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NMR of enzymatically synthesized uniformly $^{13}C^{15}N$-labeled DNA oligonucleotides

(heteronuclear/Klenow fragment/deoxynucleotide kinase/alkaline hydrolysis)

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ABSTRACT **A procedure for the enzymatic synthesis of uniformly $^{13}C^{15}N$-labeled DNA oligonucleotides in milligram quantities for NMR studies is described. Deoxynucleotides obtained from microorganisms grown on ^{13}C and ^{15}N nutrient sources are enzymatically phosphorylated to dNTPs, and the dNTPs are incorporated into oligonucleotides using a 3′-5′ exonuclease-deficient mutant of Klenow fragment of DNA polymerase I and an oligonucleotide template primer designed for efficient separation of labeled product DNA from unlabeled template. The labeling strategy has been used to uniformly label one or the other oligonucleotide strand in the DNA duplex dGGCAAAACGG·dCCGTTTTGCC in order to facilitate assignment and structure determination by NMR. Application of ^{15}N and ^{13}C heteronuclear NMR experiments to isotopically labeled DNA is presented.**

Multidimensional heteronuclear NMR experiments can provide a wealth of spectral information in the form of heteronuclear chemical shifts, scalar couplings, and relaxation parameters, which yield structural and dynamic information for macromolecules. In addition, the spectral dispersion provided by the addition of a heteronuclear dimension extends the limit to the size of the macromolecule that can be studied by NMR.

^{13}C and ^{15}N heteronuclei are now routinely incorporated into proteins (1) for use in NMR structure determinations. Recently, the uniform incorporation of these heteronuclei into RNA oligonucleotides has been achieved (2–4). NMR experiments have also been done recently on oligodeoxynucleotides labeled uniformly in the deoxyribose at a single nucleotide (5) and uniformly in a nucleotide (6), but heteronuclear NMR studies of uniformly labeled oligodeoxynucleotides have not been reported because of the technical challenge and the cost of chemical syntheses and polymerization of labeled phosphoramidites.

Here we describe an enzymatic synthesis of $^{13}C^{15}N$ uniformly labeled oligodeoxynucleotides and present some preliminary heteronuclear spectra of a DNA duplex dGGCAAAA CGG·dCCGTTTTGCC (A_4·T_4), in which either the A_4 or T_4 strand is uniformly labeled with ^{13}C and ^{15}N. The synthesis procedure involves production of uniformly $^{13}C^{15}N$-labeled deoxynucleotides from enzymatic hydrolysis of the DNA of bacteria grown on 99% $^{13}CH_3OH$ and >98% $^{15}NH_4Cl$ as sole carbon and nitrogen sources, respectively. The labeled deoxynucleotides are then converted enzymatically to the triphosphates and used in a DNA polymerization reaction that utilizes an oligonucleotide hairpin primer-template containing a ribonucleotide at the 3′ terminus. Alkaline hydrolysis of the ribonucleotide linkage between the labeled DNA and the unlabeled primer-template followed by purification yields the labeled DNA. The utility of the heteronuclear labels in DNA is illustrated by heteronuclear NMR spectra of the oligodeoxynucleotide duplex A_4·T_4.

MATERIALS AND METHODS

Materials. Both 99% $^{13}CH_3OH$ and >98% $^{15}NH_4Cl$ were obtained from Cambridge Isotope Laboratories (Cambridge, MA). RNase-free DNase I was obtained from Boehringer Mannheim. Nuclease P_1, phospho*enol*pyruvate (PEP), myokinase from chicken muscle, guanylate kinase from porcine brain, and pyruvate kinase from rabbit muscle were obtained from Sigma. Centricon and Centriprep 3-kDa cutoff centrifugal concentrators were obtained from Amicon. Affi-Gel 601 boronate-derivatized polyacrylamide gel was from Bio-Rad. The Vydac nucleotide analysis column was from Rainin (Woburn, MA). DEAE-Sephadex G25 was from Pharmacia. Oligonucleotide synthesis columns were from Glen Research (Sterling, VA). Levigated alumina was obtained from Buehler (Lake Bluff, NY). NMR tubes were from Shigemi (Tokyo).

