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# Minor groove hydration of DNA in aqueous solution: sequence-dependent next neighbor effect of the hydration lifetimes in d(TTAA)$_2$ segments measured by NMR spectroscopy

**Anders Jacobson, Werner Leupin[1,+], Edvards Liepinsh and Gottfried Otting***

Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden and [1]F. Hoffmann-La Roche Ltd, Pharma Research Preclinical Gene Technologies, CH-4070 Basel, Switzerland

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## ABSTRACT

**The hydration in the minor groove of double stranded DNA fragments containing the sequences 5′-dTTAAT, 5′-dTTAAC, 5′-dTTAAA and 5′-dTTAAG was investigated by studying the decanucleotide duplex d(GCATTAATGC)$_2$ and the singly cross-linked decameric duplexes 5′-d(GCATTAACGC)-3′–linker–5′-d(GCGTTAATGC)-3′ and 5′-d(GCCTTAAAGC)-3′–linker–5′-d(GCTTTAAGGC)-3′ by NMR spectroscopy. The linker employed consisted of six ethyleneglycol units. The hydration water was detected by NOEs between water and DNA protons in NOESY and ROESY spectra. NOE-NOESY and ROE-NOESY experiments were used to filter out intense exchange cross-peaks and to observe water–DNA NOEs with sugar 1′ protons. Positive NOESY cross-peaks corresponding to residence times longer than ~0.5 ns were observed for 2H resonances of the central adenine residues in the duplex containing the sequences 5′-dTTAAT and 5′-dTTAAC, but not in the duplex containing the sequences 5′-dTTAAA and 5′-dTTAAG. In all nucleotide sequences studied here, the hydration water in the minor groove is significantly more mobile at both ends of the AT-rich inner segments, as indicated by very weak or negative water–A 2H NOESY cross-peaks. No positive NOESY cross-peaks were detected with the G 1′H and C 1′H resonances, indicating that the minor groove hydration water near GC base pairs is kinetically less restrained than for AT-rich DNA segments. Kinetically stabilized minor groove hydration water was manifested by positive NOESY cross-peaks with both A 2H and 1′H signals of the 5′-dTTAA segment in d(GCATTAATGC)$_2$. More rigid hydration water was detected near T4 in d(GCATTAATGC)$_2$ as compared with 5′-d(GCATTAACGC)-3′–linker–5′-d(GCGTTAATGC)-3′, although the sequences differ only in a single base pair. This illustrates the high sensitivity of water–DNA NOEs towards small conformational differences.**

## INTRODUCTION

NMR spectrocopy offers unique possibilities to study the hydration of DNA in aqueous solution at high resolution. By measuring the NOE between water protons and DNA protons, individual hydration water molecules of the innermost layer of hydration can be detected. The measurements are sensitive to the residence times of the water molecules at their hydration sites in the 0.1–1 ns time regime (1,2). Based on a simple model of cross-relaxation, the sign of the water–DNA cross-peaks observed in NOESY spectra inverts for residence times of ~0.5 ns, while shorter and longer residence times result in negative and positive NOESY cross-peaks respectively (1). Data are now available for several DNA fragments with B-type conformation (3–7) and for non-canonical DNA structures (8). NOE measurements performed with B-DNA indicated that the spine of hydration in the minor groove of AT-rich DNA segments, which had been observed by X-ray crystallography in single crystals (9–11), is characterized by water residence times >1 ns, while at all other hydration sites the water molecules seem to exchange much faster (4,7). A comparison of the dodecamers d(GTGGAATTCCAC)$_2$ and d(GTGGTTAACCAC)$_2$ showed that an ordered spine of hydration with water residence times >0.5 ns is associated with the sequence 5′-dAATT, whereas the sequence 5′-dTTAA kinetically destabilizes hydration in the minor groove.

