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Efficient enzymatic synthesis of ^{13}C,^{15}N-labeled DNA for NMR studies

Deborah E. Smith, Jin-Yuan Su* and Fiona M. Jucker**

NeXstar Pharmaceuticals Inc., 2860 Wilderness Place, Boulder, CO 80301, U.S.A.

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Summary

The power of heteronuclear NMR spectroscopy to study macromolecules and their complexes has been amply demonstrated over the last decade. The obstacle to routinely applying these techniques to the study of DNA has been the synthesis of ^{13}C,^{15}N-labeled DNA. Here we present a simple and efficient method to generate isotope-labeled DNA for NMR studies that is as easy as that for isotope labeling of RNA. The method was used to synthesize a uniformly ^{13}C,^{15}N-labeled 32-nucleotide DNA that binds to human basic fibroblast growth factor with high affinity and specificity. Isotope-edited experiments were applied to the ^{13}C,^{15}N-labeled DNA bound to unlabeled protein, and the ^{13}C,^{15}N-labeled DNA was also examined in complex with ^{15}N-labeled protein. The NMR experiments show that the DNA adopts a well-defined stable structure when bound to the protein, and illustrate the potential of ^{13}C,^{15}N-labeled DNA for structural studies of DNA–protein complexes.

Introduction

NMR spectroscopy of larger macromolecules and macromolecular complexes relies on the availability of isotope labeling to resolve the crowded proton spectrum. ^{13}C,^{15}N labeling of proteins was introduced many years ago and dramatically changed the way in which protein structures are determined by NMR spectroscopy (Fesik and Zuiderweg; 1990; Clore and Gronenborn, 1994). In recent years, methods have been developed to efficiently synthesize isotope-labeled RNA (Batey et al., 1992,1995; Nikonowicz et al., 1992; Michnicka et al., 1993; Hines et al., 1994). This has tremendously simplified structural studies of RNA and RNA–protein complexes (Battiste et al., 1995; Greenbaum et al., 1995; Hall, 1995; Jucker and Pardi, 1995; Pardi, 1995; Avis et al., 1996; Dieckmann et al., 1996). The availability of isotope-labeled RNA has tremendously facilitated resonance assignments (Kellogg et al., 1992; Pardi and Nikonowicz, 1992; Farmer et al., 1993; Kellogg and Schweitzer, 1993; Nikonowicz and Pardi, 1993; Sklenář et al., 1993; Legault et al., 1994; Marino et al., 1994,1995; Simorre et al., 1995,1996a,b; Wijmenga et al., 1995). In addition, heteronuclear NMR experiments are being designed to access additional structural information from nucleic acids (Schwalbe et al., 1994; Legault et al., 1995; Mueller et al., 1995).

Isotope labeling of DNA has been difficult and only few attempts have been made to uniformly isotope-label DNA (Ono et al., 1994; Zimmer and Crothers, 1995). Chemical synthesis has been achieved, but the multistep process is laborious and requires a certain level of chemical expertise to be successfully completed (Ono et al., 1994; Quant et al., 1994; Tate et al., 1994,1995; Fujiwara et al., 1995). Enzymatic synthesis of DNA oligonucleotides can easily be accomplished with a variety of polymerases, but requires isotope-labeled deoxyribonucleotide triphosphates (dNTPs) as precursors. Isotope-labeled deoxyribonucleotide monophosphates (dNMPs) can readily be isolated from biological materials with procedures similar to those used for obtaining ribonucleotide monophosphates (rNMPs) (Chandrasegaran et al., 1985; Batey et al., 1992; Nikonowicz et al., 1992; Zimmer and Crothers, 1995). However, phosphorylation of dNMPs to dNTPs has been a problematic step in the synthesis of isotope-labeled DNA. It is possible to use chemical phosphorylation methods, but we obtained large variations in

*Present address: Department of Life Sciences, National Yang-Ming University, Shipai, Taipei, Taiwan.
**To whom correspondence should be addressed.

yields (between 30% and 70%) (Ott and Hoard, 1965; Simon et al., 1990). Alternatively, cell extracts have been used to enzymatically phosphorylate the dNMPs (Hurlbert and Furlong, 1967; Zimmer and Crothers, 1995). The kinase activity of such cell extracts was not easily reproducible in our hands and we could not achieve quantitative phosphorylation of the monophosphates to the triphosphates. Due to the variability in yields, we do not find these methods adequate for phosphorylating milligram quantities of ^{13}C,^{15}N-labeled nucleotides. Since DNA is less abundant in the cell than RNA, high yields are required for all steps to make isotope labeling of DNA practical.

In this paper we describe an efficient method to isotope label DNA in milligram quantities. Quantitative phosphorylation of dNMPs to dNTPs is achieved with a mixture of purified enzymes. The method was applied to synthesize a ^{13}C,^{15}N-labeled DNA oligonucleotide that binds with high affinity and specificity to human basic fibroblast growth factor (bFGF).

Materials and Methods

Materials

All chemicals were purchased from JT Baker unless otherwise stated. RNase free DNase I, and P1 nuclease were from Boehringer Mannheim, pyruvate kinase (rabbit muscle), myokinase (chicken muscle), guanylate kinase (porcine brain) and nucleoside-monophosphate kinase were obtained from Sigma. The 3'-5' exo$^-$ Klenow fragment of DNA polymerase I was purchased from New England BioLabs. Phenylmethylsufonyl fluoride (PMSF), aprotinin, leupeptin and pepstatin were obtained from Sigma. DNA oligonucleotides were acquired from Operon. Thin-layer chromatography was carried out on polyethyleneimine cellulose coated plates (Aldrich). The *E. coli* strain expressing deoxythymidylate kinase can be obtained by contacting the authors by e-mail at fiona@shaman.nexstar.com.