Preparation of $^{13}C^{15}N$ dNMPs. These procedures (Fig. 1) are based largely on the NMP biosynthetic procedures of Batey *et al.* (2), with the exceptions that cells were grown to stationary phase, DNase I was used, and the nucleotides were put over an anion exchange column prior to separation of the dNMPs from the NMPs.

Growth of Methylotrophic Bacteria on $^{13}CH_3OH$ and $^{15}NH_4Cl$. *Methylophilus methylotrophus* were grown in minimal medium as described (2). After growth to stationary phase and harvesting by centrifugation, a yield of 2 g of wet packed cells per liter was obtained when *M. methylotrophus* were grown on $^{13}CH_3OH$ and $^{15}NH_4Cl$.

Isolation of Nucleic Acids. According to the published procedure (2), 4 g of cells was resuspended in an equal volume of STE (100 mM NaCl/10 mM Tris·HCl/1 mM EDTA, pH 8.0). The cell suspension was added to 100 ml of STE containing 1% SDS and incubated at 37°C for 10 min with shaking. The lysed cells were added to a 200-ml 25:24:1 mixture of STE equilibrated phenol/chloroform/isoamyl alcohol preheated to 65°C. The mixture was incubated at 65°C for 30 min with periodic vigorous shaking followed by centrifugation at 6000 rpm in a Beckman JA10 rotor for 10 min. The aqueous phase was removed and the organic phase was extracted once with 200 ml of STE, using a blender to mix the organic and aqueous phases. The aqueous phases were pooled and extracted once with an equal volume of 24:1 chloroform/isoamyl alcohol. Nucleic acids were precipitated by the addition of 1/10th vol of 3 M sodium acetate (pH 5.2) and 3 vol of ice-cold 100% ethanol. The dried pellet was resuspended in 25 ml of TE (10 mM Tris·HCl, pH 8.0/1 mM EDTA). For *M. methylotrophus,* a purified nucleic acid yield of 1100 A_{260} units was obtained for 4 g of wet packed cells.

Enzymatic Hydrolysis of Nucleic Acids. PAGE analysis with UV shadowing and ethidium bromide staining was used to monitor the degradation of DNA to mononucleotides by the enzymes DNase I and nuclease P_1. The purified nucleic acids

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Abbreviations: PEP, phospho*enol*pyruvate; HMQC, heteronuclear multiple quantum correlation; HSQC, heteronuclear single quantum correlation; 2D, two dimensional.

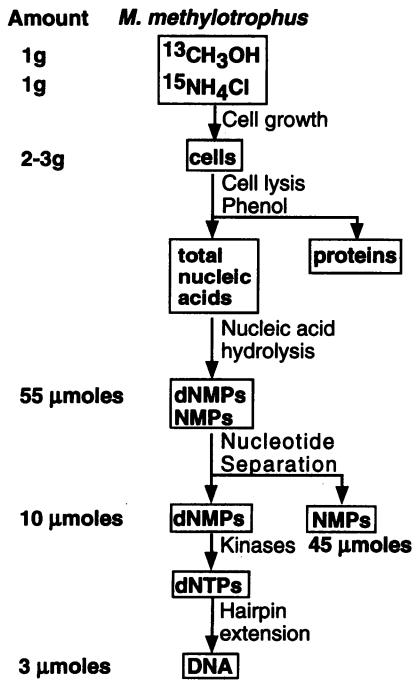

FIG. 1. General procedure and yields for the biosynthetic production of $^{13}C^{15}N$-labeled deoxynucleotides and their incorporation into oligodeoxynucleotides [adapted from Batey *et al.* (2)]. [Reproduced by permission of Oxford University Press (copyright 1992, Oxford Univ. Press).]

were brought to 10 mM $MgCl_2$; 100 units of RNase-free DNase I was added and incubated for 1 hr at 37°C. The DNase I-treated nucleic acids were denatured by heating in a boiling water bath for 10 min and immediately chilling in an ice bath for 10 min. The solution was brought to 50 mM sodium acetate, pH 5.2/0.1 mM $ZnCl_2$. Six units of nuclease P_1 was added and the reaction mixture was incubated at 55°C for 1 hr. The nucleic acids were denatured a second time, another 6 units of nuclease P_1 was added, and the reaction was allowed to proceed for a second hour at 55°C.

