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Improvements for Measuring $^1H-^1H$ Coupling Constants in DNA via New Stripe-COSY and Superstripe-COSY Pulse Sequences Combined with a Novel Strategy of Selective Deuteration

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Three bond proton-proton vicinal coupling constants are of potential value for analyzing sugar conformations in DNA. However, self-cancellation in antiphase cross peaks and modulation of peak splittings by transverse cross relaxation can alter the apparent coupling constants such that they do not accurately reflect the sugar conformations. Transverse cross relaxation is most effective between strongly coupled geminal proton pairs. Here we report the use of stereospecific deuteration at the H2″ position in the A5 and A6 residues in the 12 base pair DNA sequence [*d*(CGCGAATTCGCG)2] as a means of investigating the effect of transverse cross relaxation on P.E.COSY type cross peaks. Deuteration of the H2″ proton is expected to reduce the transverse cross relaxation rate by the square of ratio of the proton to deuteron gyromagnetic ratios, i.e., by a factor of 42. Additionally, a striking eight- to ninefold increase in the signal intensity was observed for cross peaks involving the remaining H2′ proton resulting from diminished dipolar relaxation. Further improvements in signal-to-noise ratio were realized by collecting P.E.COSY spectra in strips, using an experiment referred to as stripe-COSY, employing selective excitation pulses which reduced the number of required t_1 increments by a factor of four. A final improvement was achieved by employing selective time-shared homonuclear decoupling during the acquisition period, in an experiment referred to as superstripe-COSY, to collapse splittings due to passive couplings. Collectively, these approaches provide P.E.COSY-type spectra with two to three orders of magnitude increased sensitivity per unit time and that are relatively free from artifacts. © 1997 Academic Press

This Communication reports new approaches for improving the measurement of proton–proton coupling constants in DNA and for diminishing the effects of transverse cross relaxation on P.E.COSY (*1, 2*) type cross peaks. The methods are demonstrated for measuring $^3J_{H1'-H2'}$ coupling constants in a 12-base-pair DNA oligonucleotide. We introduce two modified pulse sequences: the stripe-COSY and the superstripe-COSY. The stripe-COSY is essentially a phase-sensitive COSY-35 (*3, 4*) collected in narrow strips. The stripe-COSY requires significantly shorter total experimental time than a standard COSY-35 spectrum. In the example presented here, the total experimental time was reduced by a factor of 4.9. The superstripe-COSY is a stripe-COSY experiment with additional elements for eliminating remaining unwanted passive splittings. Finally, selective deuteration was used to further improve the spectral quality and to effectively eliminate the deleterious effects of transverse cross relaxation (*5, 6*). In this example, the H2″ proton of the deoxyribose sugar was replaced with a deuteron in all adenosine nucleotides. Consequently, the magnitude of transverse cross relaxation normally present between the geminal H2′ and H2″ protons, which is determined by the square of the dipolar Hamiltonian, was reduced by the square of the ratio of the gyromagnetic ratios, i.e., $(\gamma_D/\gamma_H)^2$ or about 42-fold. Altogether, in the example presented here, the superstripe-COSY combined with selective H2″ deuteration produced a spectrum in ~1/5 the time required for the COSY-35 and with about eight to nine times greater signal-to-noise. Using a constant signal-to-noise comparison, the superstripe-COSY combined with selective deuteration can potentially reduce the total experimental time by a factor of 200–300 compared to a standard COSY-35 experiment.

Proton–proton coupling constants are very useful in determining the conformations of organic compounds and biomolecules since dihedral angles can be determined based on measured coupling constants via Karplus relationships (*7, 8*). In DNA, the coupling constants among H1′, H2′, H2″, H3′, and H4′ protons are of foremost interest for determining the sugar conformation (*9*). Although high-resolution correlation experiments such as phase-sensitive COSY (*10, 11*) and DQF-COSY (*12, 13*) in principle allow the measurement of all coupling constants in systems of limited complexity, they are less useful for complex spin systems and for large molecules. In larger molecules, the broad lines associated with short correlation times lead to the overlap of numerous multiplet components and to the self-cancellation of antiphase multiple components (*14, 15*).