Cell growth and isolation of isotope-enriched nucleic acid

These methods are analogous to published procedures (Batey et al., 1992; Nikonowicz et al., 1992; Zimmer and Crothers, 1995). A wild-type strain of *E. coli*, MY285, was grown on M9 minimal media (Sambrook et al., 1989) containing 0.2% ^{13}C-labeled glucose (99% isotope-enriched, Cambridge Isotope Laboratories) and 5 mM 98% ^{15}N-enriched ammonium sulfate (Aldrich Chemical) as the only carbon and nitrogen sources in the media. The ^{13}C, ^{15}N-labeled nucleic acids were isolated from the cells by phenol extraction as described previously (Batey et al., 1992; Zimmer and Crothers, 1995). The nucleic acids were degraded to the monophosphates at ~1.2 mg/ml in 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ and incubated with RNase free DNase I (2.7 U/mg nucleic acid) at 37 °C for 4 h. The buffer was then adjusted to 50 mM sodium acetate pH 5.2, 0.1 mM $ZnCl_2$ and incubated overnight at 55 °C with P1 nuclease (0.5 U/mg nucleic acid). The progress of degradation was monitored by thin-layer chromatography (Nikonowicz, 1992), and the products were analyzed by reverse-phase HPLC as described below.

Reverse phase HPLC analysis

Analytical reverse-phase HPLC was used to monitor (i) the enzymatic degradation of polymeric nucleic acid to the 5' monophosphate nucleotides; (ii) the separation of dNMPs from rNMPs; and (iii) the phosphorylation of dNMPs to dNTPs. The reactions were analyzed on a Vydac C_{18} column (Alltech) and separated with a gradient from 98 mM triethylammonium acetate pH 6.5, 2% methanol to 70 mM triethylammonium acetate pH 6.5, 30% methanol over 61 min with a flow rate of 0.5 ml/min.

Separation of deoxyribonucleotides from ribonucleotides

Deoxyribonucleotides were separated from ribonucleotides on an Affigel 601 (Biorad) *cis*-boronate affinity column as described previously (Rosenberg et al., 1972; Batey et al., 1992; Nikonowicz et al., 1992; Zimmer and Crothers, 1995). The column was equilibrated in 1 M triethylammonium bicarbonate (TEABC) pH 8.0. The mixture of 5' monophosphate ribo- and deoxyribonucleotides was lyophilized and dissolved in 1 M TEABC before it was applied to the column. The column was washed with 1 M TEABC and the dNMPs were collected in the flow-through. The rNMPs were eluted with acidified H_2O (pH ≈ 4.5) as described previously (Batey et al., 1992; Nikonowicz et al., 1992). TEABC was removed from the dNMP fraction by the addition of 3 volumes of methanol and repeated rotary evaporation in the presence of methanol until no triethylamine could be detected by smell. The dNMP and rNMP fractions were lyophilized, resuspended in 10 mM Tris-HCl pH 7.5, and analyzed by reverse-phase HPLC.

Expression and purification of deoxythymidylate kinase

Yeast deoxythymidylate kinase (TMPK) was expressed in *E. coli* DH 10B as a glutathione-S-transferase fusion protein (GST-TMPK) from a GEX-2 vector (Pharmacia). *E. coli* cells were grown in LB media (Sambrook et al., 1989) containing 100 μg/ml carbenicillin (Sigma). LB/carbenicillin medium (250 ml) was inoculated with 1/10 volume of overnight culture and grown at 37 °C until the A_{600} reached 0.8. Isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 1 mM to induce production of the GST-TMPK fusion protein and the culture was incubated for 4 h at 37 °C to allow protein expression. The cells were harvested by centrifugation at 5500 × g for 10 min, and all the following steps were carried out on ice or at 4 °C. The cells

were washed in cold phosphate buffer (20 ml of 20 mM NaH_2PO_4 pH 7.0, 150 mM NaCl), resuspended in 20 ml of phosphate buffer containing various protease inhibitors (0.5 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin), and then lysed by sonication (Virtis Virsonic 475). After the addition of 1.0% Triton X-100 (Sigma), the cell lysate was clarified by centrifugation at 12 000 × g for 15 min. Three milliliter of 50% S-linked glutathione/agarose beads (Sigma), previously equilibrated in phosphate buffer, were then added to the clarified cell lysate and incubated overnight at 4 °C. The beads were collected by centrifugation (10 s at 1000 × g) and washed 4 times with phosphate buffer in the presence of protease inhibitors. The bound GST-TMPK protein was eluted from the beads with an equal volume of 15 mM glutathione in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM DTT, 0.05% NP-40 (Sigma) for 30 min, and separated from the beads (15 s centrifugation at 1000 × g). After a second elution, both eluents were combined and extensively dialyzed against 25 mM HEPES pH 7.4, 100 mM NaCl, 2 mM DTT, 1 mM PMSF, 10% glycerol, 0.05% NP-40 at 4 °C. The purity was checked by SDS-PAGE and the yields were determined by Bradford reagent (Sigma). The GST-TMPK was stored at –80 °C.

Enzymatic phosphorylation of dNMPs to dNTPs

Enzymatic phosphorylation was achieved by a method similar to that described for ribonucleotides, using phosphoenolpyruvate as a phosphate donor to regenerate rATP (Simon et al., 1990; Batey et al., 1992; Nikonowicz et al., 1992). The deoxyribonucleotides were converted to their triphosphates in a one-pot reaction mixture containing all four dNMPs at 1.2 mM in 80 mM Tris-HCl pH 7.5, 20 mM KCl, 20 mM $MgCl_2$, 0.5 mM rATP and 10 mM of phosphoenolpyruvate. The pH was adjusted to 6.9 with KOH prior to the addition of the enzymes. Pyruvate kinase (10 U), myokinase (3 U), guanylate kinase (0.02 U), nucleoside-monophosphate kinase (0.05 U) and GST-TMPK fusion protein (3 μg) were added per mg of dNMPs. The reaction was incubated at 37 °C for 6–8 h and analyzed by reverse-phase HPLC. The triphosphates were concentrated ~5 fold by rotary evaporation and stored at pH 6.5 at –80 °C.