Separation of dNMPs from NMPs. The nucleotides were exchanged into 1 M triethylammonium bicarbonate (TEABC) at pH 9.2 by loading onto a DEAE-Sephadex G25 column (bicarbonate form) (8 × 2.5 cm). All nucleotides were eluted with 1 M TEABC (pH 9.2). The nucleotide fraction was loaded onto a 1 M TEABC (pH 9.2) equilibrated Affi-Gel 601 *cis*-boronate affinity column (20 × 2.5 cm) at 4°C. The flow-through (dNMP fraction) was collected and rotary evaporated at 40°C under reduced pressure. The column was washed with 1 M TEABC (pH 9.2). The NMPs were eluted by treating the column with CO_2-acidified H_2O (pH 4.5).

Isolation of Deoxynucleotide Kinasing Enzymes from *Escherichia coli*. Five liters of *E. coli* JM101 was grown to logarithmic phase as described by Hurlbert and Furlong (7). Cells were harvested by spinning at 5000 rpm for 15 min in a Beckman JA10 rotor. Cells (10.8 g) were lysed by grinding with levigated alumina (2.5 g/g of cells) and extracted two times with 35 ml of 10 mM K_2HPO_4, pH 7.2/2 mM glutamine/5 mM EDTA per g of cells. The alumina and cell debris were separated from the lysate by centrifugation at 8000 rpm for 30 min in a Beckman JA20 rotor. The cell lysate was collected and subjected to a 45,000 rpm spin in a Beckman ultracentrifuge type 60 Ti rotor for 1 hr. The ribosomes were precipitated from the supernate with ≈500 mg of streptomycin sulfate and pelleted by spinning at 15,000 rpm for 10 min in a Beckman JA20 rotor. A slurry of 0.1% Polymin P, 1% Celite, and the supernate was spun at 15,000 rpm for 20 min in a JA20 rotor. The resulting supernate was fractionated at ammonium sulfate concentrations of 0.21, 0.30, and 0.35 mg/ml; each fractionation was spun at 8000 rpm for 10 min in a JA20 rotor. The ammonium sulfate fraction (0.30–0.35 mg/ml) was resuspended in 11 ml of 50 mM glycine, pH 8.0/2 mM glutamine/0.2 mM EDTA and labeled as the *E. coli* kinasing fraction. The protein concentration was determined as 3.1 mg/ml; the *E. coli* kinasing fraction was stored in 1-ml aliquots at −20°C.

Phosphorylation of dNMPs. The phosphorylation reactions were monitored with a Vydac nucleotide analysis anion exchange HPLC column. A combination of commercial kinases and the *E. coli* kinasing fraction were used to phosphorylate the dNMPs to dNTPs using PEP as the phosphate donor. The kinasing reactions were done in a vol of at least 1 ml with 80 mM Tris·HCl, pH 7.5/20 mM KCl/20 mM $MgCl_2$/10 mM dithiothreitol/10 mM $^{13}C^{15}N$ dNMPs/50 mM PEP/100 μM ATP/50 units of pyruvate kinase per ml/20 units of myokinase per ml/0.1 unit of guanylate kinase per ml. The phosphotransferase reactions were allowed to proceed at 37°C for 2 hr before the *E. coli* kinasing fraction (0.31 mg/ml), an additional 25 units of pyruvate kinase per ml, and 25 mM PEP was added. The reaction was allowed to proceed for another 4 hr at 37°C. At this time, the reaction mixture was extracted with 1 vol of phenol, three times extracted with 1 vol of chloroform, and extracted once with 1 vol of diethyl ether. The $^{13}C^{15}N$-labeled dNTPs were stored in 1-ml aliquots at −20°C.

