field compared to the free DNA. This shift direction is opposite to the ^{31}P shifts in DNA caused by intercalating agents (Wilson & Jones, 1982). Upfield shifts of ^{31}P resonances in DNA were also reported to result from interactions with the outside binders tetralysine (Wilson & Jones, 1982) and netropsin (Patel, 1979).

Chemical shifts to lower field upon complexation were also observed for the nonlabile protons of **1** (Figure 2), the largest shifts being of the order 1.2 ppm. Interestingly, the protons in the meta positions with respect to the aza nitrogen are most distinctly shifted in both the pyridine and the quinoline moieties of the drug (Figure 1). Finally, the low-field chemical shifts in the range 9.5–10.5 ppm for all three labile protons in **1** are indicative of hydrogen-bond formation. This is further corroborated by the slow exchange of these protons with the solvent: In the free drug the exchange rates are so fast that these resonances are not observed in the ^1H NMR spectrum in H_2O obtained with selective excitation, while the amide resonance in the complex is even observable when the H_2O line is suppressed by saturation. Inspection of B DNA and the models resulting from the X-ray study of the netropsin–d-$(CGCGAATTCGCG)_2$ complex (Kopka et al., 1985) indicate that only N3 of adenine and the 2-keto group of thymine can act as hydrogen-bond acceptors in the minor groove. The occurrence of such hydrogen bonds cannot be directly determined by ^1H NMR but may be inferred from observation of ^1H–^1H NOEs involving DNA protons near these hydrogen-bond acceptors, as will be further discussed below.

The most direct and precise information on intermolecular contacts came from ^1H–^1H NOEs. At the present stage no quantitative distance measurements were attempted, and the experiments focused on observing the absence or presence of intermolecular NOEs. On this level the effects of spin diffusion were not critical, and therefore a mixing time of 150 ms was used for most of these studies. (Intramolecular spin diffusion could be followed in the quinoline moiety of the drug, so that there was an internal check on the presence of spin diffusion effects.) The schematic drawing of the orientation of **1** relative to d-$(GCTAATTACG)_2$ in Figure 9 is compatible with all NOEs observed (Table II). The drug molecule is aligned along the minor groove of the DNA. Inspection of the constraints imposed on the structure of the complex by the NOEs using a Labquip model built with the assumption that the DNA forms a regular B type duplex indicates some special features that are described in the following.

The intermolecular NOEs between the amide proton of **1** and $T_5 1'H$, $A_6 1'H$, and $A_7 2H$ make it most likely that this amide proton is hydrogen bonded to the 2-keto group of T_5. It appears further that the amino proton between BQ and Q could be hydrogen bonded to the 2-keto group of T_4. Similar hydrogen-bonding interactions were reported for the netropsin

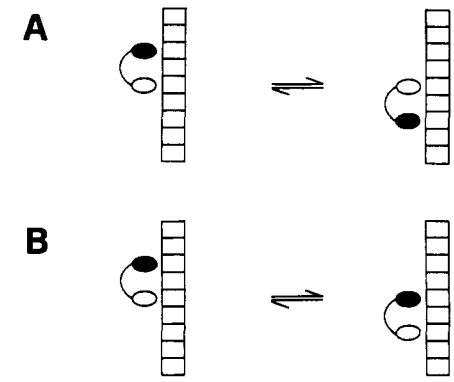

FIGURE 11: Schematic presentation of two possible types of dynamic processes in the complex formed between d-$(GCATTAATGC)_2$ and the drug **1**. The DNA duplex is symbolized by a ladder with 10 rungs and the drug by two linked ovals, where the filled and empty ovals represent the quinoline moiety and the pyridine moiety, respectively. A. Flipping motion of **1** relative to the DNA duplex. B. Sliding motion of **1** relative to the DNA.

complexes with d-$(CGCGAATTCGCG)_2$ (Patel, 1982), d-$(GGAATTCC)_2$, or d-$(GGTATACC)_2$ (Patel & Shapiro, 1985).

The length of the extended form of drug **1** is approximately 18.5 Å, which corresponds to the length of about five to six stacked base pairs. Since intermolecular NOEs and chemical shift changes were observed over the length of about five to six base pairs exclusively along the minor groove (Figures 7 and 9), it is then apparent that **1** is curved into a crescent shape when binding to d-$(GCATTAATGC)_2$. A drug conformation of this type would be reminiscent of the netropsin–d-$(CGCGAATTCGCG)_2$ complex, where in addition the two pyrrole rings of the drug were found to be twisted by 33° out of coplanarity (Kopka et al., 1985).