Enzymatic synthesis of DNA oligonucleotides

The 3'-5' exo$^-$ Klenow fragment of DNA polymerase I was used to synthesize a 32-nucleotide DNA oligonucleotide (5'GCGGGGCTATGTAAATTACTGCTGTACTACGC) from a template consisting of a primer and a coding strand. An 18-nucleotide DNA primer (5'TAATACGACTCACTATArG), with a ribonucleotide at the 3' end, was annealed to a 50-nucleotide coding strand complementary to the 32-nucleotide DNA on the 5' end, and complementary to the DNA primer on the 3' end. A 150 μM template was formed in 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ by heating the two strands at 90 °C, followed by equilibrating at 37 °C. Enzymatic DNA synthesis was carried out with the 3'-5' exo$^-$ Klenow fragment of DNA polymerase I (40 U/mg dNTPs) from a 25 μM template and 1.3 mM ^{13}C,^{15}N-labeled dNTPs for 1 h as described previously (Zimmer and Crothers, 1995). The mixture was diluted 10-fold and brought to 0.3 M KOH and incubated at 70 °C for 1 h to hydrolyze the ribo-linkage between the primer and the desired isotope-labeled DNA product (Zimmer and Crothers, 1995). The reaction was neutralized with HCl, brought to 20 mM EDTA, ethanol precipitated, and the pellet was dissolved in 10 mM Tris-HCl pH 7.5. At this point the unincorporated dNTPs can be recovered for use in another Klenow reaction. In this case the mixture is subjected to dialysis in a Filtron 1K Centrifugal Concentrator (Filtron) and the unincorporated dNTPs are collected in the flow-through (Zimmer and Crothers, 1995). It is recommended that the recovered dNTPs be analyzed by HPLC, and either re-phosphorylated or directly used to supplement another Klenow reaction. The oligonucleotides (template, primer and ^{13}C,^{15}N-labeled oligonucleotide) were separated on a 15% denaturing polyacrylamide gel and the ^{13}C,^{15}N-labeled DNA was excised and eluted with an Elutrap Electro-separation apparatus (Schleicher and Schuell). The ^{13}C,^{15}N DNA was purified on a Sep-pack C-18 cartridge (Waters) and extensively dialyzed against 10 mM KH_2PO_4 pH 6.5, 150 mM NaCl, 0.05 mM EDTA, 10 mM 98% deuterated DTT (Isotec), 10 μM sodium azide, 10% D_2O in a Filtron 1K Centrifugal Concentrator (Filtron).

Small-scale Klenow extensions (40 μl reactions) were carried out using a mixture of commercial dNTPs and a trace amount of α ^{32}P-labeled dCTP. Identical conditions were used as described above, with the exception that the dNTP concentration was 0.2 mM containing the following ratio of individual nucleotides: 28% dGTP, 22% dCTP, 22% dATP, 28% dTTP. In addition, 0.3% (mol/mol) α ^{32}P-labeled dCTP was added to the reaction. Alkaline hydrolysis was carried out as described above, and the volume was reduced in a MicroCon 10 concentrator (Amicon) before the reaction products were analyzed on a denaturing polyacrylamide gel. The distribution of radioactivity in the reaction products was determined on a phosphor imager (Fuji).

Expression and purification of human basic fibroblast growth factor

Human basic fibroblast growth factor (bFGF) was produced in *E. coli* BL 21. The protein was expressed with a 15-amino acid C-terminal tail containing a thrombin-cleavable histidine tag (G. Waksman, personal communication). The cells were grown at 37 °C in LB for unlabeled protein or in M9 minimal media containing 0.2% glucose and 5 mM 98% ^{15}N-enriched ammonium sulfate (Aldrich Chemical) for ^{15}N-labeled protein (Sam-

