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Stereospecific assignment of H5′ and H5″ in a (5′*R*)-/(5′*S*)-deuterium-labeled DNA decamer for $^{3}J_{HH}$ determination and unambiguous NOE assignments

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Abstract

The stereoselective deuterium labeling at the 5′ methylene protons of the ribose ring recently developed by Kawashima et al. [1995, *Tetrahedron Lett.*, **36**, 6699–6700] enabled the assignment of pro-*R* and pro-*S* protons at the 5′ position. The deuterium-labeled nucleotides, [(5′*S*)-^{2}H]- and [(5′*R*)-^{2}H]-diastereomers, in an approximate ratio of 2:1, were incorporated in the decamer 5′-d(GCATTAATGC)-3′. Thus, both pro-*R* and pro-*S* methylene proton signals without geminal coupling appeared in the NOESY and DQF-COSY spectra. Complete stereospecific assignments and simplified spin systems enabled the determination of 15 ^{3}J coupling constants between H4′ and H5′/H5″, and the unambiguous assignment of 135 NOESY cross peaks originating from H4′/H5′/H5″ resonances.

The stereospecific assignment of H5′ and H5″ protons in the NMR spectra of nucleic acids leads to significant improvement in the use of NOE distance and torsion angle constraints for structure determination (Van de Ven and Hilbers, 1988; Varani and Tinoco, 1991; Wijmenga et al., 1993). In particular, it is necessary to determine the backbone torsion angles, β (P5′-O5′-C5′-C4′) and γ (O5′-C5′-C4′-C3′), by means of J coupling constants (Tate et al., 1995; Marino et al., 1996). For such stereospecific assignments, several methods have been proposed, involving proton chemical shifts (Remin and Shugar, 1972), NOEs (Blommers et al., 1991), homonuclear isotropic mixing correlations (Glaser et al., 1989), heteronuclear coupling constants (Schmieder et al., 1992; Hines et al., 1993), carbon chemical shifts (Marino et al., 1996), and stereoselective deuterium labeling (Kline and Serianni, 1990; Kawashima et al., 1995,1997; Ono et al., 1996). The proton chemical shift method assumes that the H5′ (pro-*S* proton) resonance is the downfield one. This works well for the double helical region of A-form RNA, but not for DNA, nor for loop regions of RNA (Blommers et al., 1991; Varani and Tinoco, 1991; Weisz et al., 1992; Marino et al., 1996). The conventional NOE method is ambiguous because of spin diffusion and overlapping of broad peaks caused by J splitting and the fast relaxation of the 5′ methylene protons. This fast relaxation affects many homo- and heteronuclear 2D experiments, e.g. COSY, TOCSY, dual CT-HSQC (Tate et al., 1995), and so on. Hines et al. (1993) pointed out that the sign of two-bond ^{13}C-^{1}H scalar coupling constants is useful for stereospecific assignment. Very recently, Marino et al. (1996) found a strong correlation between the ^{13}C5′ chemical shift and the difference between the H5′ and H5″ chemical shifts. These ^{13}C-related methods are

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becoming more useful (Schmieder et al., 1992; Hines et al., 1993, 1994; Marino et al., 1996). However, it is clear that stereoselective deuterium labeling at the 5′ position can solve both the problems of ambiguous assignment and fast relaxation.

For explicit stereospecific assignment and exact determination of vicinal proton–proton coupling constants, 100% stereoselective deuterium labeling (Hangeland et al., 1992; de Voss et al., 1994) is better. However, we need two (pro-*R* and pro-*S*) syntheses and two independent NMR measurements. Recently, Kawashima et al. (1995) developed one of the most efficient stereoselective deuterium labeling methods for the 5′ site. Each nucleoside obtained by the method is a mixture of 5′*S* and 5′*R* deuterium-labeled isotopomers in an approximate ratio of 2:1. This deuterium labeling method has already been applied to determine $^{3}J_{PH5'}$ and $^{3}J_{PH5''}$ of a single thymidine in a 12-mer DNA duplex (Tate et al., 1995). Here we prepared a 10-mer DNA which has 5′ stereoselective deuterium in all residues, and evaluated the utility of NOEs, and J coupling constants originating from the H5′ and H5″ protons.