NOEs between the methyl protons of Q and $A_7 2H$ and between the methyl protons of P and $A_3 2H$ (Figures 1 and 9) define the locations of the ends of drug molecule **1** in the minor groove of a single, isolated complex. In such isolated species with the asymmetric drug bound (Figure 9A or B), the magnetic equivalence of symmetrically disposed DNA protons would be lost, for example $A_6 2H$ on the + strand would not be equivalent to $A_6 2H$ on the – strand. The NMR spectra of the complex of **1** with d-$(GCATTAATGC)_2$ at temperatures above ca. 300 K are thus clearly not compatible with the existence of kinetically stable structures of the type shown in Figure 9, since for most spins they manifest the twofold symmetry observed also in the free DNA (Figure 1). At lower temperature the asymmetry of the DNA in the complex was revealed for several distinct hydrogen atoms (e.g., Figures 3 and 10). Overall, the implication is that, in any distinct species of the complex, the drug **1** must undergo motions relative to the DNA that are sufficiently fast on the NMR time scale to average out the effects of the drug binding on the ^1H chemcial shifts of the DNA. As was previously explained, the data of Figures 3 and 10 indicate that the rate for this kinetic process must be of the order of 200 s^{-1} at 300 K.

Consideration of the structural symmetry in the complex of d-$(GCATTAATGC)_2$ with **1** (Figures 1 and 9) shows that the observed preservation of the magnetic equivalence of the two DNA strands above 300 K must result from an equilibrium between symmetrically equivalent drug binding sites on the DNA (Figure 11A). [On the basis of similar chemical exchange phenomena, Patel and Shapiro (1985) have also proposed such a dynamic equilibrium for the complexes between netropsin and d-$(GGAATTCC)_2$ or d-$(GGTATACC)_2$,

respectively.] The simplest model that could explain the experimental observations is a *flip-flop process* (Figure 11A) between two bindig sites corresponding to the two species shown in Figure 9. (Note that these two species are not distinguishable, unless one of the DNA strands would be modified, e.g. by chemical modification of a base.) More generally, each of these two interchanging binding modes could include an ensemble of rapidly exchanging conformers. This might be generated, for example, by sliding movements of the drug along the minor groove between different, distinct binding sites (Figure 11B). However, on the basis of symmetry arguments (Figures 1, 9, and 11), it is clear that such sliding motions alone could not result in the observed NMR equivalence of the two DNA strands above 300 K, independent of the rate of the sliding motions.

The flip-flop process of Figure 11A might occur within the complex of **1** and d-$(GCATTAATGC)_2$, or it might involve transient dissociation of the complex, perhaps involving exchange of drug molecules between different DNA duplexes. While no definite mechanistic statements can presently be made, there is an observation that would appear to support an intramolecular rearrangement mechanism, without dissocation of the complex. This is the kinetic stabilization of the Watson–Crick base pairs as judged from the slow exchange of the imino protons in the complex, which indicates that the drug reduces the solvent accessibility of the imino protons of the DNA. A similar observation was reported by Pardi et al. (1983) for the complex between netropsin and d-$(CGCGAATTCGCG)_2$. A possible structural basis for stabilization of the labile DNA protons was proposed by Kopka et al. (1985) for a crystalline netropsin–DNA complex, i.e. that the spine of H_2O molecules along the minor groove is disturbed, or displaced entirely, by the drug molecule in the complex.

The present paper is one of the initial applications of 2D NMR techniques for studies of DNA–drug complexes. Further refinement of this approach should in the near future enable studies of details that have been discussed by others on the basis of different experimental methods such as the apparent specificity of minor-groove binders for A-T base pairs or the role of electrostatic forces in the complex formation [e.g., Braithwaite and Baguley (1980) and Kopka et al. (1985)]. Already at the present stage, this study presents another nice illustration of the potentialities of NMR for providing data on both the structure and the kinetic properties of intermolecular complexes with DNA.

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Registry No. SN 6999, 68772-09-8; d$(GCATTAATGC)_2$, 103980-80-9.