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The E.COSY (*14, 16, 17*) and P.E.COSY (*1, 2*) pulse sequences were developed to minimize overlap by reducing the number of multiplet components in each cross peak. For an ABX spin system, the number of multiplet components of a cross peak is reduced from 16 to 8 in the form of two quartets displaced by passive coupling constants. So both active and passive coupling constants can be measured directly from an E.COSY or P.E.COSY spectrum. The disadvantage of E.COSY lies in its complicated data acquisition process, which requires the linear combination of several phase-shifted data sets. This increases the minimum acquisition time and the susceptibility to long-term spectrometer instabilities. The P.E.COSY experiment is much easier to run in practice since it requires only two pulses. However, the 35° mixing pulse and the alternating use of two phase cycling schemes to remove the dispersive character of diagonal peaks make the P.E.COSY experiment lengthy, especially when significant signal averaging is required to produce a spectrum with a good signal-to-noise ratio. Still, self-cancellation remains a serious problem. As the linewidth approaches or becomes larger than the value of the coupling constant, which occurs in larger molecules such as DNA oligonucleotides or proteins, the self-cancellation of the antiphase of multiplet components reduces the intensity of peaks and modulates the apparent separation of the multiplets (*18*). Consequently, the measured or apparent coupling constants do not necessarily reflect the actual sugar conformation (*19, 20*). Accordingly, spectra of molecules that fall into this category must be carefully manipulated to correct the apparent separation in order to get useful coupling constant values (*2*).

In addition to the problems mentioned above, transverse cross-relaxation effects can also potentially limit one's ability to accurately measure $^3J_{H1'-H2'}$ coupling constants. It has been suggested that the effects of cross relaxation may lead to misinterpretation of the sugar dynamics (*6*). The percentage of north conformation in a N−S two-site equilibrium may be overestimated, as a result of the modulation of the frequency and phase of the individual multiplet components. To directly address the effects of transverse cross relaxation, we introduce a strategy of selectively deuterating the H2″ position in the sugar ring which efficiently eliminates the primary source of cross relaxation. The synthesis of the (2′-*R*)-N^6-benzoyl-2′-deoxy[2′-^{2}H]adenosine was accomplished using the McLaughlin (*21*) procedure: adenosine is N^6-benzoylated in 83% yield (4 g scale). Using the Robins procedure, treatment of the N^6-benzoyladenosine with the bidentate 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TPDS) reagent for the selective protection of the 3′ and 5′ hydroxyls was accomplished in 88% yield (4 g scale) (*22*). Attempted conversion of the 2′-hydroxyl group using trifluoromethanesulfonyl chloride to the sulfonate proved to be problematic. The use of trifluoromethanesulfonyl anhydride in 1:1 pyridine and methylene chloride gave the sulfonate in 92% purified yield. Treatment of the 2′-sulfonate with 13.2 equivalents of LiBr (freshly dessicated) in hexamethylphosphoramide (distilled from calcium hydride) afforded the 2.35 g of the bromide. Using the recent procedure of Ishido and colleagues (*23*) radical reduction of the bromide was effected with triethylborane and tri-*n*-butyltin deuteride. Purification was accomplished using flash silica gel chromatography (1/1 ethylacetate/toluene). The reaction was run three times affording 67, 74, and 82% yields of the product. Cleavage of the silyl protecting groups with ammonium fluoride in methanol, at room temperature, afforded a 98% yield of (2′-*R*)-N^6-benzoyl-2′-deoxy[2′-^{2}H]adenosine. The phosphoramidite of (2′-*R*)-N^6-benzoyl-2′-deoxy[2′-^{2}H]-adenosine, (2′-*R*)-5′-*O*-(4,4′-dimethoxytrityl)-N^6-benzoyl-2′-deoxy[2′-^{2}H]adenosine, was constructed using pyridine and freshly precipitated 4,4′-dimethoxytrityl chloride. Purification of the crude reaction mixture gave a 71% yield. Conversion to the phosphoramidite was accomplished using 2-cyanoethyldiisopropylchlorophosphoramidite, diisopropylethylamine, and methylene chloride as the solvent. The crude material was purified twice on silica gel (40*g* 2×, pretreated with 5% triethylamine/ethylacetate, v/v; 200 mL) and eluted with 5% triethylamine/ethylacetate (v/v) using a gravity column. The purified yield was 85%. The chemistry just described is general for the preparation of purine nucleotides (*23*). We are currently evaluating the generality of the method of Ishido and colleagues (*23*) for construction of ^{2}H-labeled 2′-deoxynucleic acids containing pyrimidine bases.