Chemical Synthesis of Oligonucleotide Templates. The oligonucleotide primer-template hairpins were synthesized on an Applied Biosystems DNA synthesizer (model 380B) using four 1-μmol scale U and A columns (Glen Research) in which the ribonucleotide derivative is covalently attached to the aminopropyl-CPG via a succinyl linkage on the 2′ or 3′ O and capped with acetate on the unattached 2′ or 3′ O. Hydrolysis of the succinyl linkage and all deprotection are achieved by using concentrated ammonium hydroxide, whereas use of a silyl 2′ protecting group on the ribonucleotide requires an additional desilylation step using tetrabutyl ammonium fluoride. The oligodeoxynucleotides were synthesized using cyanoethyl phosphoramidite chemistry and standard coupling protocols, cleaved from the support with concentrated NH_4OH, and deprotected overnight at 55°C. The samples were purified by denaturing PAGE, and sample concentrations were determined by measuring absorbance at 260 nm. The oligonucleotide templates and products are shown in Fig. 2.

Purification of Cloned DNA Polymerase Klenow Fragment Deficient in 3′-5′ Exonuclease. The D355A, E357A 3′-5′ exonuclease-deficient double mutant of DNA polymerase I Klenow fragment was purified as described (8) from *E. coli*.

Synthesis of $^{13}C^{15}N$ Uniformly Labeled Oligodeoxynucleotides. The oligonucleotide hairpins, A_4TEMP and T_4TEMP, served as templates for the enzymatic syntheses of A_4 and T_4, respectively. The A_4 hairpin extension reaction was done in 4.9 ml containing 10 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM A_4TEMP, 3 mM $^{13}C^{15}N$ dNTPs (30 oligodeoxynucleotides), and 490 units of Klenow fragment (3′-5′ exonuclease deficient). The reaction was allowed to proceed at 37°C for 2 hr and stopped by heating to 75°C for 10 min; the dNTPs were

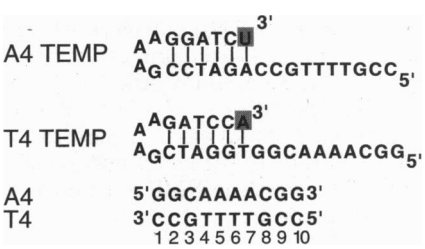

FIG. 2. Oligonucleotide templates and products used in the hairpin extension method for synthesis of uniformly labeled $^{13}C^{15}N$ DNA. Ribonucleotides are shaded.

separated from the products and template using a Centriprep-3. The buffered Centriprep filtrate containing the unincorporated dNTPs from the A_4TEMP extension was combined with T_4TEMP in a 6-ml reaction mixture containing 0.08 mM T_4TEMP and 1.2 mM recycled dNTPs as well as 500 units of newly added Klenow fragment (3′-5′ exonuclease deficient). The synthesis of T_4 by hairpin extension was carried out in the same manner as A_4 synthesis. For both extensions, DNA was desalted with the Centriprep-3, and the volumes were reduced to 0.2 ml by Speed-Vac.

Alkaline Cleavage and Separation of Template from Product. The extended hairpins, both in volumes of 0.2 ml, were brought to 0.3 M KOH by the addition of 0.1 ml of 1 M KOH. Alkaline hydrolysis was allowed to proceed for 2 hr at a temperature of 55°C. The samples were diluted to 0.5 ml with formamide gel loading buffer. The template and $^{13}C^{15}N$-labeled product were separated from each other by preparative denaturing PAGE, visualized by UV shadowing, cut from the gels, and either electroeluted (template) or crushed and soaked (product). The template DNA was recovered by ethanol precipitation and the labeled DNA product was purified from residual polyacrylamide by anion exchange.

NMR Sample Preparation. Chemically synthesized oligodeoxynucleotides A_4 and T_4 were purified by anion exchange HPLC. After anion exchange, chemically and enzymatically synthesized oligonucleotides were concentrated to 0.2 ml and microdialyzed against 5 mM sodium phosphate (pH 7.0), 37.5 mM NaCl, and 0.125 mM EDTA. Oligonucleotide concentrations were determined by absorbance at 260 nm, and each enzymatically synthesized $^{13}C^{15}N$ DNA strand was combined 1:1 with its chemically synthesized complement. Two NMR samples of duplex DNA were made: A_4T (≈2 mM) contained $^{13}C/^{15}N$-labeled A_4 in a duplex with unlabeled T_4, and T_4A (≈1 mM) contained $^{13}C/^{15}N$-labeled T_4 in a duplex with unlabeled A_4. Final samples were in 20 mM sodium phosphate, 200 mM NaCl, 0.5 mM EDTA (pH 7.0) in a vol of 180 μl in a Shigemi tube.