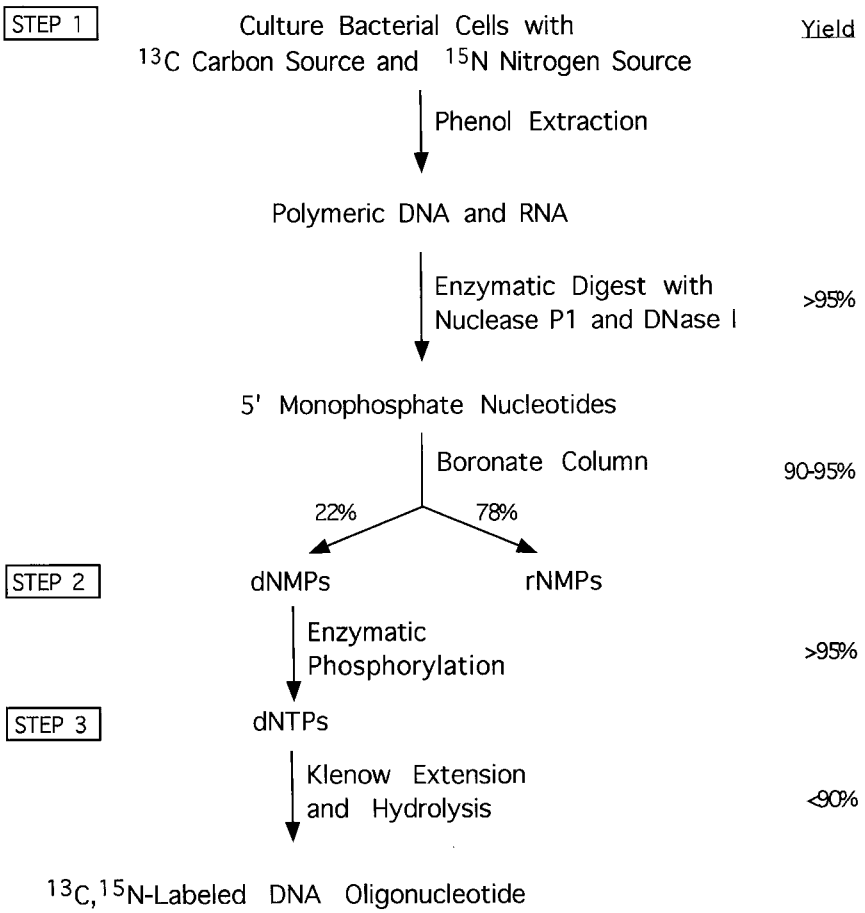

Fig. 1. Flow chart illustrating the synthesis of isotope-labeled DNA. The procedure can be divided into three steps: (1) isolation of $^{13}C,^{15}N$-labeled deoxyribonucleotides; (2) phosphorylation of the deoxyribonucleotides to the triphosphates; and (3) synthesis of $^{13}C,^{15}N$-labeled DNA oligonucleotides of defined sequence. The yields for the individual procedures are given on the right side in % of the expected theoretical yield.

brook et al., 1989). At an A_{600} of ~1.0, the cultures were transferred to 30 °C and protein synthesis was induced with IPTG (Sigma). After 5–6 h the cells were harvested and resuspended in phosphate-based saline buffer (PBS) (Sambrook et al., 1989) in the presence of protease inhibitors. The cells were homogenized and ruptured by sonication with a Virtis Virsonic 475 sonicator and lysozyme was added. The cell debris was removed by centrifugation at 25 000 × g for 15 min. The supernatant was filtered through a Nalgene 0.45 µm cellulose acetate filter. The cleared cell lysate was incubated with nickel-NTA agarose beads (Quiagen) for 2 h at room temperature. The beads were extensively washed with PBS, followed by PBS, 50 mM imidazole, and PBS, 0.4 M NaCl. The protein was eluted in PBS, 0.25 M imidazole, 0.4 M NaCl. The bFGF-containing fractions were pooled, the volume doubled with PBS, 0.4 M NaCl, 10 mM DTT and 400 U of thrombin was added to the protein fraction obtained from 1 l *E. coli* growth. Thrombin cleavage was performed overnight at room temperature, and SDS-PAGE was used to verify that complete cleavage of the histidine tag had occurred. A second purification step was added to remove thrombin and other contaminants. The reaction mixture was diluted to 0.1 M NaCl with 20 mM Tris-HCl pH 7.5, 10 mM DTT, and further purified by FPLC (Pharmacia) over a HiTrap heparin sepharose affinity column (Pharmacia). Thrombin eluted at approximately 0.8 M NaCl and bFGF was eluted with 1.8 M NaCl.

Sample preparation

Two samples were prepared: a 0.2 mM $^{13}C,^{15}N$-labeled DNA with unlabeled bFGF, and a 0.6 mM $^{13}C,^{15}N$-labeled DNA with ^{15}N-labeled bFGF. The $^{13}C,^{15}N$-labeled DNA and bFGF were repeatedly dialyzed against 10 mM KH_2PO_4 pH 6.5, 150 mM NaCl, 0.05 mM EDTA, 10 mM 98% deuterated DTT (Isotec), 10 µM sodium azide, 10% D_2O. The $^{13}C,^{15}N$-labeled DNA was heated to 95 °C and cooled on ice. The $^{13}C,^{15}N$-labeled DNA–bFGF complex was formed by adding small aliquots of bFGF protein to the $^{13}C,^{15}N$-labeled DNA and the volume was reduced to 50 µl in a Filtron 1K Centrifugal Concentrator (Filtron).

NMR spectroscopy

All NMR experiments were performed on a Varian VXR 500 MHz spectrometer using an indirect detection probe equipped with pulse field gradients. Spectra were

collected at 15 and 25 °C. One-dimensional (1D) spectra were collected with a jump-return sequence to suppress the solvent (Hore, 1983). The (^{15}N,^{1}H) HSQC-WATERGATE experiment was collected on a 0.6 mM sample of ^{13}C,^{15}N-labeled DNA and ^{15}N-labeled bFGF, with the ^{15}N carrier at 116 ppm (Bodenhausen and Ruben, 1980; Piotto et al., 1992). The data were processed with FELIX 95.0 (Molecular Simulations) on a silicon graphics extreme workstation.

Results and Discussion

The synthetic scheme used here for isotope labeling of DNA is summarized in Fig. 1. The method is an alternative to that previously published for the enzymatic synthesis of isotope-labeled DNA (Zimmer and Crothers, 1995). In this procedure only purified enzymes are used for dNMP phosphorylation, producing essentially quantitative yields.

The procedures can be divided into three major steps: isolating the ^{13}C,^{15}N-labeled dNMPs from natural sources, phosphorylating the nucleotides to obtain the ^{13}C,^{15}N-labeled dNTPs, and synthesis of polymeric ^{13}C,^{15}N-labeled DNA. The first step can be achieved by a variety of methods (Chandrasegaran et al., 1985; Batey et al., 1992; Hoffman and Holland, 1995; Zimmer and Crothers, 1995). Total nucleic acid can readily be isolated from cells by phenol extraction and ethanol precipitation. This separates the nucleic acids from proteins and other cell debris. If cells were grown to express ^{13}C,^{15}N-labeled protein for structural studies, ^{13}C,^{15}N-labeled nucleic acids can still be isolated by these procedures, as long as no DNase or RNase was added. In this case, the cell debris and the discard products from the first protein purification step can be subjected to phenol extraction in order to obtain the ^{13}C,^{15}N-labeled DNA and RNA.