The 5′ deuterium-labeled and non-labeled DNA decamers, 5′-^{1}G^{2}C^{3}A^{4}T^{5}T^{6}A^{7}A^{8}T^{9}G^{10}C-3′, were chemically prepared with a conventional solid phase automated DNA synthesizer. For the deuterium-labeled one, phosphoramidite was prepared in the previously reported manner (Kawashima et al., 1995), which enabled stereoselective 5′ deuterium labeling. Both oligomers were deblocked and purified by our standard method (Kyogoku et al., 1995). The sample pH was adjusted to 7.0 with 20 mM sodium phosphate buffer and 50 mM sodium chloride, and the temperature was 303 K (30 °C). The strand concentration was 3 mM in a micro tube (Shigemi) with 250 μl D_2O. All spectra were measured with Bruker AMX600 and ARX500 NMR spectrometers for the deuterium-labeled and the non-labeled sample, respectively. The spectral width was 4000 or 5000 Hz with 2K recording points for both dimensions. 90° shifted sine or sine squared window functions, and once or twice zero-filling were applied prior to Fourier transformation. The mixing time for NOESY was 100 ms. The DQF-COSY spectra were recorded with ^{31}P decoupling. The pulse repetition delay for all 2D spectra was 2 s.

The deuterium fraction of each nucleotide was determined as the relative intensity of the residual ^{1}H peak in the ^{1}H 1D NMR spectrum (Kawashima et al., 1995). The ratio of (5′*S*)-^{2}H to (5′*R*)-^{2}H was 61:39, 80:20, 76:24, and 69:31 for A, G, C, and T, respectively, with more than 90 atom% deuterium. In Figure 1, parts of the DQF-COSY spectra including the sugar 4′, 5′, and 5″ resonances are shown for the 5′ deuterium-labeled DNA (left) and the non-labeled one (right). The complex J splitting and line shape distortion have disappeared in the left spectrum. Non-labeled species (less than 10%) were expected to be observed in the left spectrum, but were not seen there. The difference between the deuterium fractions of pro-*S* and pro-*R* is not clear in the DQF-COSY spectrum. In Figure 2, the NOESY (left) and DQF-COSY (right) spectra of the 5′ deuterium-labeled DNA are shown for the whole sugar 3′, 4′, 5′, and 5″ regions. In both spectra the 5′ and 5″ signals show simpler splitting and a narrower line width than those of non-labeled DNA.

For DNA with this base sequence, most proton signals have already been assigned, and it has been characterized as having the canonical B DNA conformation (Chazin et al., 1986). However, the H5′ and H5″ resonance assignments were not reported. We assigned all the non-exchangeable protons using the spectra shown in Figures 1 and 2 at a slightly different temperature, 303 K (Chazin et al. used 300 K). The chemical shift values are given in Table 1. The protocol used for the assignment was as follows. First, the H4′ resonances were assigned based on the scalar connectivities of H3′ in a DQF-COSY spectrum. In the same DQF-COSY spectrum, 16 of 20 H5′ or H5″ resonances were found as cross peaks originating from the assigned H4′ signals. This means that both the H5′ and H5″ resonances of six residues (G1, C2, A3, A6, A7, and G9) could be identified in a single DQF-COSY spectrum. The stereospecific assignment of these peaks was performed by means of a NOESY spectrum instead of DQF-COSY, because the DQF-COSY cross peaks of H4′/H5′ and H4′/H5″ exhibited similar intensities and J coupling constants. The deuterium-enriched sample has about 30% of pro-*S* (H5′) protons and 60% of pro-*R* (H5″) protons, thus, in most cases the H5″ peak intensity is expected to be stronger than that of H5′. For six residues (G1, C2, A3, A6, A7, and G9), six sets of two NOESY cross peaks corresponding to either intraresidue H4′/H5′ or H4′/H5″ correlations were identified in the DQF-COSY spectrum, and relatively intense cross peaks were assigned to H4′/H5″. The assignments were confirmed by the difference in the peak intensity between the H3′/H5′ and H3′/H5″ resonances, i.e., relatively intense cross peaks corresponded to H3′/H5″. In particular, the stereospecific assignment based on H3′ was useful for the H5′ and H5″ resonances of the three