NMR. Spectra were acquired on a GE Omega 500 spectrometer using a 5-mm triple resonance (^1H, ^{13}C, ^{15}N) probe (Bruker, Billerica, MA) equipped with *X, Y, Z* pulsed field gradient coils. For all spectra, the proton carrier frequency was set at 4.75 ppm. GARP-1 (globally optimized alternating-phase rectangular pulses) (9) decoupling of the heteronucleus was used during acquisition. NMR data were processed on a Silicon Graphics Indigo using the NMR software program FELIX 2.3 (Biosym Technologies, San Diego). FIDs (free induction decoys) were sine-bell apodized and zero-filled prior to Fourier transformation to obtain real matrices of dimensions 1024 × 1024.

RESULTS AND DISCUSSION

Synthesis. Procedures for the biosynthetic production of isotopically labeled nucleotides from *E. coli* (3, 4), *M. methylotrophus* (2), and *Methylophilus extorquens* (10) have been reported, and methods for the production of isotopically labeled deoxynucleosides from algae are available (11). We have introduced minor modifications into the procedures of Batey *et al.* (2); these or similar modifications can be introduced into most of the other nucleotide biosynthetic preparative methods in order to obtain purified deoxynucleotides. Using 1 g of $^{13}CH_3OH$ and 1 g of $^{15}NH_4Cl$ as sole carbon and nitrogen sources, respectively, for 1-liter *M. methylotrophus* cultures, a 2-g wet cell pellet was obtained, which yielded 10 μmol of dNMPs and 45 μmol of NMPs.

Methods for the large-scale enzymatic phosphorylation of NMPs to NTPs have been published (12) and chemical methods for the conversion are available for both NMPs and dNMPs (13). We have found that a combination of commercially available kinases (12) and a crude *E. coli* pyrimidine kinase fraction (7) can be used to efficiently phosphorylate the dNMPs to dNTPs. The *E. coli* crude kinase fraction provides the pyrimidine kinase activity that is necessary to convert TMP to TDP. Greater than 90% of the dNMPs were converted to dNTPs in the enzymatic phosphorylation reactions reported here.

Although a number of reports describing the uniform isotopic labeling of RNA oligonucleotides have recently been published (2–4), uniform isotopic labeling of DNA oligonucleotides in NMR-scale quantities has not yet been reported. The procedure described here uses an oligonucleotide hairpin primer template, which contains a single ribonucleotide at its 3′ end, which enables separation of primer from product by alkaline hydrolysis. The hairpin loop joining the template to primer was designed in order to ensure a 1:1 primer/template stoichiometry and thereby minimize any nonspecific annealing of templates or primers that might result in undesired polymerization products. The 3′-5′ exonuclease-deficient DNA polymerase I Klenow fragment has the ability to polymerize DNA from both RNA and DNA primers, has no exonuclease activities, and was easily obtained in large quantities. It should be noted that in this particular synthesis, the exonuclease-deficient mutant form of the Klenow enzyme was used because it provided higher yields of product than the wild type. For subsequent syntheses of different template primers, presumably the template-independent polymerase activity (14) of the mutant Klenow enzyme resulted in products that were longer than the desired length. For these cases, the wild-type Klenow fragment seems to be a better choice of enzyme. Following enzymatic extension of the template primer with 3′-5′ exonuclease-deficient mutant DNA polymerase I Klenow fragment and $^{13}C^{15}N$ uniformly labeled dNTPs, the 2′-hydroxyl of the ribonucleotide at the end of the primer template provides for base-catalyzed hydrolysis of the labeled product from the template primer. The procedure is schematized in Fig. 3.

