Site-specific ^{15}N-labelling of oligonucleotides for NMR: the *trp* operator and its interaction with the *trp* repressor

Vasudevan Ramesh[a], Yao-Zhong Xu[b], Gordon C.K. Roberts[a,*]

[a]*Department of Biochemistry and Biological NMR Centre, Medical Science Building, University of Leicester, University Road, Leicester, UK*
[b]*Cancer Research Campaign Nitrosamine-induced Cancer Research Group, Department of Biochemistry and Molecular Biology, University College London, London, UK*

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Abstract A convenient and economical method is described for the site-specific ^{15}N-labelling of the 4-amino group of individual cytidine residues in oligonucleotides. This is applied to a 20 base-pair oligonucleotide corresponding to the *trp* operator; this oligonucleotide and its complex with the *E. coli trp* repressor are studied by NMR, using ^{1}H-^{15}N HMQC and ^{15}N-edited ^{1}H-^{1}H NOESY experiments.

Key words: NMR; Stable isotope; Protein–DNA interaction

1. Introduction

The use of stable isotopes (^{2}H, ^{13}C, ^{15}N) incorporated biosynthetically is well established as an invaluable aid to the resolution and assignment of protein NMR spectra [1,2]. By growing *E. coli* cells in appropriate media, it is possible to label the protein either uniformly or selectively with the desired isotope (e.g. [3–6]). Recently, RNA molecules isotopically labeled with ^{13}C and/or ^{15}N have been successfully produced by using labeled nucleotides isolated from ribosomal RNA of *E. coli* cells grown in media containing [^{13}C]glucose and/or $^{15}NH_4Cl$ [7,8]. The availability of such labeled RNAs has made it possible to use NMR for detailed studies of the conformation of functionally important RNA motifs [9,10].

The isotopic labelling of oligodeoxyribonucleotides for NMR still depends on total chemical synthesis [11,12]. A significant advantage of chemical synthesis over biosynthetic methods is that labelling of the oligonucleotide can be achieved in a site specific manner, leading to unambiguous NMR resonance assignments [13–15]. In addition, the specific isotopic label on the oligonucleotide can be very valuable in studies of its interaction with another molecule such as protein or drug by using isotope-edited NOE experiments [16]. Although chemical methods have been available for a number of years [17], they have not found general and routine application due to the complexity of the chemical synthesis.

In view of the high costs involved in the preparation of sufficient quantities (~5 mg) of pure DNA oligomer, in addition to the expensive isotope used for labelling, it is necessary to develop an economical route to the synthesis of isotopically labelled DNA oligomers. In this communication we report the application of a simple procedure [18,19] for introducing ^{15}N isotopic labels at the 4-position of any specified cytosine. The synthetic approach depends on the fact that it is relatively easy to introduce a leaving group such as the triazolo group onto the 4-position of pyrimidines and subsequently replace it with ammonia. While this paper was in preparation, a similar chemical approach to ^{15}N labelling was reported by Acedo et al. [15].

We have used this method to ^{15}N label the exocyclic amino nitrogen (N^4) of cytidine C(−6) in a 20 bp oligodeoxyribonucleotide corresponding to a consensus *trp* operator (the numbering starts at the centre of the palindromic sequence, with bases in the 5′ direction being given negative numbers):

d 5′-CGAACTAGTTAACTAGTTCG-3′

We chose to introduce the label at this position because the complementary base G6 plays a role in the specificity of the interaction between this operator sequence and the *trp* repressor [20], forming a water-mediated hydrogen bond to the protein as illustrated in Fig. 1.

