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# Imino Proton Assignments in the Proton Nuclear Magnetic Resonance Spectrum of the λ Phage $O_R3$ Deoxyribonucleic Acid Fragment[†,1]

Eldon L. Ulrich, Eeva-M. M. John,[‡] Geoffrey R. Gough, Mary J. Brunden, Peter T. Gilham, William M. Westler, and John L. Markley*

ABSTRACT: The 17 base pair duplex d(TATCACCGCAAGGGATAp)·d(TATCCCTTGCGGTGATAp) corresponding to the $O_R3$ operator site of λ phage has been synthesized and studied by $^1H$ nuclear magnetic resonance spectroscopy at 470 MHz. The 13 imino proton resonances observed at 20 °C have been assigned to specific base pairs at positions 3-15 on the basis of nuclear Overhauser effect measurements and studies of the temperature dependence of peak intensities. Resonances from the A-T base pairs at positions 1, 2, 16, and 17 are assumed to be absent from the spectrum because of terminal fraying. Resonances from many of the base pairs suggested by Ohlendorf et al. [Ohlendorf, D. H., Anderson, W. F., Fisher, R. G., Takeda, Y., & Matthews, B. W. (1982) *Nature (London) 298*, 718-723] to be involved in specific binding of the λ phage cro repressor are well resolved.

A λ phage infection of *Escherichia coli* may follow either a lytic or a lysogenic life cycle (Ptashne et al., 1980; Echols, 1980; Herskowitz & Hagen, 1980). The choice depends on a delicate balance between the binding of the λ cI and cro repressors to the phage right-hand operator and several other phage and host factors (Herskowitz & Hagen, 1980; Little & Mount, 1982). Three repressor binding sites exist in the λ right-hand operator, $O_R1$, $O_R2$, and $O_R3$ (Ptashne et al., 1980). The cro repressor (as a dimer) binds preferentially to the $O_R3$ site (Ptashne et al., 1980) and represses transcription of genes from the $P_{rm}$ promoter. Repression at $P_{rm}$ turns off transcription of the cI gene and other λ genes required for a lysogenic life cycle. Transcription from the $P_r$ promoter is allowed and leads to a lytic λ infection. Biophysical studies of this system are of great interest since they may illuminate how specific DNA-protein interactions occur and how they can serve as a switch for the control of biological functions.

The cro repressor structure has been determined by X-ray crystallography (Anderson et al., 1981). Through extensive model building exercises, Ohlendorf et al. (1982) have proposed several DNA-amino acid interactions they believe are involved in the specific binding of the cro repressor to the $O_R3$ DNA site. Their model is consistent with DNA chemical modification experiments (Ptashne et al., 1980; Johnson et al., 1979) and other studies of cro repressor binding to DNA (Boschelli, 1982; Boschelli et al., 1982). Four NMR studies of the cro repressor (Kirpichnikov et al., 1982; Kurochkin & Kirpichnikov, 1982) and its nonspecific binding to DNA have been published (Iwahashi et al., 1982; Arndt et al., 1983). We report here assignments for the imino proton resonances in the $^1H$ NMR spectrum of the λ phage $O_R3$ DNA fragment. A preliminary report of these data has been presented (Ulrich et al., 1983). The assignments were made through an analysis of imino-imino proton nuclear Overhauser enhancement (NOE) experiments, a technique originally developed for studies of tRNA (Roy & Redfield, 1981) and recently applied to duplex DNA molecules (Patel et al., 1982a,b, 1983; Scheek et al., 1983) and the temperature dependence of the imino proton peak intensities.

## Materials and Methods

*Chemical Synthesis of Oligodeoxyribonucleotides.* The two fully protected oligonucleotides d(TATCACCGCAAGGGATA)rC and d(TATCCCTTGCGGTGATA)rC were synthesized by sequential condensations of tetramer and pentamer

[†] From the Departments of Chemistry (E.L.U., E.-M.M.J., W.M.W., and J.L.M.) and Biological Sciences (G.R.G., M.J.B., and P.T.G.), Purdue University, West Lafayette, Indiana 47907. *Received February 2, 1983.* This work was supported by research grants from the National Institutes of Health (RR 01077, GM 11518, and GM 19395).

[‡] Present address: Department of Chemistry, Addis Ababa University, Addis Ababa, Ethiopia.