In a reaction that used 15 μmol of $^{13}C^{15}N$ dNTPs and 0.49 μmol of gel-purified A_4TEMP oligonucleotide, $^{13}C^{15}N$ dNTPs were incorporated into 0.23 μmol of purified A_4 oligonucleotide; 7 μmol of the unincorporated $^{13}C^{15}N$ dNTPs were recycled and used in an extension reaction with 0.47 μmol of T_4TEMP in which 0.21 μmol of $^{13}C^{15}N$ T_4 oligonucleotide was purified. These quantities of labeled oligodeoxynucleotide

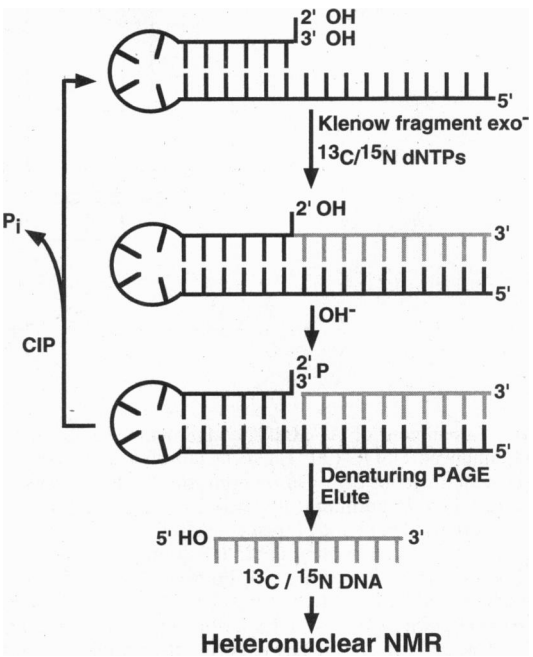

FIG. 3. Hairpin extension method for enzymatic synthesis of uniformly $^{13}C^{15}N$-labeled oligodeoxynucleotides from $^{13}C^{15}N$ dNTPs.

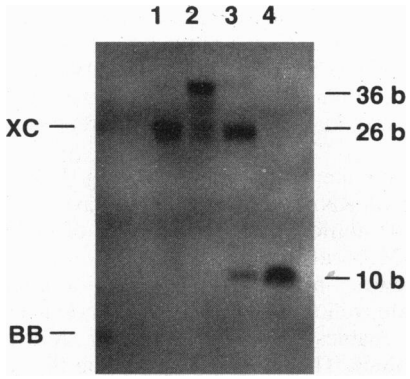

FIG. 4. UV shadow PAGE analysis of species present at different stages of the hairpin extension reaction. A_4TEMP (lane 1) was extended with $^{13}C^{15}N$ dNTPs (lane 2), and the product was hydrolyzed with KOH (lane 3). Lane 4, chemically synthesized A_4.

were sufficient to make ≈1 mM NMR samples in 180 μl in Shigemi NMR tubes. With an additional alkaline phosphatase step, the recovered templates can be regenerated for use in additional extensions. UV shadow PAGE analysis of the species present at different stages of the enzymatic synthesis of A_4 is shown in Fig. 4. In the syntheses reported here, a $^{13}C^{15}N$ dNTP incorporation yield of 29% was observed. The high incorporation yield offsets the low biosynthetic yield of dNMPs from bacterial cultures, which is 10–20% of the biosynthetic yield of NMPs. Thus, the amount of ^{13}C and ^{15}N culture medium required to uniformly label oligodeoxynucleotides is comparable to the amount required to label oligoribonucleotides.

NMR. As has been observed for proteins and RNA, uniform isotopic labeling of DNA should aid in the assignment and structure determination of oligodeoxynucleotides as single strands, in complexes with other strands, and with ligands such as proteins, RNA, and drugs. Here, we demonstrate NMR experiments on labeled DNA that provide heteronuclear chemical shift assignments and confirm by scalar coupling