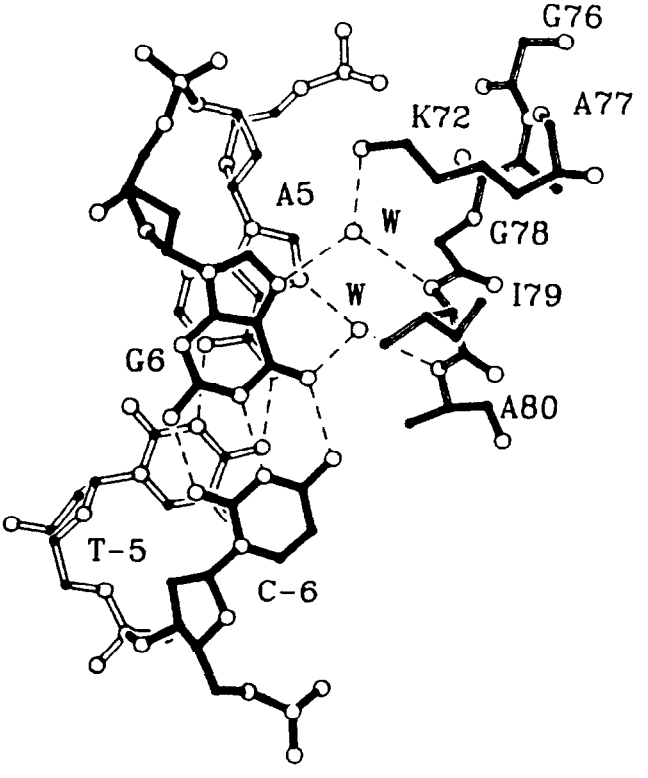

Fig. 1. The environment of the G(6)–C(−6) base pair of the *trp* operator in its complex with the *trp* repressor, as seen in the crystal structure of the complex [20].

*Corresponding author. Fax: (44) (116) 525526.

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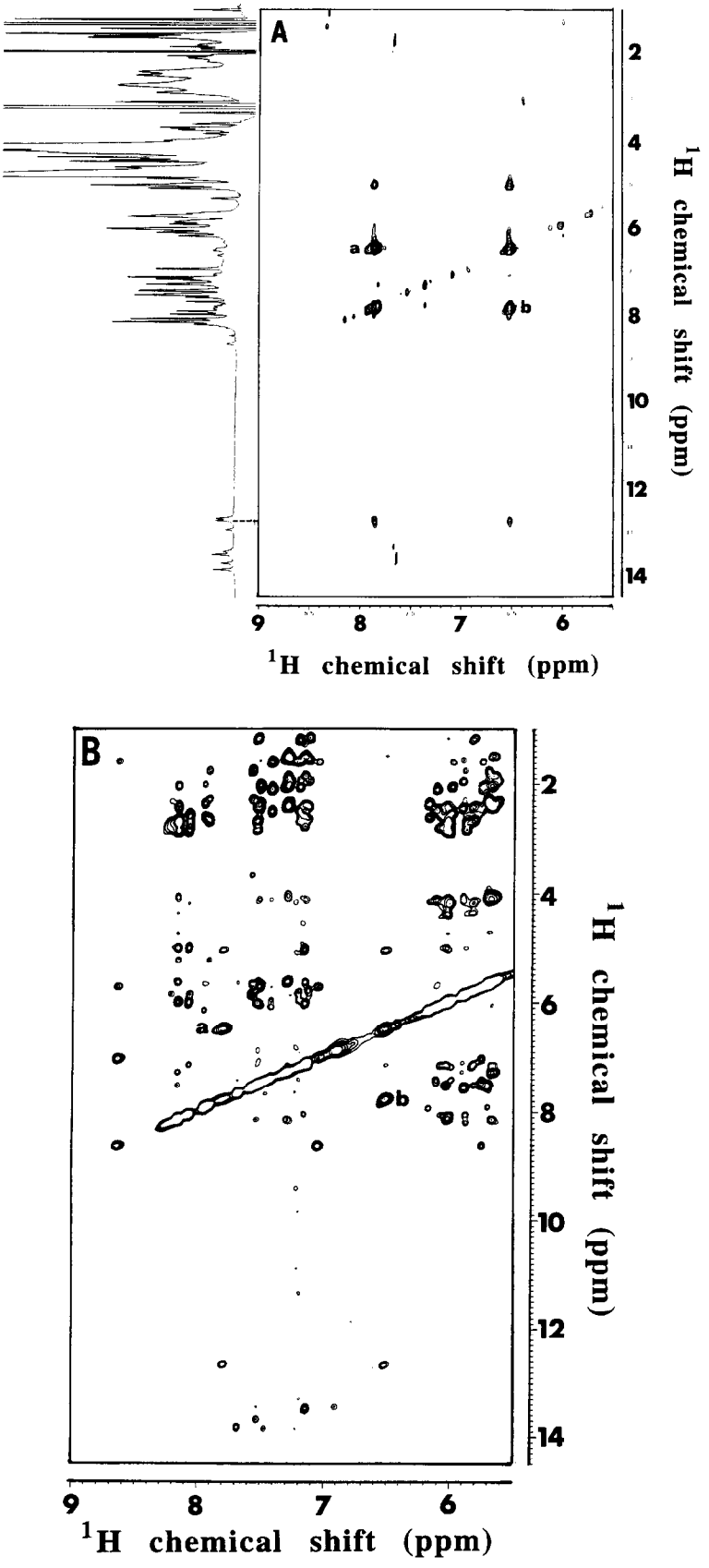

Fig. 2. (A) ^{15}N-edited ^{1}H-^{1}H NOESY spectrum (mixing time 100 ms) of specifically ^{15}N-labelled DNA in 90% H_2O + 10% ^2H_2O, recorded at a ^{1}H frequency of 600 MHz, and a sample temperature of 300K. The one-dimensional ^{1}H spectrum is shown along the F1 dimension for reference. (B) Conventional ^{1}H-^{1}H NOESY spectrum of unlabelled DNA recorded under the same experimental conditions.