All spectra shown in this Communication were obtained with either the stripe-COSY or the superstripe-COSY pulse sequence. The stripe-COSY is a modified version of the COSY-35 sequence in which a hard 90° pulse is replaced by an *e*-BURP pulse (*24*). The main purpose of employing an *e*-BURP pulse is to selectively excite a narrow spectral width (∼820 Hz for the H1′ region) rather than the entire spectral width (4000 Hz). This provides the flexibility in running 2D NMR experiments to optimize either minimum total acquisition time or maximum instrinsic digital resolution. Since chemical shifts of the H1′ and the H2′ and H2″ protons on the sugar ring are well separated, 1.8−3.0 ppm for H2′ and H2″ protons and 5.3−6.3 ppm for H1′ protons, it is possible to selectively excite either the H1′ or the H2′ and H2″ region. For a given resolution, the number of time increments is reduced by the relative spectral width excited (164 increments for 820 Hz instead of 800 increments for 4000 Hz). Therefore, the total acquisition time is reduced by a factor of 4.9 in the above example. For a given total acquisition time, the time saved can be invested either for high sensitivity (more transients) or for high resolution (more time increments) provided that the spin−spin relaxation time is favorable.

An application of the stripe-COSY pulse sequence is demonstrated for the dodecamer $d(CGCGAATTCGCG)_2$ in Fig.