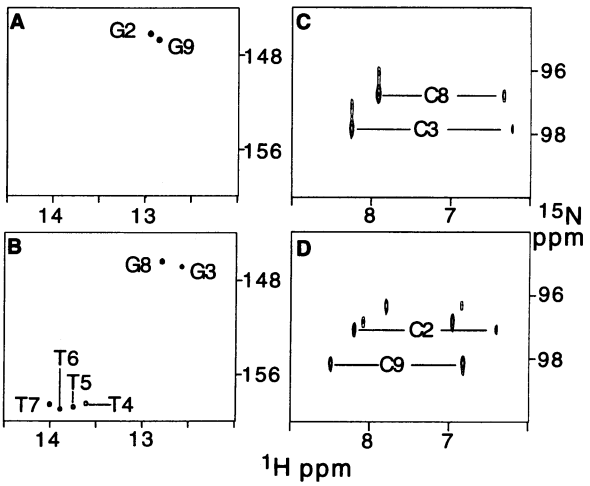

FIG. 5. Imino (*A* and *B*) and amino (*C* and *D*) regions of A_4T (*A* and *C*) and T_4A (*B* and *D*) from 2D gradient-enhanced reverse-detected $^{15}N^1H$ HMQCs with spin-echo water suppression (16, 17). Spectra were recorded at 15°C in 10% ^2H_2O/90% H_2O with spectral widths of 10 kHz in t2 and 5 kHz in t1. The ^{15}N frequency was set to 120 ppm. Four scans of 1024 complex points were collected in t2 and 256 complex points were collected in t1 using the States-TPPI (time proportional phase incrementation) (18) method for quadrature detection. Experiment time was 45 min. ^{15}N decoupling was applied during acquisition. Assignments are indicated. In *C*, the H-bonded amino proton resonances (downfield) are of higher intensity than their non-H-bonded partners. The ^{15}N chemical shift of NH^2H is upfield of that for NH_2 and can be observed at this contour level for the H-bonded resonances. In *D*, the terminal C amino resonances are observed but are not assigned.