The study of DNA hydration is limited by the number of non-exchanging DNA protons with which NOEs with the hydration water can be observed. The conclusions on the water residence times in DNA minor grooves have mainly been based on NOEs observed with adenine 2 protons. The water–DNA NOEs with the 1′ desoxyribose protons, which also point into the minor groove of B-type DNA structures, are usually obscured by overlap with very big exchange cross-peaks from the hydroxyl protons from the 3′- and 5′-ends of the DNA fragments. The exchange cross-peaks can be removed by increased temperature or the addition of exchange catalysts, like phosphate or ammonia, which cause coalescence of the signals from the exchanging protons with the water resonance (3). However, under these conditions the exchange rates of the imino protons are also increased (12), which makes it difficult to distinguish direct

* To whom correspondence should be addressed

+Present address: Gymnasium Liestal, Department of Chemistry, CH-4410 Liestal, Switzerland

water–A 2H NOEs from imino proton–A 2H NOEs appearing at the water chemical shift because of rapid exchange between imino and water protons. Here we applied recently developed NMR experiments (13) which enable the observation of water–DNA NOEs with the 1′ protons by separating the hydroxyl proton exchange peaks from the NOEs in the absence of exchange catalysts.

The present study started with the observation of minor groove hydration water with residence times >0.5 ns in the 5′-dTTAA segment of d(GCATTAATGC)$_2$, which shows that a 5′-dTTAA sequence does not automatically infer a kinetic destabilization of the spine of hydration in the minor groove. To investigate the effect of the nucleotides surrounding the 5′-dTTAA segment, the two DNA fragments 5′-d(GCATTAACGC)-3′–linker–5′-d(GCGTTAATGC)-3′ and 5′-d(GCCTTAAAGC)-3′–linker–5′-d(GCTTTAAGGC)-3′ were synthesized, which contain all four possible sequences 5′-dTTAAT, 5′-dTTAAC, 5′-dTTAAG and 5′-dTTAAA. The linker between the two DNA strands consists of six ethyleneglycol units connected to the two DNA strands via a phosphate group: –$PO_2$–O–$(CH_2CH_2O)_6$–$PO_2$–. The use of such a synthetic linker enables one to synthesize the DNA duplex in one single strand, resulting in an accurate 1:1 ratio of the complementary strands and in thermally stabilized duplexes (14,15). It has been demonstrated that the linker employed does not significantly alter the structure of the surrounding stretch of DNA duplex. The three oligonucleotides were used to investigate the sequence dependence of the minor groove hydration. The results indicate that the mobility of the minor groove hydration water molecules, as measured by the sign and size of water–DNA NOEs, reports on small conformational differences with a sensitivity comparable with that of chemical shift measurements.

## MATERIALS AND METHODS

### NMR detection of hydration water

NOEs between the $^1$H NMR signal of the water and the DNA are used to probe the presence of hydration water molecules near the DNA protons. The NOEs give rise to an exchange of magnetization between water and DNA protons which is manifested as cross-peaks in two-dimensional NOESY (16) and ROESY (17) spectra. Positive NOESY cross-peaks are observed if the dipole–dipole interaction lasts for longer than ~500 ps (2; see Discussion). Negative water–DNA NOESY cross-peaks indicate exchange of the hydration water within ~100 ps. The NOE cross-peaks are always negative in ROESY. Cross-peaks due to proton exchange between the water and the DNA are positive both in NOESY and ROESY. Note that a negative NOE leads to a positive NOESY cross-peak and vice versa. In the following we always refer to the sign of the cross-peaks rather than the sign of the NOEs.

### NMR sample preparation

The self-complementary DNA sequence 5′-d($G_1C_2A_3T_4T_5A_6A_7T_8G_9C_{10}$)-3′ and the singly cross-linked DNA sequences 5′-d($G_1C_2A_3T_4T_5A_6A_7C_8G_9C_{10}$)-3′–linker–5′-d($G_{11}C_{12}G_{15}T_{14}T_{15}A_{16}A_{17}T_{18}G_{19}C_{20}$)-3′ (decamer 1) and 5′-d($G_1C_2C_3T_4T_5A_6A_7A_8G_9C_{10}$)-3′–linker–5′-d($G_{11}C_{12}T_{13}T_{14}T_{15}A_{16}A_{17}G_{18}G_{19}C_{20}$)-3′ (decamer 2) were synthesized on a Pharmacia DNA synthesizer. The linker group –$PO_2$–O–$(CH_2CH_2O)_6$–$PO_2$– was introduced as a phosphoramidite building block. It is commercially available from Glen Research (Serling, VA) and was used as supplied. After cleavage of the protecting groups by heating the protected oligonucleotides in concentrated ammonium hydroxide, the samples were purified by anion exchange chromatography on Q-Sepharose columns with a 0.5–2.0 M NaCl gradient. To remove any salts which could catalyze proton exchange, the samples were desalted by repeated dialysis against water and subsequently ultrafiltrated with a 200 mM NaCl solution and finally pure water to remove excess salt. The lyophilized samples were dissolved in a mixture of 90% $H_2O$/10% $D_2O$ and the pH adjusted to 7.0. The final concentration of the samples was 1.2 mM DNA duplex for d(GCATTAATGC)$_2$, 2 mM for decamer 1 and 1.8 mM for decamer 2.