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phosphotriester blocks in the 3′ to 5′ direction according to the schemes TATC + [ACCG + (CAAGG + GATArC)] and TATC + [CCTT + (GCGGT + GATArC)].

For this purpose fully protected tetradeoxyribonucleotide blocks d(TATC), d(ACCG), d(CCTT), and d(GATA) were each prepared by two successive additions of appropriate phosphodiester barium salts $[(MeO)_2Tr]dN'\text{-}(ClPh)\cdot{}^1/_2Ba^{2+}$ to dinucleotides $dN'\text{-}^{\circ}dN'\text{-}^{\circ}(CNEt)$[2] (Gough et al., 1979b) according to a rapid synthetic procedure previously described (Gough et al., 1982). After purification by silica gel column chromatography, the tetramers were obtained in 67–90% yields. The fully protected pentadeoxyribonucleotides d(CAAGG) and d(GCGGT) were prepared by four additions of mononucleotide barium salts to the phosphotriesters dibG-°-(CNEt) and dT-°-(CNEt), respectively. The overall yields of pure pentamers were 35 and 41%.

In order to minimize the danger of depurination of the ultimate 3′-terminal deoxyadenosine in both oligonucleotides during chain extension (S. H. Gray and P. T. Gilham, unpublished experiments), this residue was protected by 3′ to 5′ phosphate linkage to the ribonucleoside cytidine (Gough et al., 1979a). The fully protected tetramer d(GATA) was decyanoethylated and condensed with $N^4,O^{2'},O^{3'}$-tribenzoylcytidine (2 equiv) in the presence of (toluenesulfonyl)nitrotriazole (4 equiv). The resulting pentamer d(GATA)rC was isolated in 70% yield.

The various blocks were combined in the order defined by the schemes above. With 0.068 mmol of d(GATA)rC at the start in each case, typical condensation reactions contained the hydroxyl component (1 equiv), the phosphodiester component (1.5 equiv), and (toluenesulfonyl)nitrotriazole (3.5 equiv) in pyridine (0.5 mL). After hydrolysis and extraction of the condensing agent (Gough et al., 1982), each fully protected phosphotriester intermediate was isolated by silica gel column chromatography. At the conclusion of the syntheses the two protected octadecamers were obtained in 54 and 63% overall yields, respectively.

Fully protected d(TATCACCGCAAGGGATA)rC was treated with (a) tetramethylguanidinium pyridinealdoximate (1 M in 50% aqueous dioxane, 6.7 mL, 2 days at 25 °C), (b) concentrated $NH_4OH$ (150 mL, 3 days, 25 °C) followed by evaporation to dryness, and (c) 80% acetic acid (150 mL, 25 min, 25 °C). After evaporation of the acid in vacuo, the residue was dissolved in 20% ethanol and neutralized with dilute $NH_4OH$. The resulting solution was dried on cellulose powder (Whatman CF 11, 20 g), which was then washed with ethanol to remove the deprotecting agent. The oligonucleotide was subsequently eluted with 1 M $NH_4OH$ (10 × 100 mL) and partially purified by preparative chromatography on 250 cm of Whatman 3MM paper in the solvent system *n*-PrOH–concentrated $NH_4OH$–$H_2O$ (5:1:4 v/v). The band corresponding to the 18-mer was eluted, and the 3′-terminal cytidine was removed by treatment with $NaIO_4$ (0.2 M, 3.56 mL, 1 h at 0 °C), then with L-methionine (0.2 M, 5.34 mL, 0.5 h at 0 °C), and finally with 1 M cyclohexylamine hydrochloride–0.25 M Hepes, pH 7.5 (15.13 mL, 1.5 h at 45 °C). The resulting solution of crude d(TATCACCGCAAGGGATAp) (I) was dialyzed, and about half of the material was purified by HPLC using a 1 × 25 cm column of cross-linked poly(ethylenimine) on microparticulate silica (Lawson et al., 1983). Elutions were carried out with 400 mL of 0.05 M $KH_2PO_4$ in 30% (v/v) aqueous methanol containing a linear gradient of 0.2–0.4 M $(NH_4)_2SO_4$ (pH 6) at a flow rate of 4 mL/min. The resulting ammonium salt of the pure heptadecamer I (343 $A_{260}$ units) was desalted by dialysis.