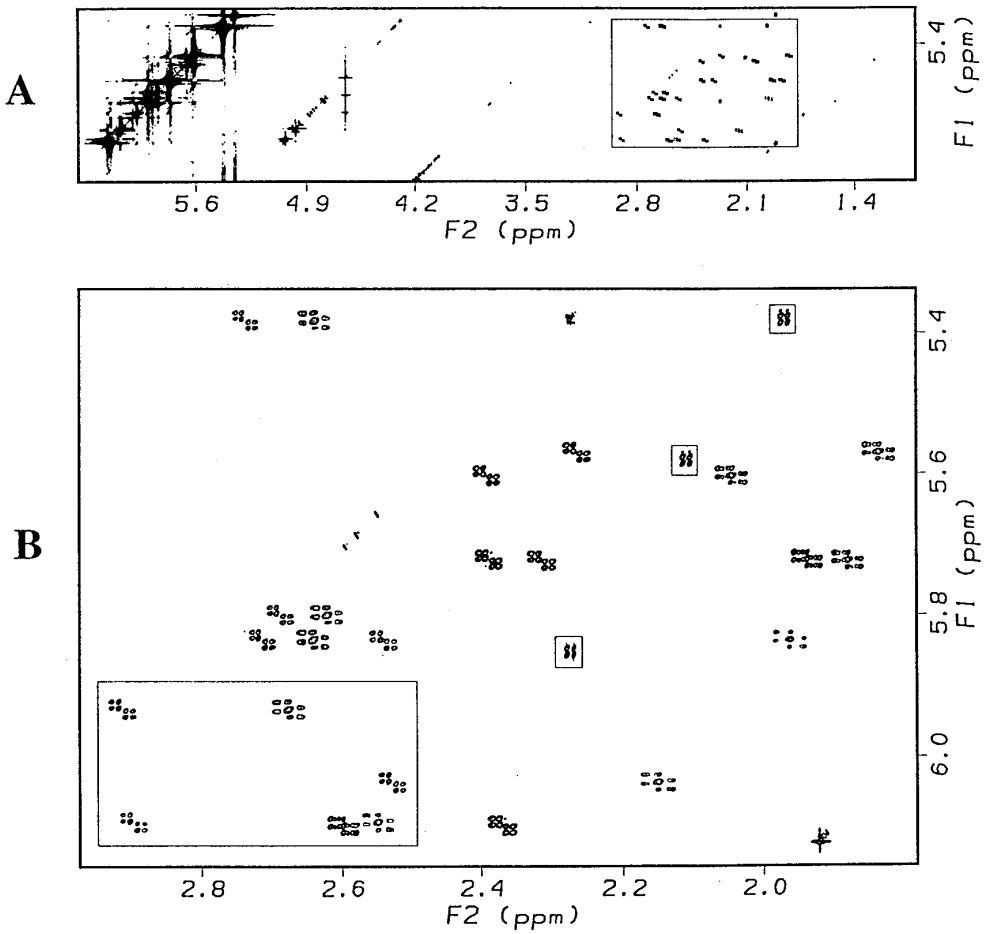

FIG. 1. (A) The full stripe-COSY spectrum for protonated $d(CGCGAATTCGCG)_2$ collected at 750 MHz. The boxed area shows the H1′ to H2′ region enlarged in (B). (B) The H1′ to H2′ region of the stripe-COSY for protonated $d(CGCGAATTCGCG)_2$. The small boxes indicate artifacts resulting from cross peaks from H5–H6 spin systems of cytosines that have been folded in from the high-frequency region of the spectrum. The transmitter offset for the selective pulse was centered at 5.8 ppm whereas the transmitter offset for the nonselective pulse was centered at 3.7 ppm. The corresponding spectral widths were 820 Hz (covering H1′ protons only) and 4003.6 Hz for f_1 and f_2, respectively. 2048 complex points were collected during the acquisition time (1.95 Hz/point) and 164 t_1 increments (5 Hz/point) with zero-filling to 1024 and 4096 along f_1 (0.8 Hz/point) and f_2 (0.97 Hz/point). 128 transients were collected per increment. A 40° and 20° shifted sine-bell apodization function was used in direct and indirect dimensions, respectively, to enhance spectral resolution. The total relaxation delay was 2.512 s per transient. The total acquisition time is 27 h for each spectrum.

1. Figure 1A shows the entire spectrum representing its strip-like character where the directly detected dimension has a spectral width of 4000 Hz and the indirectly detected dimension has a spectral width of 820 Hz. The primary artifact present in the spectrum is caused by the folding of the low-frequency region of the diagonal back into the spectrum. The unfolded diagonal is strong between 5 and 6 ppm in F_2. As the low-frequency diagonal is folded in once (4.2 to 5.0 ppm) and twice (3.4 to 4.2 ppm), its intensity is rapidly diminished. The H1′ to H2′/H2″ region of interest is indicated in a box in Fig. 1A and enlarged in Fig. 1B. In Fig. 1B, additional folding produces cross peaks due to the three-bond coupling of the H5 and H6 protons of the cytosine bases which are shown in the small boxes. In practice, the location of these artifacts can be manipulated by slight adjustments in the reduced spectral width or the transmitter offset for the selective pulses so as to minimize unfavorable overlap between peaks of interest and artifact cross peaks. Otherwise, all 12 pairs of cross peaks among H1′, H2′, and H2″ protons are observed with well-resolved multiplet components. The largest boxed area in Fig. 1B indicates the region of the spectrum containing the A5 and A6 residues.

The effect of selective deuteration of H2″ protons of the sugar rings of A5 and A6 on the resonances of H1′–H2′ is shown in Fig. 2. Inspection of Fig. 2A (protonated) in comparision to Fig. 2D (deuterated) shows that the H1′–H2″ cross peaks have completely disappeared in the deuterated sample, indicating that the H2″ protons have been essentially 100% deuterated. Figures 2B and 2C and Figs. 2E and 2F show slices through the A5 and A6 cross peaks for the protonated and