### NMR measurements

All experiments were recorded at 4°C on a Bruker DMX-600 NMR spectrometer equipped with a Q-switch probe head (18). Two-dimensional NOESY, ROESY, NOE-NOESY and ROE-NOESY experiments were recorded for each of the three DNA fragments. NOESY and ROESY spectra were recorded using previously published pulse sequences, where the water signal is suppressed before acquisition by a spin-lock pulse (19,20). In addition, the quality factor of the probe head was switched low during the evolution time and during the NOESY mixing time, to optimize the sensitivity and resolution of the water–DNA cross-peaks (18). The NOE-NOESY and ROE-NOESY pulse schemes were modified from previously published sequences (13).

Figure 1 shows the pulse sequences of the NOE-NOESY and ROE-NOESY experiments. The water is selectively excited by a 90° Q-switched selective pulse (13). The following 90° pulse in the NOE-NOESY sequence (Fig. 1A) converts the water magnetization into longitudinal magnetization. A homospoil pulse is used to suppress radiation damping (21) during the mixing time $\tau_{m1}$. During this mixing time, magnetization is transferred from the water protons to the DNA protons by NOE or chemical exchange. The following pulses represent a conventional NOESY pulse sequence, where the water signal is left unexcited by the jump–return sequence (22) after the mixing time $\tau_{m2}$. This scheme assumes that the water magnetization has largely returned to equilibrium by the end of $\tau_{m2}$. To support the return of the water magnetization by radiation damping, a relatively long mixing time $\tau_{m2}$ is used (200 ms in the present experiments), no homospoil pulse is applied during $\tau_{m2}$ and the phase of the 90° pulse after $t_1$ is shifted by 45° with respect to the phase of the 90° pulse before $t_1$ (23). In the ROE-NOESY experiment, the water magnetization is spin-locked by the spin-lock pulse together with the flanking 90° pulses (24) for the duration of the mixing time $\tau_{m1}$ during which magnetization is transferred to the DNA protons by the water–DNA NOEs (Fig. 1B). In both experiments, the Q-factor of the probe head is switched low during $t_1$ to avoid broadening of the water signal in the $F_1$ dimension by radiation damping. The results of the NOE-NOESY and ROE-NOESY experiments are two-dimensional spectra in which the water–DNA NOE and exchange cross-peaks are on the diagonal and off-diagonal peaks arise from a second NOE transfer step to further DNA protons. The off-diagonal peaks aid in the assignment of overlapping direct water–DNA NOE cross-peaks on the diagonal.

In the present work, the NOE-NOESY and ROE-NOESY experiments were primarily used to filter out the large exchange cross-peaks between water and DNA. The chemical exchange rate of the DNA protons involved is sufficiently rapid that the magnetization transferred from the water to the DNA during $\tau_{m1}$