2. Materials and methods

2.1. DNA synthesis and purification

Synthesis of the oligodeoxynucleotide containing ^{15}N-labeled cytosine was carried out using a modification of the method previously described [18]. In brief, the oligonucleotide was synthesized on an 8.5 micromol scale using an automated DNA synthesizer (Applied Biosystems 391), using Expedite amidites of the normal bases and Expedite guanine CPG-support (from Millipore). The portion of the oligonucleotide 3′ to the modified cytosine was synthesized on the machine, then a versatile monomer 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-triazolo-2′-deoxyuridine-3′-*O*-(2-cyanoethyl-*N,N*-diisopropyl)-phosphoramidite was added manually as follows. 50 mg of the required monomer (synthesized in our laboratory or purchased from Glen Research, USA) was dissolved in 0.5 ml of anhydrous CH_3CN and 0.5 ml of 0.5 M tetrazole in anhydrous CH_3CN added. The bottom end of the cartridge containing the support with the partially synthesized oligomer attached was disconnected from the machine and the solution of monomer and tetrazole injected from a gas tight syringe. The syringe was used to draw the solution in and out of the cartridge several times over a period of 5 min, and the cartridge was then immediately reconnected to the synthesizer and the synthesis completed.

After synthesis, the CPG-support bearing the synthetic oligomer was transferred to a tube, to which 2 ml of 6 M ^{15}N-ammonia (ISOTEC Inc., USA) was added. The tube was tightly sealed, occasionally shaken and left for 36 h at 25°C to cleave the oligomer from the support, remove all protecting groups and to substitute the 4-triazolo function with $^{15}NH_3$ to produce the desired (N^4-^{15}N)-cytosine at position −6. Reaction at this moderate temperature minimises the possibility of hydrolysis of the 4-triazolo group to yield uracil.

The product was purified by using a Nensorb (DuPont) nucleic acid purification cartridge (8 columns) according to the manufacturers protocol. The resultant oligomer was checked by FPLC [19]; this chromatography can clearly distinguish the oligomer containing converted base (i.e. ^{15}N-cytosine) from that containing hydrolysed base (i.e. uracil). The latter was the most probable impurity, but was not detected in the chromatograph. About 500 OD units of pure oligonucleotide were obtained, corresponding to a 20% isolated yield based on the first nucleoside attached to the CPG support.

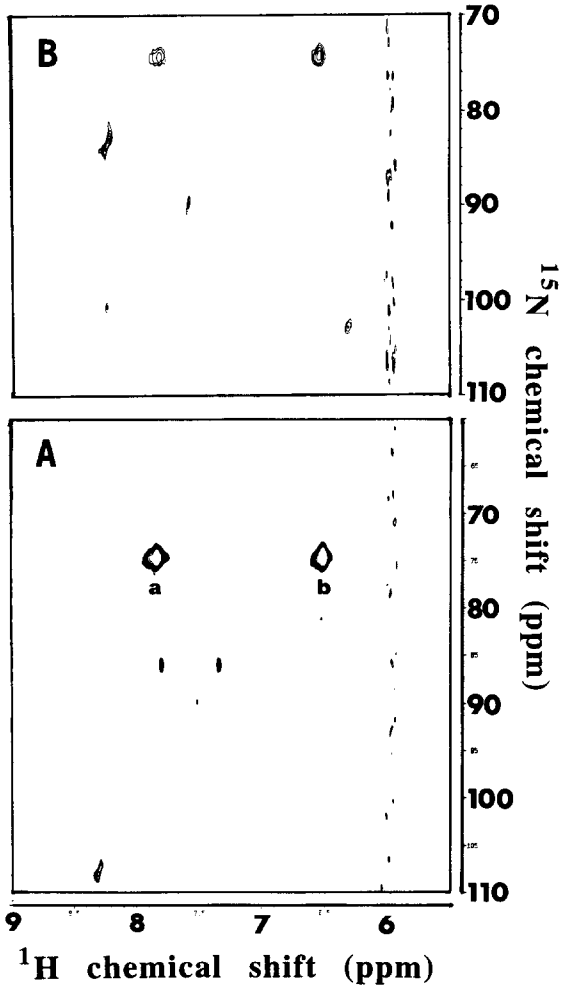

Fig. 3. ^{15}N-^{1}H correlated HMQC spectra, recorded at a sample temperature of 300K. (A) Free ^{15}N-labelled oligonucleotide; (B) ^{15}N-labelled oligonucleotide in its 1 : 1 complex with *E. coli trp* repressor.