The phenol-extracted nucleic acids, a mixture of ^{13}C, ^{15}N-labeled DNA and RNA, were enzymatically degraded to the 5' monophosphates, and the dNMPs were separated from the rNMPs (Rosenberg et al., 1972; Batey et al., 1992; Nikonowicz et al., 1992; Zimmer and Crothers, 1995). The isotope-enriched dNMPs are the building blocks for synthesizing ^{13}C,^{15}N-labeled DNA oligonucleotides, whereas the rNMPs can be used for the synthesis of ^{13}C,^{15}N-labeled RNA.

The second step in the procedure is the phosphorylation of the dNMPs to the triphosphates. Phosphorylation of dTMP has been a major stumbling block for the enzymatic synthesis of ^{13}C,^{15}N-labeled DNA. As an alternative to already published methods for enzymatic phosphorylation (Hurlbert and Furlong, 1967; Zimmer and Crothers, 1995), we use yeast deoxythymidylate kinase (TMPK) in a fusion protein with glutathione-S-transferase (GST) to phosphorylate dTMP (Su and Sclafani, 1991). This GST-TMPK fusion protein is expressed at high levels in *E. coli* (16 mg/l, enough to phosphorylate 1.3 g of dTMP), and presents the advantage of an easy one-step purification on glutathione beads. The TMPK enzyme possesses a dual activity, carrying out the phosphorylation of both monophosphate and diphosphate nucleotides in vitro. As shown in Fig. 2A, dTMP can be quantitatively converted to dTTP with GST-TMPK.

For our application it was not necessary to separate the mixture of deoxyribonucleotides into the individual bases. Therefore, the phosphorylation of the deoxyribonucleotides was achieved in a one-pot reaction, containing phosphoenolpyruvate to regenerate the phosphate donor rATP. In addition to the enzymes routinely used to phosphorylate rNMPs (Simon et al., 1990; Batey et al., 1992; Nikonowicz et al., 1992), GST-TMPK was added to allow the reaction to go to completion for all four deoxyribonucleotides (Fig. 2B). This reaction resulted in >95% yields and was significantly simpler to perform than chemical phosphorylation methods or enzymatic methods using cell extracts. The use of purified enzymes helps to ensure reproducibility and high yields, and makes the synthesis

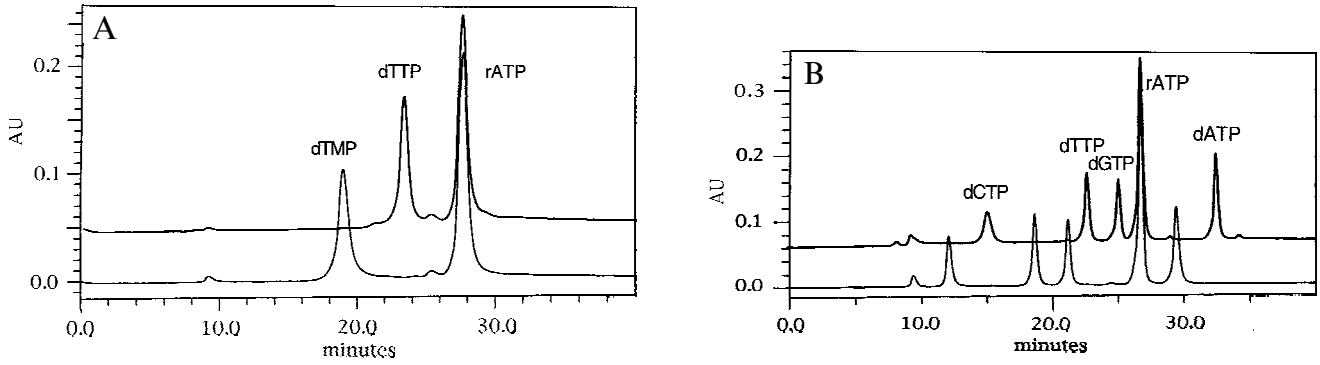

Fig. 2. Phosphorylation of the dNMPs to dNTPs as monitored by reverse-phase HPLC. (A) Phosphorylation of dTMP to dTTP with GST-TMPK. The reaction mixture was injected before (lower trace) and after enzymatic phosphorylation (upper trace). The HPLC trace shows that quantitative phosphorylation of dTMP to dTTP was achieved with GST-TMPK. (B) One-pot phosphorylation of dNMPs to dNTPs. ^{13}C,^{15}N-labeled dNMPs are shown before enzymatic phosphorylation (lower trace) and after phosphorylation (upper trace). The HPLC trace shows that phosphorylation of all four ^{13}C,^{15}N-labeled dNMPs to the triphosphates was quantitative.