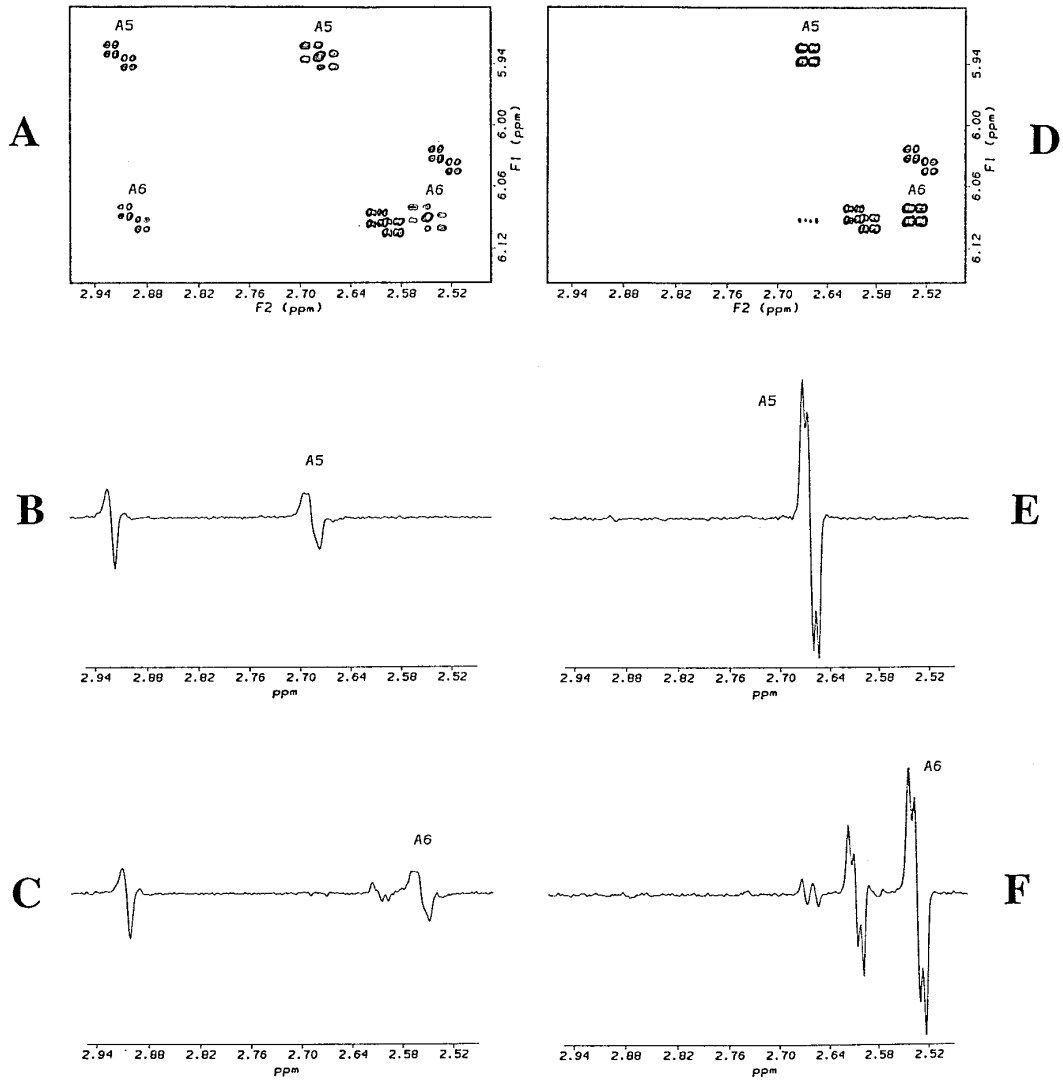

FIG. 2. Stripe-COSY spectra of protonated $d(CGCGAATTCGCG)_2$ and A5–H2″ and A6–H2″ deuterated $d(CGCGAATTCGCG)_2$ collected at 750 MHz. (A) Expanded contour plot of the region showing only the H1′ to H2′ and H1′–H2″ cross peaks of the A5 and A6 nucleotides for protonated $d(CGCGAATTCGCG)_2$. (B) 1D slice taken at the low-frequency H1′ resonance through the cross peaks of A5 in the protonated sample. (C) 1D slice taken at the low-frequency H1′ resonance through the cross peaks of A6 in the protonated sample. (D) Expanded contour plot of the region showing only the H1′ to H2′ cross peaks for the A5 and A6 nucleotides for the A5–H2″ and A6–H2″ deuterated $d(CGCGAATTCGCG)_2$ sequence. (E) 1D slice taken at the low-frequency H1′ resonance through the cross peaks of A5 in the deuterated sample. (F) 1D slice taken at the low-frequency H1′ resonance through the cross peaks of A6 in the deuterated sample. Note that in the trace through A6, the intensity of the cross peak immediately deshielded relative to A6 is more intense in (F) than in (C). This does not reflect a change in the intensity of the neighboring cross peak, but rather the position of the slice has changed due to the collapse of the doublet of quartets for A6. Consequently, the slice through the low-frequency H1′ component of A6 passes through the most intense region of the adjacent cross peak in (F).