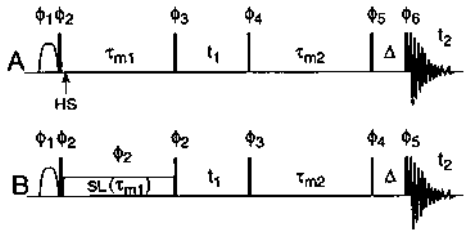


**Figure 1.** Pulse sequences of NOE-NOESY and ROE-NOESY. All pulses are 90° pulses. The first pulse is a shaped Q-switched pulse for selective water excitation with suppression of radiation damping (13). Other than during the Q-switched pulse, the quality factor of the probe head is switched low only during the evolution time $t_1$ and, in the NOE-NOESY experiment, during the mixing time $\tau_{m1}$. The water signal is suppressed by the jump–return sequence with the delay Δ which results in an excitation profile along the $F_2$ frequency axis described by $\sin(2\pi\nu\Delta)$, where ν is the offset from the carrier frequency in Hz. The water suppression relies on radiation damping during the mixing time $\tau_{m2}$ to return the water magnetization back to equilibrium before the jump–return sequence. Therefore, the quality factor of the probe head must be switched high during $\tau_{m2}$ and no homospoil pulse must be used during $\tau_{m2}$. (**A**) NOE-NOESY. HS denotes a homospoil pulse which supports the selection of longitudinal magnetization. Phase cycle: $\phi_1 = 16(x,–x)$; $\phi_2 = 8(x,x,–x,–x)$; $\phi_3 = 4[4(x),4(–x)]$; $\phi_4 = 2[8(x),8(–x)]$; $\phi_5 = 16(x),16(–x)$; $\phi_6 = 16(–x),16(x)$; receiver = x,–x,–x,x,–x,x,x,–x,2(–x,x,x,–x,x,–x,–x,x),x,–x,-x,x,–x,x,x,–x. The radiation damping of the water magnetization during $\tau_{m2}$ is supported by adding a 45° phase shift to the phases $\phi_4$, $\phi_5$ and $\phi_6$ (23). (**B**) ROE-NOESY. SL denotes the spin-lock pulse of the ROE mixing time $\phi_{m1}$. Phase cycle: $\phi_1 = 8(x,–x)$; $\phi_2 = 4(y,y,–y,–y)$; $\phi_3 = 2[4(x),4(–x)]$; $\phi_4 = 8(x),8(–x)$; $\phi_5 = 8(–x),8(x)$; receiver = x,–x,x,–x,–x,x,–x,x,–x,x,–x,x,x,–x,x,–x. The radiation damping of the water signal is supported by adding a 45° phase shift to the phases $\phi_3$, $\phi_4$ and $\phi_5$ (23).

is almost completely transferred back to the water by the end of the second mixing time $\tau_{m2}$. Therefore, the diagonal of the NOE-NOESY and ROE-NOESY experiments is free of big exchange peaks. In the conventional two-dimensional NOESY and ROESY spectra, the exchange cross-peaks with rapidly exchanging DNA protons occur in the same cross-section as the water–DNA NOEs.

The mixing times for the intermolecular water–DNA NOEs were 50 ms in NOESY, ROESY and ROE-NOESY and 100 ms in NOE-NOESY. The second mixing time $\tau_{m2}$ in the NOE-NOESY and ROE-NOESY experiments was set to 200 ms. NOESY and ROESY spectra of each DNA fragment were recorded under identical conditions. Similarly, the NOE-NOESY and ROE-NOESY experiments were recorded with identical parameter settings. The NOE-NOESY spectra were recorded with a shortened phase cycle, including the first eight steps of the phase cycle of Figure 1A. The spin-lock pulse in the ROE-NOESY experiments was applied as a train of 4 µs hard pulses spaced by 16 µs delays (25). The acquisition parameters varied slightly between the different DNA samples: $t_{1max}$ = 53–68 ms, $t_{2max}$ = 164 ms, except for the NOESY and ROESY spectrum of $d(GCATTAATGC)_2$ where $t_{2max}$ = 328 ms. The total experimental times were 20–22 h each for the NOE-NOESY and ROE-NOESY experiments, 30 h for the NOESY and ROESY spectra of $d(GCATTAATGC)_2$ and about 3–6 h for the NOESY and ROESY spectra of the other two DNA fragments. The delay Δ in the jump–return sequence of the NOE-NOESY or ROE-NOESY experiments (Fig. 1) or before the spin-lock purge pulse in the NOESY and ROESY experiments (19) was set to 142 µs, resulting in a spectral excitation function described by $\sin[0.53(\delta - 5.0)]$, where δ is the chemical shift in p.p.m. in the $F_2$ dimension. The maxima of this excitation profile are at 2.1, 7.9 and 13.8 p.p.m. This excitation profile resulted in <30% intensity variation over the spectral range for the signals of the base protons between 6.5 and 8.5 p.p.m. To improve the spectral appearance, baseline corrections were applied in both dimensions after Fourier transformation and the spectral region between 5.0 and 10.8 p.p.m. was inverted to compensate for the sign inversion by the spectral excitation profile.