The complementary oligomer II, d(TATCCCTTGCGGTGATAp), was deprotected and purified similarly, and the two molecules were characterized and analyzed by methods previously described (Gough et al., 1982). Base ratio (A:C:G:T) analyses gave 6.0:4.0:4.0:3.0 for I and 3.0:4.2:4.0:5.9 for II. Hyperchromicities (25 °C) of 38% and 23% and $\epsilon_{260}$ values (25 °C) of 142 800 and 144 400 were determined for oligomers I and II, respectively. The absorbances were used to combine the two species in equimolar proportions, and the resulting duplex ($T_m$ = 62 °C at 4 μM total strand concentration in 1 M NaCl) was converted to its sodium salt by addition of the appropriate amount of dilute NaOH and isolated by lyophilization.

*NMR Spectroscopy.* The solution used for NMR spectroscopy consisted of 15 mg of duplex at 3 mM concentration in 0.1 M sodium phosphate buffer (pH 7)–1.5 mM EDTA and contained 10% $^2H_2O$ to provide for the NMR field/frequency lock. $^1H$ NMR spectra were obtained with a Nicolet Magnetics 11.3 T spectrometer using the Redfield 214 pulse sequence (Redfield et al., 1975) to suppress the solvent peak. A total of 8192 time-domain data points were collected during an acquisition time of 0.45 s; 1024 time-domain scans were normally averaged. The following procedure was used to determine the parameters used in the Redfield experiment: (i) The reciprocal of the offset of the spectrometer frequency from the water resonance (3950 Hz) was set as the pulse length for a low-power, one-pulse experiment. (ii) The spectrometer power was set to the value required for an on-resonance low-power 90° pulse. (iii) A final value for the total Redfield pulse length was obtained by multiplying the on-resonance, low-power 90° pulse length by $^5/_3$ and then empirically adjusting the total Redfield pulse length until the best suppression of the water signal was observed. The spectrometer frequency was placed in the open region between the A-T and G-C imino proton resonances. For NOE experiments, the decoupler was gated on during the 500-ms relaxation delay between observe pulses. NOE difference spectra were obtained by subtracting the experimental spectrum taken with the decoupler on resonance from the control spectrum with the decoupler frequency set off resonance. Chemical shifts are reported in parts per million (ppm) from internal sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_2$]propionate (TSP).

## Results

The imino proton region of the $^1H$ NMR spectrum of the 17 base pair $O_R3$ DNA fragment is shown in Figure 1. Two sets of peaks are distinguished in the spectrum. In agreement with model studies (Crothers et al., 1973), the low-field peaks (a–e) are assigned to A-T base pairs, and the high-field peaks (j–q) are assigned to G-C base pairs. On the basis of the combined intensity of peaks a and b, which is assumed to represent two protons, the resonance at 13.6 ppm has an intensity of approximately two protons; it is labeled with two letters, c and d. Similarly, the peak at 12.87 ppm with an approximate three-proton intensity is labeled with three letters, k, l, and m.

*Temperature Effects.* $^1H$ NMR spectra of the imino proton region of the $O_R3$ DNA fragment taken over the temperature range 5–68 °C are shown in Figure 2. At 5 °C the A-T peaks have a combined intensity of about seven protons when the

[2] Abbreviations: dN′ represents dT, dbzC, dbzA, or dibG; the symbol -° denotes a *p*-chlorophenyl phosphotriester linkage; EDTA, ethylenediaminetetraacetic acid; NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; ppm, parts per million; TSP, sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_2$]propionate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography.

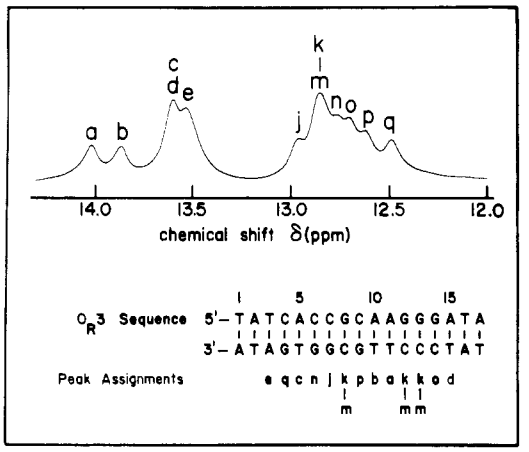


FIGURE 1: The imino proton spectrum at 470 MHz and resonance assignments for the λ phage $O_R3$ DNA fragment. Peaks in the NMR spectrum are arbitrarily labeled a–q. The spectrum was obtained with a sample 3 mM in DNA and 100 mM in sodium phosphate buffer, pH 7 at 20 °C. The sequence of the $O_R3$ DNA fragment is given below the spectrum with the letter designation for each peak aligned with the base pair assigned to that peak.