deuterated samples, respectively. In comparison to the slices through cross peaks of the protonated sample, the deuterated DNA exhibits a signal increase of about 5.8 times in the H1′–H2′ cross peak. Three factors contribute to the increase in intensity. A twofold increase in the intensity results from the elimination of the H2′–H2″ passive couplings when the H2″ proton is replaced by a deuteron. This causes a collapse of the doublet of the quartets into a single quartet. The remaining intensity increase is due to (1) the reduction of the H2′ linewidth, and to a lesser extent the H1′ linewidth, and (2) diminished decay of the H1′–H2′ antiphase coherence during the t_1 evolution time. Both of these effects result from the weaker dipolar coupling of the H–D geminal pair which leads to less efficient spin–spin relaxation. In addition to the improvement in the signal-to-noise ratio, the self-cancellation of antiphase multiplet components is significantly reduced because of the narrower linewidths. Similar improvements are also observed for the A6 residue shown in Figs. 2C and 2F.

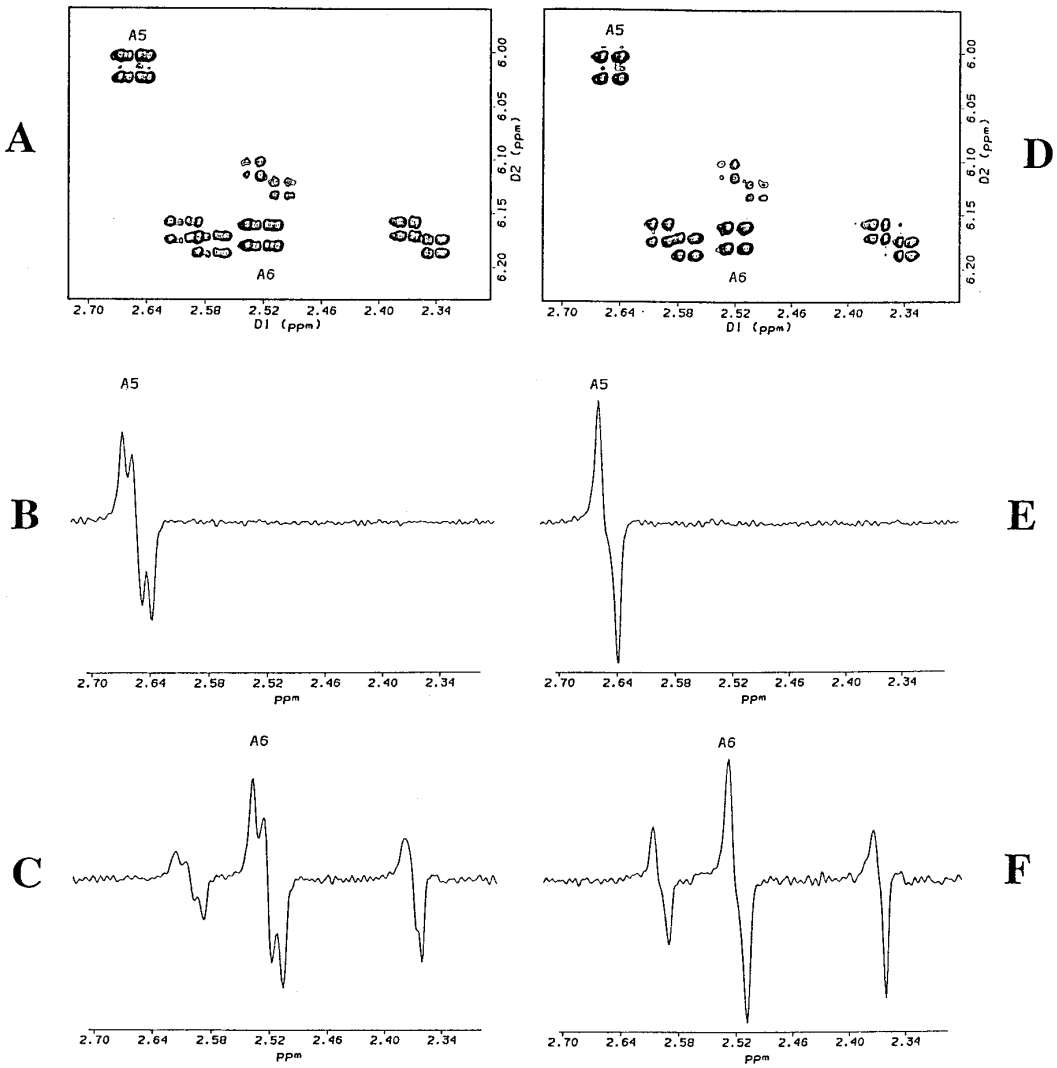

FIG. 3. (A) Expanded contour plot from a stripe-COSY spectrum of A5–H2″ and A6–H2″ deuterated d(CGCGAATTCGCG)$_2$ collected at 500 MHz showing the H1′ to H2′ and H1′–H2″ cross peaks of the A5 and A6 nucleotides. (B) 1D slice taken from (A) at the low-frequency H1′ component of the A5 cross peak. (C) 1D slice taken from (A) at the low-frequency H1′ resonance of the A6 cross peak. (D) Expanded contour plot from a superstripe-COSY spectrum of A5–H2″ and A6–H2″ deuterated d(CGCGAATTCGCG)$_2$ collected at 500 MHz showing the H1′ to H2′ and H1′–H2″ cross peaks of the A5 and A6 nucleotides. (E) 1D slice taken from (D) at the low-frequency H1′ component of the A5 cross peak. (F) 1D slice taken from (D) at the low-frequency H1′ resonance of the A6 cross peak. The transmitter offset for the selective pulse was centered at 5.8 ppm whereas the transmitter offset for the nonselective pulse was centered at 3.5 ppm. The corresponding spectral widths were 550 Hz (covering H1′ protons only) and 3000.1 Hz for f_1 and f_2 (2.92 Hz/point), respectively. 