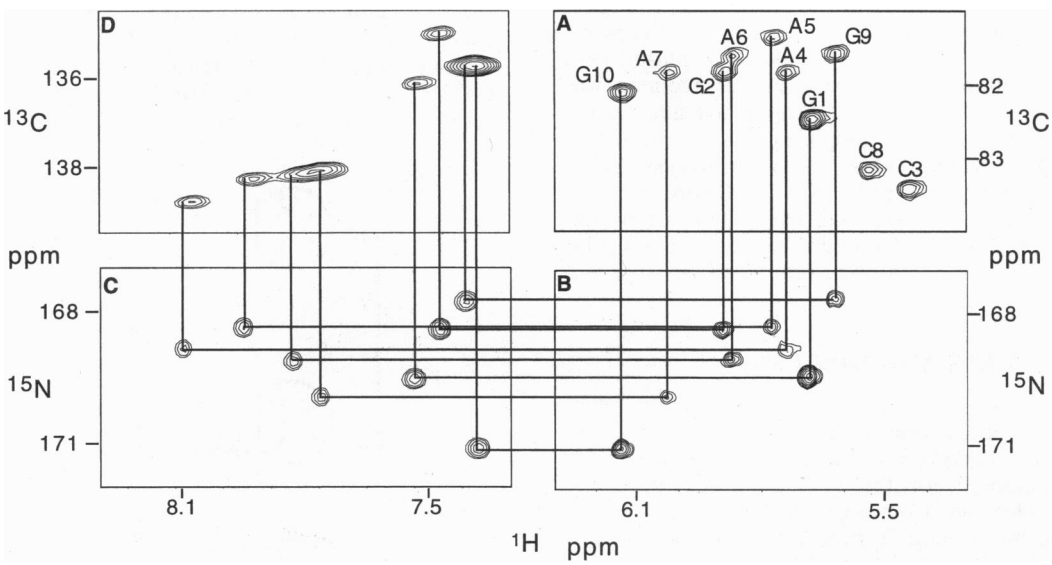

FIG. 6. Assignment of H1′, C1′, N9, H8, and C8 in purines. (*A*) The H1′–C1′ region of a 2D $^{13}C^1H$ 52-msec constant time gradient-selected (19) and enhanced HSQC (20, 21) spectrum of A_4T in ^2H_2O at 25°C. The experiment was collected with a spectral width of 5 kHz in t2 and 10 kHz in t1 with a carrier offset of 60 ppm for ^{13}C. Four transients and 512 complex points were taken in t2 with ^{13}C decoupling; 400 complex t1 points were taken. Experiment time was 2 hr. (*B*) The H1′–N9 region from the HCN experiment (22, 23) at 25°C in ^2H_2O. Spectral widths of 4 and 3 kHz were used in t2 and t1, respectively. Carrier offsets were 85 ppm for carbon and 165 ppm for ^{15}N. Sixty-four scans and 512 complex points were taken with ^{13}C decoupling in t2; 128 complex t1 points were taken. Experiment time was 6 hr. (*C*) The H8–N9 region from the refocused $^{15}N^1H$ 2-bond HMQC experiment (4) done on A_4T at 25°C in ^2H_2O. The experiment was collected with spectral widths of 4 and 6 kHz in t2 and t1, respectively and a carrier frequency of 146 ppm for ^{15}N. Sixteen scans and 512 complex points were acquired in t2 with ^{13}C and ^{15}N decoupling; 256 complex t1 points were taken. Experiment time was 3 hr. (*D*) The H8–C8 region from the 2D gradient-selected (19) and enhanced $^{13}C^1H$ HSQC (21) of A_4T at 25°C in ^2H_2O. The experiment was taken with the ^{13}C carrier set to 120 ppm. Spectral widths of 4 and 10 kHz were used for ^1H and ^{13}C, respectively. Four scans and 512 complex points were taken with ^{13}C decoupling in t2, and 256 complex points were taken in t1. Experiment time was 1 hr.

methods proton connectivities previously established by nuclear Overhauser effect measurements (J. G. Nadeau and D.M.C., unpublished data).

The ^{15}N–^{1}H gradient-enhanced heteronuclear multiple quantum correlation (HMQC) (15, 16) can be used to assign ^{1}H and ^{15}N chemical shifts of slowly exchanging base-paired imino protons in G and T nucleotides and amino protons in G, C, and A residues. Any ambiguity in base type from imino proton chemical shift (G, 10–13 ppm; T, 12–15 ppm) is resolved by determining the nitrogen chemical shift (G, 146 ppm; T, 160 ppm). Base-specific assignment of amino protons (6.6–9 ppm) can also be made based on amino nitrogen chemical shifts (G, 75 ppm; A, 81 ppm; C, 98 ppm). By labeling only one strand in the duplex, we have been able to halve the spectral complexity. In this report, we show the imino and C amino regions of the two-dimensional (2D) HMQC spectra of A_4T and T_4A (Fig. 5).

Fig. 6 shows four experiments on A_4T that were used to determine carbon (C1′ and C8) and nitrogen (N9) chemical shifts and to confirm proton (H1′ and H8) chemical shifts assigned by conventional proton NMR methods. Fig. 6*A* shows the C1′–H1′ cross peaks in a 2D constant time heteronuclear single quantum correlation (HSQC) (20, 21). The proton chemical shifts are correlated to the glycosidic nitrogens in a HCN (22, 23) experiment (Fig. 6*B*), which uses the two consecutive transfers of magnetization from ^{1}H to ^{13}C to ^{15}N and then back. Fig. 6 shows an expansion of the region corresponding to the H1′–N9 correlation for purines. The N9 chemical shift is correlated to the H8 chemical shift in a refocused $^{15}N^{1}H$ 2-bond HMQC (4) experiment (Fig. 6*C*). The two experiments in Fig. 6 *B* and *C* can be combined into one experiment in order to get a through-bond correlation of the well-dispersed H1′ to H8 (6, 23), thus eliminating the requirement for good ^{15}N chemical shift dispersion for connecting the two protons. Fig. 6*D* shows the aromatic region of the $^{13}C^{1}H$ HSQC (24), which allows correlation of H8 to C8.

Here we have used 2D heteronuclear NMR experiments to illustrate the utility of heteronuclear labels in DNA. A number of 2D and 3D experiments for assignment and structure determination are made possible with the incorporation of heteronuclear labels into DNA. Due to the similar chemical nature of DNA to RNA, many of the heteronuclear experiments that have been recently developed for the assignment of uniformly labeled RNA resonances will be directly applicable to uniformly labeled DNAs. In addition, the labels provide access to heteronuclear relaxation information, which is rich in dynamics information for the molecule. In addition, further spectral simplification can be achieved by separation of the four deoxyribonucleotides and selective incorporation of each residue type into oligonucleotides.

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