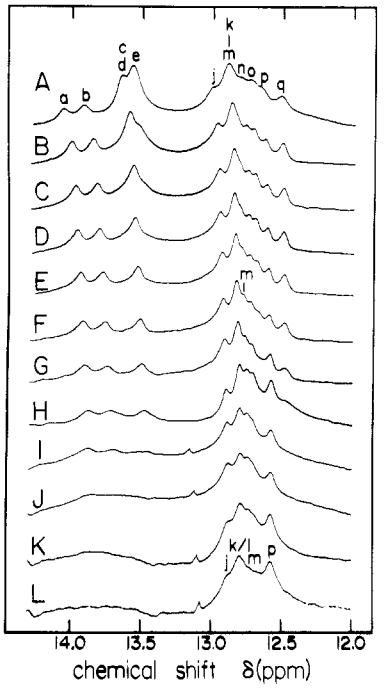


FIGURE 2: Temperature dependence of the imino proton region of the $^1H$ NMR spectra of the λ phage $O_R3$ DNA fragment: (A) 5, (B) 20, (C) 25, (D) 30, (E) 35, (F) 40, (G) 45, (H) 50, (I) 55, (J) 60, (K) 65, and (L) 68 °C.

total G-C peak intensity is defined as eight. Since the fragment contains nine A-T pairs, two apparently are missing from the spectrum. It is assumed that resonances from the A-T pair at each end of the DNA fragment are absent because of fraying of the ends. $^1H$ NMR studies of smaller, helical DNA molecules (Patel et al., 1982a,b) have shown that imino protons of terminal base pairs are in rapid exchange with the bulk water at 5 °C and above, and hence their NMR intensity also averages with that of water. As the temperature is increased, the intensity in the A-T region decreases until only three resonances (a, b, and c) are observed at 30 °C (Figure 2F). Above 30 °C, two G-C imino peaks, o and q, also broaden and decrease in intensity. At 50 °C, peaks o and q are nearly absent, while the three A-T resonances are still present. The sequential loss from the spectrum of four A-T resonances, followed by two G-C peaks, with increasing temperature is

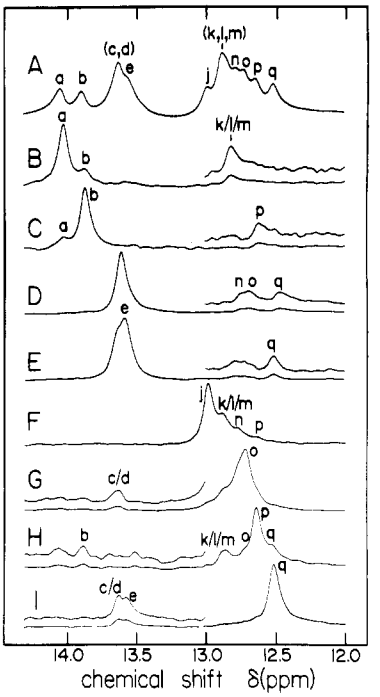


FIGURE 3: NOE difference spectra: (A) normal spectrum of the λ phage $O_R3$ DNA fragment imino protons at 20 °C; (B–I) difference spectra obtained by subtracting the spectrum taken with the irradiating frequency on resonance from the control spectrum taken with the irradiating frequency set off resonance. The largest peak in each difference spectrum was the one irradiated. Peaks in several of the difference spectra do not align exactly with the corresponding resonances in spectrum A because the NOE experiments were carried out at 16 °C. Peak assignments enclosed in parentheses indicate that two or more resonances (according to the letters) are observed at that position. Letters separated by a slash indicate that the peak has a single-proton intensity, which corresponds to one of the assignments listed.

consistent with fraying of the DNA starting from both ends and proceeding toward the middle of the sequence. Thus it appears reasonable to assign the three A-T peaks present at high temperatures (a, b, and c) to the internal A-T base pairs (positions 5, 10, and 11) and the first G-C peaks to disappear (o and q) to positions 4 and 14. The spectrum obtained at 68 °C contains two major resonances (p and k/l/m) plus resonances with reduced intensity (peaks j and m). Assignments of these peaks will be discussed below. All of the imino proton resonances shift slightly to higher field with increasing temperature; G-C peak m shows a relatively large upfield shift with temperature.