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Solid-Phase Processing of U2 snRNA Precursors†

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ABSTRACT: HeLa cell cytoplasmic extracts contain both precursors to small nuclear RNA (snRNA) U2 and an activity that is capable of trimming these snRNA precursors to the size of mature U2. The substrate for this RNA processing reaction is the ribonucleoprotein complex containing pre-U2 RNA. To circumvent the difficulty of biochemically isolating pre-U2 ribonucleoprotein (pre-U2 RNP) complexes for use as substrate for the analysis of the processing activity, we have developed a procedure for the processing of pre-U2 RNP complexes that have been immobilized on anti-Sm antibody/protein A–Sepharose columns. When the immobilized [^{3}H]uridine-labeled substrate RNP complexes are incubated at 37 °C with unlabeled cytoplasmic extracts from HeLa cells, labeled molecules the size of mature U2 are produced in a linear fashion for up to 3 h. Similar results are obtained when substrate pre-U2 RNPs are immobilized with an anti-2,2,7-trimethylguanosine antibody. Thus, accurate processing of the 3′ termini of U2 precursors occurs on the antibody columns. Incubation with buffer alone does not result in the production of mature-sized U2, indicating that the processing activity is not intrinsic to the pre-U2 RNP. Using this assay procedure, we have demonstrated that the processing activity is destroyed by trypsin or by preincubation at 65 °C but is resistant to treatment with micrococcal nuclease. These results are compatible with the conclusion that the processing activity is a classical enzyme that does not contain a nuclease-sensitive essential RNA component.

U2 RNA is one of a family of small nuclear RNAs found in eukaryotic cells. Recent evidence supports the hypothesis that U2 plays an important role in RNA splicing (Black et al., 1985; Krainer & Maniatis, 1985; Calvet et al., 1982). In HeLa cells, U2 is synthesized as a precursor that is approximately 10 nucleotides longer than the mature species (Elicieri & Sayavedra, 1976; Wieben et al., 1985; Yuo et al., 1985). Since both U2 precursors and mature U2 snRNAs[1] are complexed with the proteins which confer antigenicity with respect to autoimmune sera, it seems likely that the substrate for the processing reactions is the ribonucleoprotein complex rather than naked snRNA. Our recent experiments investigating the processing of pre-U2 RNAs in vitro support this idea.

Precursors to small nuclear RNA U2 that are present in HeLa cell cytoplasmic extracts are efficiently processed to capped RNAs the size of mature U2 upon incubation of the extract at 37 °C. Under these conditions, exogenously supplied naked U2 precursors are rapidly degraded, suggesting that the ribonucleoprotein structure of the endogenous pre-U2 RNA plays at least a passive role in the correct processing of U2 (Wieben et al., 1985). While raising interesting questions concerning the role of the snRNP proteins in the processing of snRNA precursors, this finding poses a significant technical problem for the study of this activity: that of purifying unprocessed pre-U2 RNA which retains sufficient RNP structure to be accurately processed in vitro. We have circumvented this problem and developed a simple and effective assay for U2 processing activity. We have used this assay to further characterize the U2 processing activity.

Materials and Methods

Preparation of Cell Extracts and Endogenous Processing Reactions. HeLa cells were grown in suspension culture and pulse-labeled for 45 min with [^{3}H]uridine as described. Cells were fractionated in 0.01 M NaCl, 1.5 mM $MgCl_2$, and 0.01 M Tris-HCl, pH 7.5 (RSB). The cytoplasmic fraction was removed and adjusted to either 0.1 M NaCl, 2.5 mM $MgCl_2$, and 0.01 M Tris-HCl, pH 8.5 (pH 8.5 buffer), for direct antibody selection or 0.11 M NaCl, 6.5 mM $MgCl_2$, and 0.02 M Tris-HCl, pH 8.0 (adjusted RSB), for endogenous processing reactions, which were performed as described previously (Wieben et al., 1985).

Antibody Selections. Antibody selections were performed as described (Wieben et al., 1985). Anti-2,2,7-trimethylguanosine or patient anti-Sm antisera was incubated with cytoplasmic samples for 60 min on ice, followed by the addition of 50 μL of protein A–Sepharose. After an additional 60-min incubation, 0.4 mL of carrier Sepharose CL-4B was added,

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[1] Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein.

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