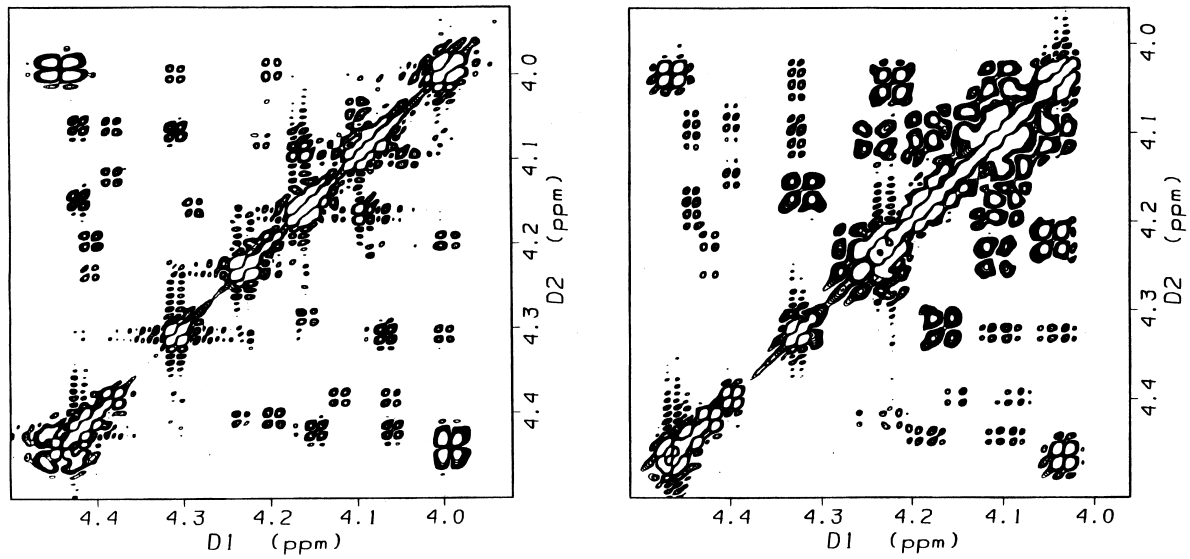

Figure 1. H4′, H5′ and H5″ resonance regions of the DQF-COSY spectra of the 5′ deuterium-labeled (left) and non-labeled (right) DNA decamers. The spectra were recorded at 303 K with Bruker AMX 600 and ARX 500 spectrometers, with 1024 and 512 hypercomplex points, respectively, for t_1, and 1024 complex points for t_2, multiplying the $\pi/2$ shifted sine-bell window function for both dimensions, and zero-filling to 4096 real points for both dimensions. The pulse repetition delay was 2 s, and the total recording time was 2 days. The phase-sensitive detection of t_1 was performed by the TPPI-States method with the half-duration shift method. The ^{31}P resonances were decoupled by means of a π pulse for t_1 and WALTZ16 for t_2. The sample solutions contained 3 mM of duplex in a 250 μl micro tube (Shigemi) with 20 mM phosphate buffer and 50 mM NaCl at neutral pH. The base sequence is 5′-GCATTAATGC-3′.