## RESULTS

Nearly all $^1H$ resonances were assigned for the three DNA sequences using conventional assignment strategies. The assignment of 3′H signals, which are degenerate with the water resonance at 4°C, was confirmed by spectra recorded at 15°C, at which temperature the water resonance was sufficiently shifted to lift the degeneracy. Based on these assignments, the water–DNA cross-peaks were assigned in the NOESY, ROESY, NOE-NOESY and ROE-NOESY cross-sections.

The assignments are available as supplementary material in the on-line version of this article.

### Water–DNA NOEs in NOESY and NOE-NOESY

Figure 2 compares the NOESY cross-section through the water line along the $F_2$ frequency axis with the cross-section taken along the diagonal of the NOE-NOESY experiment for the DNA fragment $d(GCATTAATGC)_2$. Both cross-sections display the water–DNA NOEs. The most intense cross-peaks in the NOESY cross-section (Fig. 2A) are from chemical exchange. They are from the imino proton of the terminal base pair at 13.13 p.p.m., the 5′ and 3′ hydroxyl protons at 6.03 and 6.52 p.p.m. and the labile amino protons of the terminal base pairs at 7.3 and 8.24 p.p.m. These exchange peaks are absent from the diagonal through the NOE-NOESY spectrum (Fig. 2B), because their magnetization exchanges back to the water signal during the second mixing time $\tau_{m2}$ (see Materials and Methods). All intense NOE cross-peaks from the NOESY cross-section of Figure 2A are reproduced in the diagonal cross-section through the NOE-NOESY spectrum (Fig. 2B). Different peak intensities in NOESY and NOE-NOESY are expected, because different protons relax to different extents during the evolution time $t_1$ and the mixing time $\tau_{m2}$ of the NOE-NOESY experiment. Yet, the intensities of the NOEs with the base protons vary by <3-fold and the signs of the cross-peaks are conserved. Most importantly, removal of the biggest exchange cross-peaks enables observation of water–DNA NOEs with the 2H of adenine 6 and the 1′ desoxyribose protons between 5 and 6.5 p.p.m. (Fig. 2B).

The selective pulse used in the NOE-NOESY experiment (Fig. 1) excites not only the water magnetization, but also the DNA resonances at the chemical shift of the water signal. In the DNA fragment $d(GCATTAATGC)_2$, the 3′ protons of G9 and A7 overlap with the water signal. The cross-peaks 3′H–2′H and 3′H–2″H of A7 are identified in the cross-section of Figure 2A. They are positive, like all other intra-DNA cross-peaks. The same peaks are negative or absent in the diagonal cross-section through the NOE-NOESY spectrum (Fig. 2B). Since positive NOESY cross-peaks are expected for all intra-DNA NOEs, this indicates that the selectivity for water–DNA NOEs in the diagonal cross-section through the NOE-NOESY experiment is at least as good as in the cross-section through the NOESY experiment. Similar results were obtained for the ROESY and ROE-NOESY