*NOE Experiments.* Irradiation of G-C resonance q, which has a single-proton intensity, produces an NOE in A-T peaks c and e (Figure 3I), while no effects are observed in other G-C peaks. We may conclude that the G-C base pair that gives rise to peak q has two A-T nearest neighbors, and since this characteristic is unique to the G-C base pair at position 4, it is assigned to peak q. Peaks c and e must correspond to the neighboring base pairs at positions 3 and 5. Irradiation of A-T peak e creates an NOE in only one G-C resonance, q (Figure 3E); thus peak e is assigned to position 3, which has only one G-C nearest neighbor, rather than to position 5, which has two. The assignment of resonance q to position 4 agrees with the preliminary assignment of peaks o and q to peaks 4 and 14 from the temperature data. Peak o is assigned to position 14 by difference. An NOE is observed in A-T peak d when resonance o is irradiated (Figure 3G); thus peak d is assigned to position 15. At 20 °C the A-T resonance at 13.6 ppm has an intensity of approximately two protons (peaks c and d).

Irradiating these resonances produces an NOE as expected in peaks o and q but also in G-C resonance n (Figure 3D). Since peaks o and q have been assigned to positions 14 and 4 and peak c has been assigned to the A-T at position 5, G-C resonance n is assigned to position 6. Peaks a and b are left incompletely assigned to positions 10 and 11. Resonances p and b show mutual NOEs (Figures 3C and 3H), while one proton in the peak at 12.87 ppm (k/l/m) is linked by an NOE to peak a. The two G-C resonances p and k/l/m are incompletely assigned to positions 9 and 12. The NOE experiment shown in Figure 3F indicates that resonances j and n correspond to neighboring base pairs. Since peak n corresponds to base pair 6 and peak c is already assigned to base pair 5, j can be assigned to position 7. This leaves the resonance at 12.87 ppm assigned to positions 8, 13, and 9 or 12. Further peak assignments are not possible with the NOE data, because the resonances asssigned to positions 8 and 13 overlap.

## Discussion

The NOE data presented in Figure 3 are sufficient to assign peaks c, e, and q to positions 5, 3, and 4, respectively, in the $O_R3$ DNA sequence. A combination of NOE data and temperature effects are required to assign resonances d, j, n, and o to specific positions (Figure 1). The remaining assignments of peaks a, b, k, l, m, and p can be resolved by a second consideration of the temperature data. At 65 °C (Figure 2K) there is little intensity remaining in the A-T region of the spectrum, yet five G-C imino proton peaks are visible. Two of these peaks are assigned to G-C base pairs 6 (peak j) and 7 (peak n), while the other peaks correspond to G-C base pairs at positions 8, 9, 12, or 13. In the spectrum recorded at 68 °C, peaks m and n have decreased greatly in intensity, while peaks j, p, and k or l are still present. Since peak j is assigned to position 7 and the temperature data are consistent with the DNA strand fraying from both ends toward the middle, we believe that G-C base pairs 8 and 9 are the last to dissociate and tentatively assign peak p to position 9 and the peak at 12.87 ppm (k or l) to position 8. Early et al. (1981a,b) and Patel et al. (1983) have shown that A-T imino protons of base pairs located in the interior of DNA duplex strands can exchange more rapidly with the solvent than G-C base pair imino protons located near the termini of the molecule. Therefore, resonance assignments based on the assumption that the base pairs in a DNA molecule fray sequentially from the ends are tenuous. We believe this assumption is justified, however, in assigning peaks p and k or l. The sequential fraying of the $O_R3$ DNA fragment from its termini is supported by the observed order in which imino proton peaks assigned by the NOE experiments broaden as a function of temperature. Further, we are comparing the imino proton exchange rates for two sets of G-C base pairs, not G-C base pairs vs. A-T base pairs. With the assignment of peaks p and k or l, resonances a and b can then be assigned to positions 11 and 10, respectively (parts B and C of Figure 3). Peaks k, l, and m are associated with positions 8, 12, and 13. It is not clear from our data which G-C base pair corresponds to peak m that is resolved only at temperatures above 20 °C.

After this paper was submitted, we learned that similar results had been obtained with a synthetic DNA corresponding to the $O_R3$ DNA sequence by a group at the University of Washington (Chou et al., 1983).

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