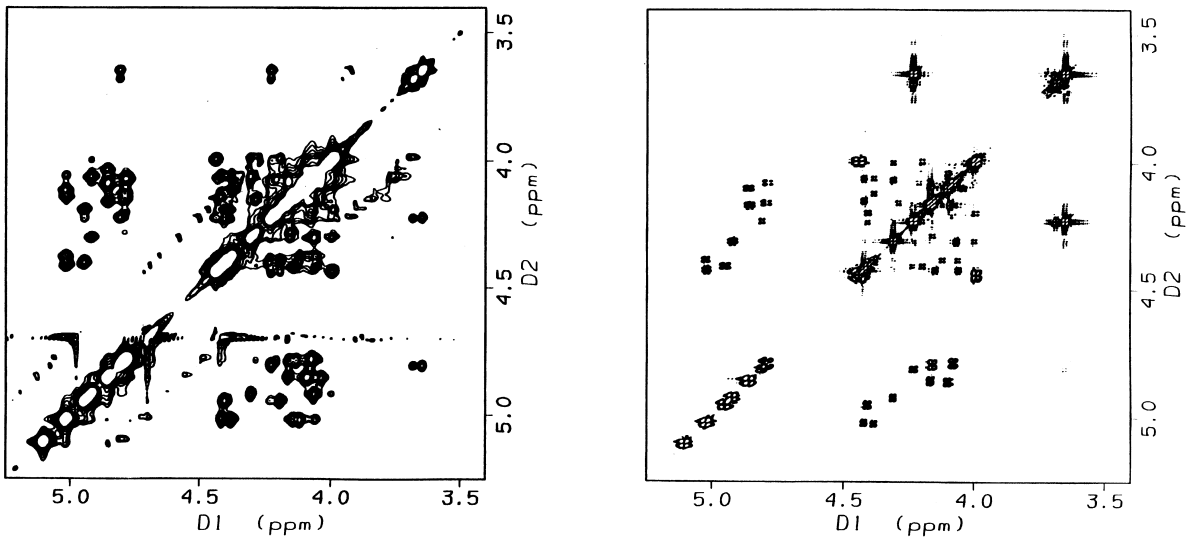

Figure 2. H3′, H4′, H5′ and H5″ spectral regions of NOESY (left) and DQF-COSY (right) spectra of the 5′ deuterium-labeled DNA decamer. The DQF-COSY spectrum is the same as that in Figure 1. The NOESY spectrum was recorded with a 100 ms mixing time without ^{31}P decoupling with a Bruker AMX 600. The total recording time was 1 day. The other parameters were the same as those for DQF-COSY described in the legend to Figure 1.

Table 1. Assignment of the non-exchangeable ^{1}H resonances of the 5′ stereoselectively deuterium-labeled DNA decamer at 303 K[a]

	G1	C2	A3	T4	T5	A6	A7	T8	G9	C10
H6/8	7.88	7.42	8.33	7.15	7.31	8.22	8.08	6.96	7.76	7.28
H2/5/M		5.36	7.64	1.41	1.60	6.69	7.52	1.27		5.09
H1′	5.90	5.64	6.29	5.87	5.69	5.93	6.08	5.67	5.87	6.10
H2′ (2′*S*)	2.55	2.10	2.73	1.93	2.05	2.71	2.49	1.84	2.51	2.18
H2″ (2′*R*)	2.74	2.44	2.94	2.46	2.43	2.88	2.84	2.27	2.63	2.18
H3′	4.82	4.87	5.03	4.80	4.87	5.03	4.96	4.79	4.93	4.45
H4′	4.24	4.17	4.43	4.17	4.11	4.39	4.42	4.09	4.32	4.00
H5′ (5′*S*)	3.72	4.01	4.08	4.30	4.11	4.08	4.25	4.20	4.00	4.32[b]
H5″ (5′*R*)	3.68	4.03	4.16	4.15	4.04	4.13	4.21	4.09	4.08	4.32[b]

[a] ppm values from DSS.
[b] Not stereospecifically assigned.

Table 2. Observed and simulated J coupling constants of the 5′ stereoselectively deuterium-labeled DNA decamer

	G1	C2	A3	T4	T5	A6	A7	T8	G9	C10
Observed values										
$J_{H4'H5'}$	4.1	4.2	5.4	5.7	[a]	5.8	5.2	5.7	5.6	[a]
$J_{H4'H5''}$	5.2	5.6	5.6	[a]	6.5	5.9	5.9	[a]	5.4	[a]
Simulated values (single rotamer model)										
$J_{H4'H5'}$	1.8	2.2	3.2			4.3	3.3		3.3	
$J_{H4'H5''}$	6.3	6.9	8.1			8.8	8.2		8.1	
γ[b]	19.9	15.7	6.3			−1.6	5.7		6.0	
Simulated values (single rotamer with the Gaussian distribution model)[c]										
$J_{H4'H5'}$	4.1	4.2	5.4			5.8	5.2		5.6	
$J_{H4'H5''}$	5.2	5.6	5.6			5.9	5.9		5.4	
γ_0[b]	24.0	17.5	2.8			−9.1	3.0		−2.4	
σ[c]	40.2	38.6	52.7			49.7	45.7		115.1	