150 t_1 increments (3.66 Hz/point) with zero-filling to 1024 and 4096 along f_1 (0.54 Hz/point) and f_2 (1.46 Hz/point). 64 transients were collected per increment. A 40° and 20° shifted sine-bell apodization function was used in the direct and indirect dimensions, respectively, to enhance spectral resolution. The total relaxation time per transient was 2.340. The total acquisition time is 12 h for each spectrum.

It is clear from the data displayed in Fig. 2 that selective deuteration can dramatically increase the signal-to-noise and reduce self-cancellation in P.E.COSY type spectra. However, selective deuteration also reduces the magnitude of transvere cross relaxation by about 42-fold. This approach, then, offers a direct method for testing the theories which predict significant modulations of the apparent coupling constants due to transverse cross relaxation. The Dickerson sequence, d(CGCGAATTCGCG)$_2$, was selected for this study because the complete set of coupling constants and a complete analysis of the ratio of %S (south) to %N (north) for each nucleotide have been previously reported (*2*). The $^3J_{H1'-H2'}$ of A5 and A6 was measured as 9.8 Hz both in the protonated sequence and in the selectively deuterated dodecamer d(GCGCAATTGCGC)$_2$. These values are in good agreement with the values reported by Bax and Lerner (*2*) but are about 1.2 Hz larger than those reported by Zhu *et al.* (*6*). Based

on these results, it appears that a significant reduction in transverse cross relaxation does not change the measured $^3J_{H1'-H2'}$ of A5 and A6 and would not alter the previous conclusion concerning the population distribution at the A5 and A6 positions in the sequence, i.e., 94% (S) with 6% (N). Consequently, the 6% deviation from pure S conformation cannot be accounted for by the effects of transverse cross relaxation. Other factors such as sugar dynamics and variations in the pucker angle should still be considered. While no change in the coupling constants was observed for the A5 and A6 residues upon deuteration, it will be interesting to examine other residues in the same sequence that have been interpreted as having large fractions of north population, such as T7 (15% N) and C9 (20% N).