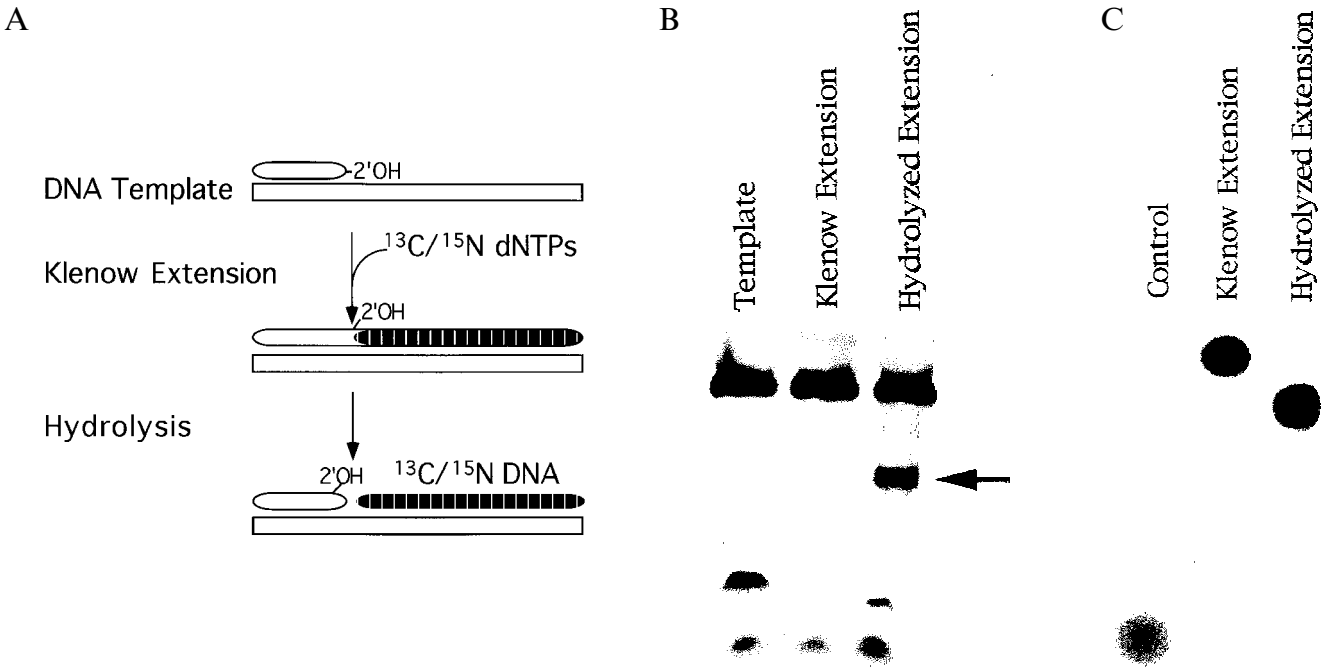

Fig. 3. $^{13}C,^{15}N$-labeled DNA was synthesized with the 3'-5' exo$^-$ Klenow fragment of DNA polymerase I. (A) Schematic representation of the DNA synthesis. The template design includes a DNA primer with a single ribonucleotide at the 3' end. After alkaline hydrolysis the $^{13}C,^{15}N$-labeled DNA product gets released. (B) Denaturing gel electrophoresis of the Klenow extension reaction and subsequent alkaline hydrolysis. The bands were visualized with stains-all (Sigma). Lane 1: Template consisting of the short primer and the longer coding strand, before DNA synthesis. Lane 2: The reaction after extension with the 3'-5' exo$^-$ Klenow fragment of DNA polymerase I. The primer is efficiently extended and only full-length product is visible, overlapping with the coding strand of the template. Lane 3: The same reaction after alkaline hydrolysis. The $^{13}C,^{15}N$-labeled DNA product is indicated by an arrow. The band at the bottom of each lane is due to bromophenol blue dye. (C) Autoradiogram of the Klenow extension and alkaline hydrolysis with commercial dNTPs, spiked with α ^{32}P-labeled dCTP. Lane 1 (control): Reaction without the addition of Klenow enzyme. Only the α ^{32}P-labeled dCTP is observable. Lane 2: Reaction after extension with the 3'-5' exo$^-$ Klenow fragment of DNA polymerase I. The $^{13}C,^{15}N$-labeled DNA-primer product is radiolabeled with ^{32}P. Lane 3: Same reaction after alkaline hydrolysis. Only the cleaved DNA oligonucleotide carries the radioisotope and is observable. The efficiency of the Klenow extension and alkaline hydrolysis become apparent. Each lane contains the same amount of radioactivity, but on film the control lane does not appear as dark as the other lanes, because α ^{32}P-labeled dCTP does not run as a sharp band. Analysis on a phosphor imager revealed that >80% of the radiolabel was incorporated into the desired DNA oligonucleotide.

of isotope-labeled DNA as easy as that of isotope-labeled RNA.

The third step is the enzymatic synthesis of polymeric $^{13}C,^{15}N$-labeled DNA of defined sequence from $^{13}C,^{15}N$-labeled dNTPs (Fig. 3). This was achieved with a system similar to that described by Crothers and co-workers (Zimmer and Crothers, 1995). A two-component template, shown in Fig. 3A, was used in a polymerization reaction with the 3'-5' exo$^-$ Klenow fragment of DNA polymerase I. The primer contained a single ribonucleotide at the 3' end. The bottom strand contained a coding region, complementary to the desired oligonucleotide, and a primer binding region. Synthesis of the complementary sequence from $^{13}C,^{15}N$-labeled dNTPs with the 3'-5' exo$^-$ Klenow fragment of DNA polymerase I yielded a DNA with a single bond that was hydrolyzed under alkaline conditions between the DNA primer and the newly synthesized $^{13}C,^{15}N$-labeled DNA (Fig. 3B).

To evaluate the purity of the $^{13}C,^{15}N$-labeled oligonucleotide, the template and the $^{13}C,^{15}N$-labeled oligonucleotide were 5' end-labeled with ^{32}P and analyzed on a sequencing gel (data not shown). Both showed the same distribution of full-length and N–1 products, and no longer products were detected. Therefore, the purity of the $^{13}C,^{15}N$-labeled product is limited by the purity of the template. In addition, electrospray mass spectrometry (ESMS) was used to analyze the molecular weight of the 99% ^{13}C-, 98% ^{15}N-labeled oligonucleotide. Both full-length and N–1 products were detected by ESMS. The molecular weight of the full-length 99% ^{13}C-, 98% ^{15}N-labeled oligonucleotide is calculated to be 10 282.02 g/mol, and the molecular weight determined by ESMS was 10 276.04 ± 1.95 g/mol, suggesting that isotope enrichment was 99.9% of the expected value.