[a] Not determined.
[b] Torsion angle γ (O5′-C5′-C4′-C3′) in degrees.
[c] Classical harmonic motion (Bruschweiler and Case, 1994) described as the Gaussian distribution of the dihedral angle centered around γ_0 with standard deviation σ (in degrees). The following generalized Karplus equations were used for these calculations: $^3J_{H4'H5'} = 5.53 - 0.99\exp(-\sigma^2/2)\cos(\gamma_0 - 120) + 4.1566\exp(-2\sigma^2)\cos 2(\gamma_0 - 120) + 0.1350\exp(-2\sigma^2)\sin 2(\gamma_0 - 120)$, $^3J_{H4'H5''} = 5.53 - 0.99\exp(-\sigma^2/2)\cos\gamma_0 + 4.1566\exp(-2\sigma^2)\cos 2\gamma_0 - 2.3761\exp(-2\sigma^2)\sin 2\gamma_0$.

residues T4, T5, and T8, whose cross peaks were not clearly visible in the DQF-COSY spectrum. They were expected to overlap with the H4′ diagonal peaks, and thus the stereospecific assignments of these three residues were confirmed using all the other NOESY spectral regions, including H5′/H5″ resonances. For the 3′ terminal residue (C10), no stereospecific assignment could be made because only one resonance was found as a H5′ or H5″ signal in all spectra. Except for this residue, we could assign all the H5′ and H5″ resonances stereospecifically.

Since these stereospecific assignments were based on distance connectivities, it is necessary to estimate the distance of each proton pair. Assuming the canonical B DNA conformation, which has a torsion angle γ of 36° (gauche plus), the intranucleotide distances of the adjacent residues were estimated for H3′/H5′, H3′/H5″, H4′/H5′ and H4′/H5″ to be 3.7, 2.9, 2.6 and 2.3 Å, respectively. All the distances involving the H5′ proton are greater than those of H5″. In particular, the H3′/H5′ distance is greater than that of H3′/H5″ between the trans and gauche plus conformations of

Table 3. Predicted and observed NOEs from the H4′/H5′/H5″ resonances of the 5′ stereoselectively deuterium-labeled DNA decamer

	Total	Aromatic	H1′	H2′/H2″	H3′	H4′/H5′/H5″
Intraresidue						
Predicted[a]	165	35	20	60	30	20
Observed	145	30	11	56	29	19
(Separated[b])	(110)	(26)	(8)	(41)	(20)	(15)
Interresidue						
Predicted[a]	90	0	27	27	18	18
Observed	51	0	25	26	0	0
(Separated[b])	(25)		(15)	(10)		

[a] Predicted NOEs: this is the number of proton pairs whose distances were expected to be within 5 Å. The canonical B DNA was used for the distance estimation.

[b] Number of well-separated peaks. 613 NOEs were predicted for each strand. 255 (= 165 + 90) NOEs, 42% of the whole strand NOEs, belong to the H4′/H5′/H5″ resonances.

the torsion angle γ. Our stereospecific assignment involving deuterium labeling is not affected by the local conformational change of the double-stranded nucleic acids.