The traces through the H1′ to H2′ cross peaks for residues A5 and A6 shown in Fig. 2 contain additional splittings of the in-phase component of the H2′ resonance in the F_2 dimension due to passive coupling between the H2′ proton and the H3′ proton. We realized that a further improvement in both sensitivity and reduced self-cancellation could be obtained by selectively decoupling the H3′ protons during the t_2 acquisition time. The superstripe-COSY experiment was performed using time-shared homonuclear decoupling selectively applied to the H3′ protons and the results are shown in Fig. 3. Figure 3A shows a contour plot of the stripe-COSY spectrum in the A5 and A6 region at 500 MHz. Figures 3B and 3C show traces from the stripe-COSY spectrum taken at the lower H1′ frequencies of the A5 and A6 cross peaks. A contour plot of the same region as in Fig. 3A is shown for the superstripe-COSY spectrum in Fig. 3D. The collapse of the in-phase doublets in the 1D of the A5, A6, and other cross peaks is apparent. Figures 3E and 3F show traces as in Figs. 3B and 3C which show essentially complete collapse of the in-phase doublets into singlets. While one might initially expect a factor of 2 increase in the signal, in practice we realize a factor of about 1.5. The failure to achieve a full factor of 2 increase is probably due to the fact that the intensity of the components of the overlapped doublet is already greater than that for the components of a fully resolved doublet. An important feature in the traces of Figs. 3E and 3F is a discontinuity in the inner lines of the antiphase components (a feature that is not present upon inspection of the partially self-canceled inner components of the antiphase peaks in the H3′-coupled spectrum). This indicates that the effects of self-cancellation have almost completely been eliminated. This has important implications for the accurate measurement of coupling constants in which serious errors can be introduced if the correction for the effects of self-cancellation are not treated properly.

While these methods were demonstrated for measuring $^3J_{H1'-H2'}$, $^3J_{H2'-H3'}$ is the coupling constant most sensitive to sugar conformations between H1′-exo and H2′-endo. In the past it has been very difficult to measure $^3J_{H2'-H3'}$ using P.E.COSY type experiments. $^3J_{H2'-H3'}$ is usually measured from a displacement of the H2″–H3′ cross peaks by the passive H2′–H3′ coupling constants. However, since $^3J_{H2''-H3'}$ is only ~1–2 Hz, the intensities of the cross peaks due to the active $^3J_{H2''-H3'}$ are very weak and suffer serious self-cancellation. The superior quality of spectra produced by the superstripe-COSY should enable measurement of $^3J_{H2'-H3'}$ directly from the splitting of the antiphase doublet in the cross peak due to the active $^3J_{H2'-H3'}$. In order to apply superstripe-COSY to measure the H2′–H3′ coupling constant, the H3′ region would be selectively excited and the H1′ region selectively time-shared homonuclear decoupled during the acquisition time. In an H2″-deuterated sample, a single splitting due to $^3J_{H2'-H3'}$ would be produced in F2.

In conclusion, we have demonstrated three strategies for improving the measurement of proton–proton coupling constants in DNA. First, the stripe-COSY pulse sequence can be used to collect P.E.COSY type spectra in ~1/5 the time of a standard COSY-35 for the same overall signal-to-noise ratio when measuring the H1′–H2″ region. Second, selective deuteration of the H2″ proton was introduced as an effective means of eliminating the effects of transverse cross relaxation in geminal proton pairs. An added benefit was that selective deuteration of the H2″ proton in the sugar moiety produced a dramatic five- to sixfold increase in the signal intensity due to the collapse of the doublet of quartets into a single quartet, reduced H2′ linewidths, and reduced decay of H2′ coherences during the t_1 evolution time because of increased T_2. The latter two effects result from weaker dipolar relaxation of the H2″ due to deuteration of its geminal proton pair. Third, the superstripe-COSY experiment was introduced as a general experiment to include elements for eliminating all other unwanted passive couplings. In the example shown here, selective time-shared homonuclear decoupling of the H3′ protons was used to collapse the passive H2′–H3′ couplings.[2] This produces another factor of 1.5 increase in the signal-to-noise ratio. Collectively, these strategies significantly reduce the total experimental time required to collect P.E.COSY type spectra. Furthermore, because of the collapse of unwanted passive couplings and the sharpening of linewidths which reduces self-cancellation, the

[2] The superstripe-COSY can be run in other ways. For example, if one does not have selectively H2″-deuterated phosphoramidites, the H2′/H2″ region could be selectively excited and selected H2″ resonances saturated during the t_1 evolution period and t_2 acquisition period. This would produce a single H1′–H2′ quartet as observed in the deuterated samples, producing a factor of 2 increase in signal-to-noise. The single quartet would result since during the high-resolution direct detection dimension, the H1′ would be coupled only to the H2′. Unfortunately, the line narrowing or reduction of transverse cross relaxation experienced with deuteration would not be realized.

measurement of coupling constants is much more straightforward and more accurate.

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