The efficiency with which dNTPs are utilized to synthesize the DNA oligonucleotides was estimated by the addition of a trace amount (0.3%) of α ^{32}P-labeled dCTP to the Klenow reaction. An autoradiogram of the gel is shown in Fig. 3C. For this experiment the dNTP composition was adjusted to match the nucleotide composition of the DNA oligonucleotide, ensuring that all four nucleotides were equally consumed during the course of the

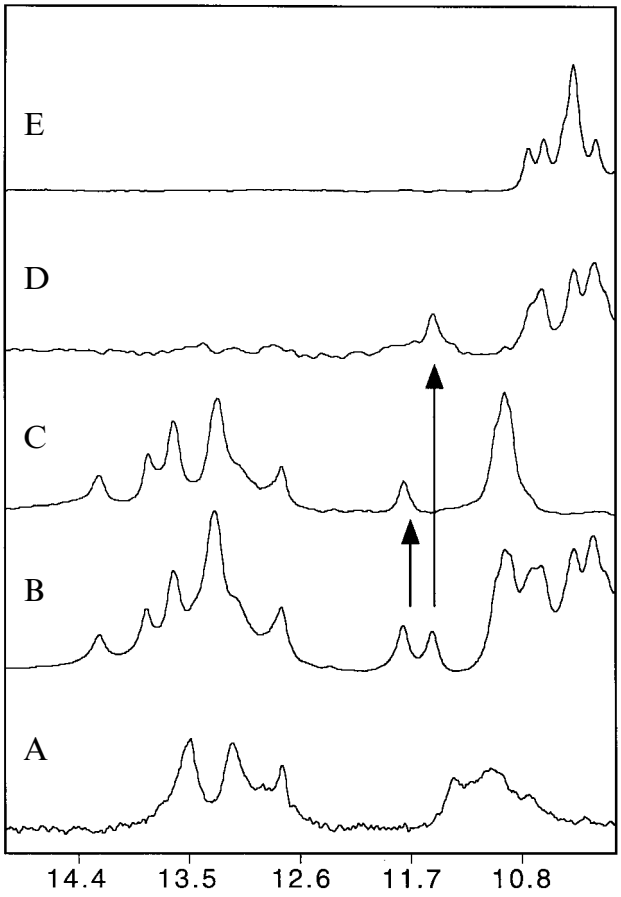

Fig. 4. 1D imino proton spectra of the $^{13}C,^{15}N$-labeled DNA, free and in complex with unlabeled bFGF. Isotope-edited experiments were used to study the $^{13}C,^{15}N$-labeled DNA–ligand complex. (A) Imino proton spectrum of the $^{13}C,^{15}N$-labeled DNA in the absence of protein, (B) same spectral region of the $^{13}C,^{15}N$-labeled DNA–bFGF complex, (C) ^{15}N-selected experiment of the $^{13}C,^{15}N$-labeled DNA–bFGF complex, (D) ^{15}N-filtered experiment of the $^{13}C,^{15}N$-labeled DNA–bFGF complex, and (E) bFGF in the absence of DNA. The arrows point to two resonances, one originating from the $^{13}C,^{15}N$-labeled DNA and the other from the protein.

Klenow reaction. The nucleotide concentration was adjusted for optimal DNA synthesis, yielding exclusively full-length product (Fig. 3C, lane 2). Reducing the nucleotide concentration would result in the synthesis of additional short products (data not shown). Analysis on the phosphor imager revealed that >80% of the radioactivity ended up in the full-length DNA oligonucleotide. After hydrolysis, the radioactivity resided on the desired oligonucleotide, demonstrating that alkaline hydrolysis results in complete release of the labeled oligonucleotide (Fig. 3C, lane 3). This experiment suggests that $^{13}C,^{15}N$-labeled dNTPs are efficiently incorporated into $^{13}C,^{15}N$-labeled DNA oligonucleotides (Fig. 3C).

The method presented here is very efficient. The yields for the steps that lead to $^{13}C,^{15}N$-labeled dNTPs are >90% and are often greater than our detection limits (see Fig. 1). The Klenow reaction is the step with the lowest and most variable yield. The dNTP incorporation can be >80% for optimized conditions. However, in a typical synthesis, the base composition of the oligonucleotide may not mirror the base composition of the dNTP mix-