The J coupling constants of H4′-H5′ and H4′-H5″ given in Table 2 were derived from the DQF-COSY spectrum in Figure 2 using the fitting tool of FELIX (Biosym Technologies, v. 2.3). Direct reading of the J splitting gave 1 or 2 Hz larger coupling constants than those obtained by means of the fitting procedure. The fitting procedure eliminates in part the contribution of the cancellation of the positive and negative broad peaks (Neuhaus et al., 1985). However, the observed J coupling constants were larger than those of the canonical B DNA (1.33 and 2.75 Hz for H4′-H5′ and H4′-H5″, respectively), and no single conformer could explain consistently both the H4′-H5′ and H4′-H5″ J coupling constants within the experimental error, 0.5 ∼ 1 Hz. In Table 2, the best fitted single rotamers are listed as a single rotamer model, the differences between the observed and simulated J values being about 2 Hz with that model. Our J values are expected to be less sensitive to the contributions of strong coupling and dipole relaxation (Harbison, 1993; Zhu et al., 1994), because one of the 5′ methylene protons is replaced by deuterium. The observed large J coupling constants may arise from the averaging of some conformations.

To explain the observed J values, we adopted a single rotamer with the Gaussian distribution model (Bruschweiler and Case, 1994). This model is based on the classical harmonic motion, and requires two parameters, γ_0 and σ, as the center and standard deviation of the Gaussian distribution, respectively. The conformational averaging effects of cos(mγ) and sin(mγ) can be expressed analytically for $\sigma \ll \pi$ as $\exp(-m^2\sigma^2/2)*\cos(m\gamma_0)$ and $\exp(-m^2\sigma^2/2)*\sin(m\gamma_0)$, respectively. Then, the generalized Karplus equation (Haasnoot et al., 1980) with the Gaussian distribution for H4′-H5′ and H4′-H5″ is written as

$$
\begin{aligned}
{}^3J_{H4'H5'} = {} & 5.53 - 0.99\exp(-\sigma^2/2)\cos(\gamma_0 - 120) \\
& + 4.1566\exp(-2\sigma^2)\cos 2(\gamma_0 - 120) \\
& + 0.1350\exp(-2\sigma^2)\sin 2(\gamma_0 - 120)
\end{aligned}
$$

and

$$
\begin{aligned}
{}^3J_{H4'H5''} = {} & 5.53 - 0.99\exp(-\sigma^2/2)\cos\gamma_0 \\
& + 4.1566\exp(-2\sigma^2)\cos 2\gamma_0 \\
& - 2.3761\exp(-2\sigma^2)\sin 2\gamma_0
\end{aligned}
$$

where γ is the dihedral angle O5′-C5′-C4′-C3′. When $\sigma = 0$, this model is identical to the simple single rotamer model. As shown in Table 2, the experimental J values were explained very well by the optimized γ_0 and σ. For G9, the Gaussian distribution model is not acceptable because the σ value is too large. The other residues also have large σ values, and this suggests the presence of a conformational distribution. Two-parameter models explain the observed values very well, but a single parameter model does not. For example, the three-site jump model and the two-site jump model with one free rotamer can do this well. Every model needs rapid conformational equilibrium on the NMR time scale. For the stem region of the A-form RNA, the torsion angle γ takes on a single