2.2. NMR sample preparation

Prior to NMR experiments, the labeled oligonucleotide was heated to 85°C for about 5 min and then very slowly cooled down to about 5°C (overnight) to ensure duplex formation. The sample contained 2 mM ^{15}N-labeled oligonucleotide, 20 mM sodium phosphate, 50 mM NaCl, pH 6.4, in 90% H_2O/10% ^2H_2O. *E. coli trp* repressor (isolated and purified as described previously [5]) was added to give a 1 : 1 complex.

2.3. NMR spectroscopy

NMR spectra were obtained using a Bruker AMX 600 spectrometer, operating at a ^{1}H frequency of 600 MHz. Spectra were measured at a sample temperature of 300K, using a spectral width of 13.2 kHz and with the carrier frequency on the water resonance. The intense signal from the water protons was minimised either by presaturation or by using the 'jump and return' pulse sequence [21] with a delay of 50 μs. Phase sensitive NOESY spectra were recorded with 2K complex points (t_2) and 256 time increments (t_1), each FID being averaged over 128 transients.

Two dimensional ^{15}N-^{1}H HMQC spectra [22] were measured with the double-quantum delay set to the calculated value of $1/2J = 5.6$ ms in the case of the free oligonucleotide, but optimised to a lower value of 4.2 ms in the case of the oligonucleotide-protein complex. The GARP pulse sequence was employed for ^{15}N-decoupling. The ^{15}N-edited ^{1}H-^{1}H NOESY experiment was implemented as described previously [23]. Proton and nitrogen chemical shifts of the spectra were calibrated with reference to the signals of dioxane and ammonium nitrate, serving as internal and external standards, respectively.

3. Results and discussion

The proton-decoupled ^{15}N-spectrum of the labeled oligonucleotide shows only a single peak at 74.72 ppm (data not shown), confirming the specificity of the ^{15}N labelling. The ^{15}N-edited ^{1}H-^{1}H NOESY spectrum of the site-specifically ^{15}N-labeled oligonucleotide is compared with the normal NOESY spectrum of the unlabelled material in Fig. 2 (spectra A and B, respectively). The considerable spectral simplification achieved by the labelling is evident from this comparison.

In the spectrum of the labeled oligonucleotide (Fig. 2A), the signals at 7.86 and 6.52 ppm (labeled a and b) correspond to the two 4-amino protons of the uniquely ^{15}N-labeled cytosine C(-6). These resonances can be individually assigned on the basis of their NOEs. In addition to the strong NOE between the two amino protons themselves, there is an NOE cross-peak between resonance b and a signal at 5.06 ppm, which can be assigned to H5 of the cytidine base. The characteristic proton chemical shifts of oligodeoxyribonucleotides in the B-DNA type conformation [24,25] suggest that the lower-field resonance, a, is likely to arise from the amino proton which is involved in the Watson-Crick hydrogen-bond. This is confirmed by the observation of an NOE cross-peak between resonance a and an imino proton resonance at 12.65 ppm, which must arise from the imino proton of G(6).

The ^{15}N-^{1}H HMQC spectra of the labeled oligonucleotide in its free state (A) and when bound to the *trp* repressor (B) are

shown in Fig. 3. There is no significant change in ^{1}H or ^{15}N chemical shift of this amino group on binding to the protein. However, the resonance linewidths are substantially increased, with the hydrogen-bonded proton (a, 7.86 ppm) showing a greater line-broadening than the non-hydrogen bonded one (b, 6.52 ppm).

This *selective* broadening cannot arise simply from the increase in overall rotational correlation time of the molecule on complex formation. This has a parallel in the line-broadening observed for the backbone peptide NH protons in the spectra of selectively labeled ^{15}N-*trp* R on binding to the operator oligonucleotide [5]. Here the broadening selectively affects amino acid residues in the N-terminal segment and, notably, in the DNA-binding helix-turn-helix domain. These *selective* changes in linewidth imply the existence of some dynamic process(es) within the complex, affecting those residues in each partner which are close to the site of interaction. We are presently carrying out additional labelling experiments to confirm and extend this observation.

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