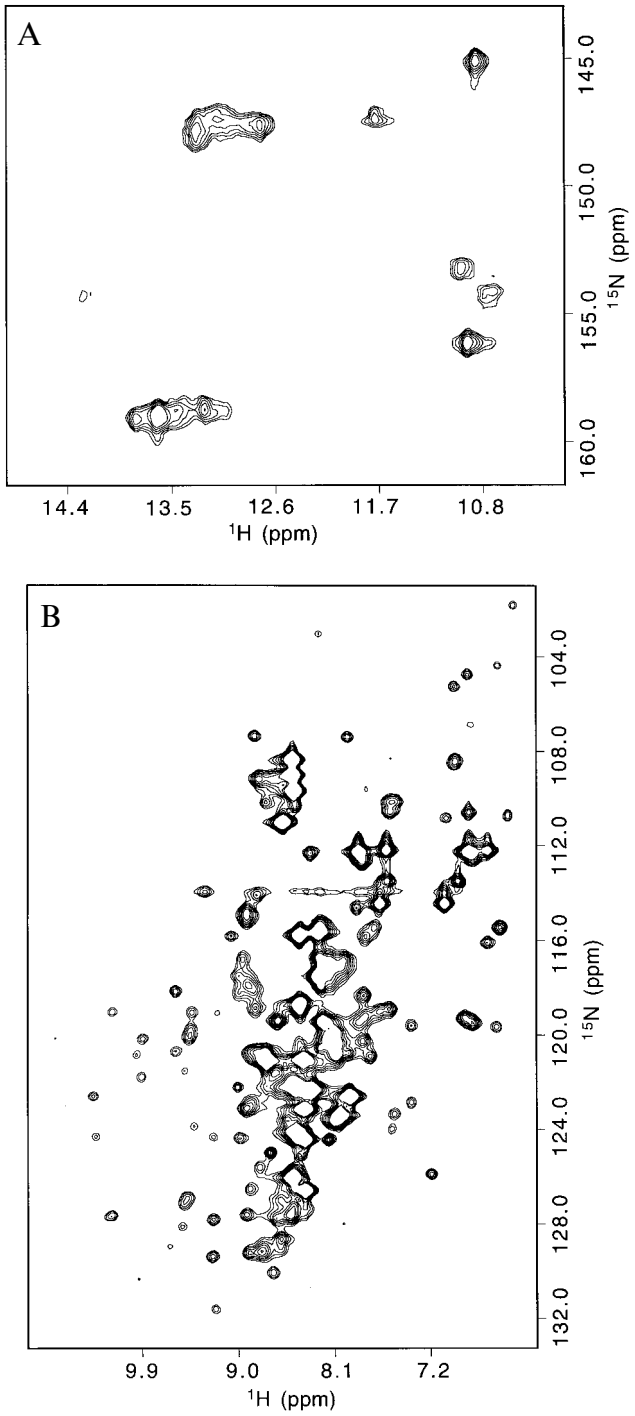

Fig. 5. $^{15}N,^{1}H$ HSQC-WATERGATE spectrum of the $^{13}C,^{15}N$-labeled DNA bound to ^{15}N-labeled bFGF. (A) The imino proton–nitrogen cross peaks of the DNA are displayed. The spectrum indicates the presence of several base pairs and suggests that a well-structured complex is formed between the DNA oligonucleotide and bFGF. (B) The amide backbone region of the $^{15}N,^{1}H$ HSQC-WATERGATE spectrum is displayed.

ture isolated from cells. Depending on the sequence of the oligonucleotide, one nucleotide will become the limiting component for oligonucleotide synthesis and not all four nucleotides will be consumed equally. Therefore, Crothers and co-workers suggested that the unused dNTPs be collected and used to supplement other Klenow reactions (Zimmer and Crothers, 1995).

To illustrate the value of the method, a ^{13}C,^{15}N-labeled DNA oligonucleotide was investigated in a complex with human bFGF. A 32-nucleotide DNA oligonucleotide, isolated by the SELEX methodology (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990), binds to human bFGF with high affinity and specificity ($K_D \sim 5$ nM), and may prove to be a potent inhibitor of angiogenesis (M. Willis, personal communication). We were especially interested in the DNA conformation in the presence of bFGF. Using homonuclear NMR techniques it would have been challenging to study this 27 kDa complex. Therefore, this 32-nucleotide DNA molecule was synthesized with >98% enrichment with ^{13}C and ^{15}N. The activity of the ^{13}C,^{15}N-labeled oligonucleotide was tested by measuring its binding affinity to bFGF by filter binding assay (Jellinek et al., 1993). The binding of the ^{13}C,^{15}N-labeled oligonucleotide to bFGF was similar to that of the chemically synthesized DNA ($K_D \sim 5$ nM, data not shown).

To unambiguously distinguish between the proton resonances from the DNA and those from the protein, a 0.2 mM complex of ^{13}C,^{15}N DNA and unlabeled bFGF was prepared. The imino proton spectrum of the DNA oligonucleotide alone is shown in Fig. 4A. Upon the addition of bFGF, the imino proton region of the spectrum changed dramatically, indicating that the DNA adopts a stable structure when bound to the target protein (Fig. 4B). A ^{15}N-selected experiment shown in Fig. 4C allowed unambiguous identification of the resonances from the ^{13}C,^{15}N-labeled DNA. All but one resonance between 11.2 and 14 ppm appears in this ^{15}N-selected spectrum (Fig. 4B). The ^{15}N-filtered experiment was used to confirm that the one missing resonance indeed originates from the protein (Fig. 4D). These simple isotope-edited 1D experiments illustrate just one of the advantages of using isotope-labeled DNA for the study of DNA–protein complexes (Griffey and Redfield, 1987; Otting and Wüthrich, 1990). The resonances of the DNA can unambiguously be separated from those of the protein and the conformation of the protein-bound DNA can be studied by heteronuclear NMR methods.

A 0.6 mM sample of ^{13}C,^{15}N-labeled DNA bound to ^{15}N-labeled bFGF was prepared and the ^{15}N,^{1}H HSQC spectra for the DNA and the protein are shown in Fig. 5. Both DNA and protein resonances exhibit the ^{15}N,^{1}H chemical shift dispersion expected for a well-structured system. The imino resonances were readily assigned to their base type in the ^{15}N,^{1}H HSQC spectrum, with thymidine imino nitrogens resonating upfield of the guanosine imino nitrogens. Several A-T base pairs and G-C base pairs are present in the DNA–protein complex, in addition to other exchange protected imino protons. These initial experiments demonstrate that the value of heteronuclear NMR techniques, powerfully demonstrated for structure determination of RNAs (Pardi, 1995; Wijmenga et al., 1995; Van Dongen et al., 1996), can now be applied to the study of DNA and DNA–protein complexes.

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