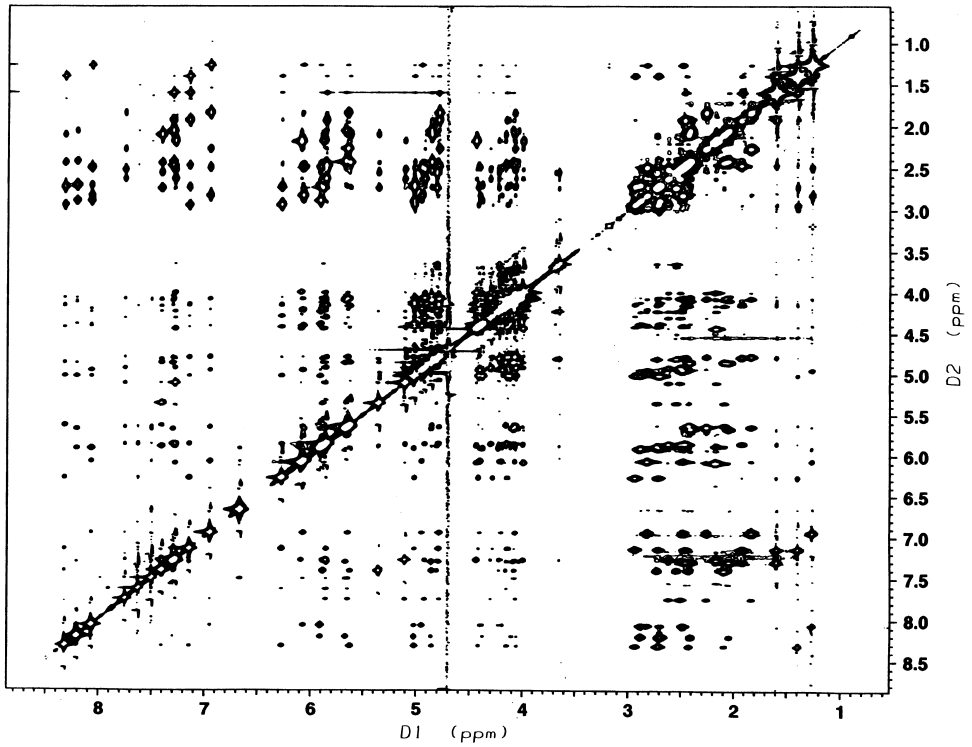

Figure 3. The whole NOESY spectrum of the 5′ deuterium-labeled DNA decamer at 303 K shown in Figure 2. For experimental parameters, see the legends to Figures 1 and 2.

rigid gauche plus conformer, and the H5′ resonance is the downfield peak of two methylene proton signals (Varani and Tinoco, 1991; Marino et al., 1996). However, this may be not true for DNA, nor for loop regions of RNA (Blommers et al., 1991; Varani and Tinoco, 1991; Weisz et al., 1992; Wijmenga et al., 1993; Marino et al., 1996; Kojima et al., this report). The possibility of a transition between the gauche plus and trans conformers has been indicated by restrained molecular dynamics calculation of DNA (Weisz et al., 1994). It seems not to be difficult to jump over the energy barrier between the two conformers in DNA.

The NOESY spectrum with a 100 ms mixing time (Figure 3) revealed many cross peaks from H4′/H5′/H5″ resonances, even in the sparse spectral regions of the H1′, H2′/H2″ and aromatic resonances. In general, it is difficult to use these peaks as distance constraints for structure calculation because of the ambiguity of the assignments and the overlapping of broader lines with many J splittings. The effects of these problems are reduced for the present deuterium-labeled sample, because some overlapping was eliminated by sharpening and decoupling of the 5′ methylene group with deuterium. In fact, the assignment problem has been solved in this study. We have determined the effect of the deuterium labeling based on the prediction of the number of NOEs originating from H4′/H5′/H5″ resonances, and compared it with the number of observed cross peaks. The number of NOEs was regarded as that of proton pairs with distances below 5 Å. The coordinate with the canonical B DNA conformation was used for the NOE prediction. These numbers of the predicted and observed NOEs are listed in Table 3, with classification of the proton types as: aromatic, H1′, H2′/H2″, H3′, and H4′/H5′/H5″. The numbers of well-separated peaks which were unambiguously assigned are given in parentheses. It should be noted that 255 (42%) of the 613 predicted intra-strand (165 intraresidue and 90 interresidue) NOEs belong to the H4′/H5′/H5″ resonances, where 169 (66%) of these 255 NOEs were expected to be observed in the sparse spectral region. In fact, 135 (53%) of the 255 NOEs were observed as well-separated peaks and thus were unambiguously assigned. The general utility of H5′ and H5″ signals has been described previously (see Wijmenga et al.,

1993), and here we point out that more than 40% NOEs originate from the H4′/H5′/H5″ resonances.

For determination of the conformation around the torsion angle γ, it is necessary not only to make stereospecific assignments, but also to maximize the utility of NOEs and J coupling constants derived from the H5′ and H5″ protons. Stereospecific deuterium labeling at the 5′ site is one of the best ways of doing this. In this report we have described the unambiguous stereospecific assignment of H5′ (pro-*S*) and H5″ (pro-*R*) signals. Additional ^{13}C labeling will increase the potential of this deuterium labeling. Such labeling research will become necessary for determination of the torsion angle γ and for structure refinement.

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