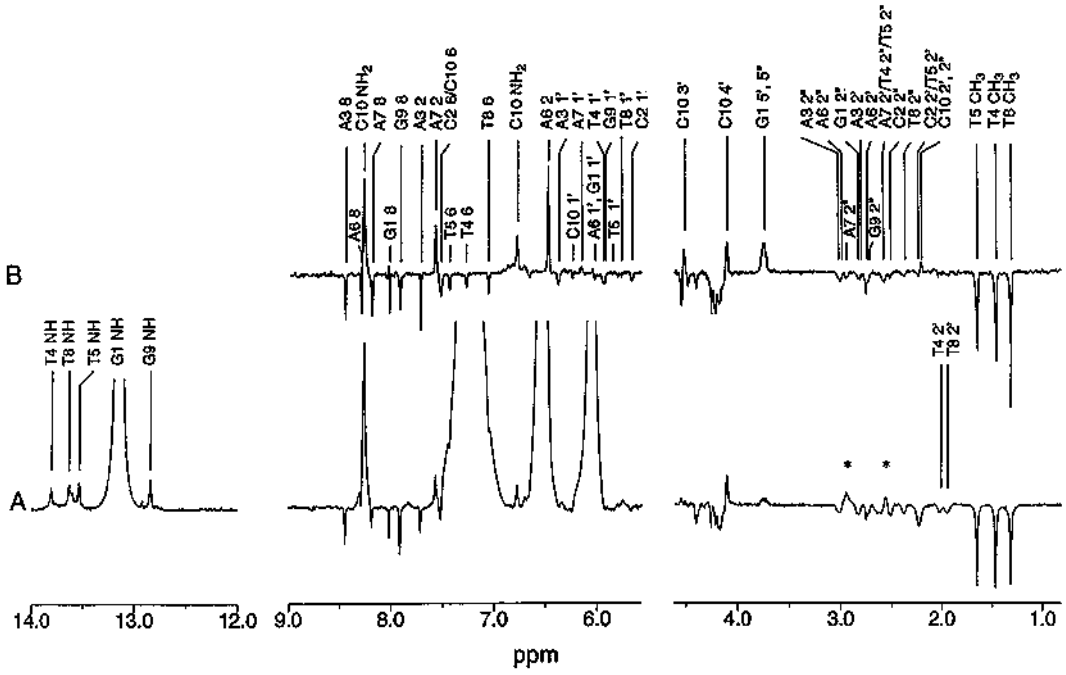


**Figure 2.** $^1$H NMR spectra of the decamer d(GCATTAATGC)$_2$ showing NOEs between protons of the DNA and water protons at 4°C, pH 7.0. (**A**) Cross-section through the NOESY spectrum taken along $F_2$ at the $F_1$ frequency of the water line. The spectrum was recorded with a mixing time of 50 ms and a total experimental time of ~30 h. Asterisks identify two intramolecular DNA cross-peaks which overlap with the cross-section through the water line (see text). (**B**) Plot through the diagonal peaks of the NOE-NOESY spectrum recorded using the pulse sequence of Figure 1A. $\tau_{m1}$ = 100 ms, $\tau_{m2}$ = 200 ms, total experimental time ~22 h. The assignments of the cross-peaks are given at the top.

experiments (data not shown). However, experience with protein samples shows that some resonances which are nearly but not exactly degenerate with the water signal may sometimes be excited by the selective pulse (Liepinsh and Otting, unpublished results) and these signals can be excited with the opposite sign to the water signal. Therefore, some of the negative NOE cross-peaks observed with the base protons could in principle arise from excited 3′ protons via intra-DNA NOEs. Yet, limited selectivity of the water excitation is not likely to interfere with measurement of water–DNA NOEs with A 2H or 1′H resonances, because neither adenine 2 protons nor 1′ protons interact with 3′ protons by sizeable direct NOEs.

The off-diagonal peaks in the NOE-NOESY and ROE-NOESY experiments are most valuable for the assignment of overlapping water–DNA NOEs on the diagonal. Figure 3 shows a selected spectral region showing off-diagonal peaks from the NOE-NOESY and ROE-NOESY spectra of d(GCATTAATGC)$_2$. Except for the peak with C10 OH, they all arise from NOEs between hydration water and 1′ protons during $\tau_{m1}$. The signs of the off-diagonal peaks reflect the signs of the water–DNA NOEs, because the sign of the magnetization is not changed by the intra-DNA NOE transfer during the NOESY mixing time $\tau_{m2}$.

Although there is no signal for the 3′OH proton on the diagonal (Fig. 2B), the intranucleotide NOE peak between the 3′OH proton and the 2′ and 2″ protons of the terminal nucleotide appears in both spectra of Figure 3. The positive sign of the peak in the ROE-NOESY spectrum identifies the interaction between water and C10 3′OH as chemical exchange (Fig. 3A). Generally, the NOE peaks are more intense in the ROE-NOESY than in the NOE-NOESY spectrum, although the mixing time $\tau_{m1}$ used for the transfer of magnetization from the water to the DNA protons was only half as long in the ROE-NOESY than in the NOE-NOESY experiment. This indicates high mobility of the hydration water molecules on the sub-nanosecond timescale (see Discussion).

The exchange peaks observed in the cross-section through the conventional NOESY experiment are important for assessing the possibility that NOEs observed in the cross-section through the water signal are exchange relayed NOEs with the labile DNA protons rather than direct NOEs with hydration water (4). Phosphate and ammonium buffers, for example, catalyze chemical proton exchange (12,26,27). In the absence of proton exchange catalysts, the largest exchange cross-peaks are expected for the terminal base pairs, because they are most exposed to the solvent. The weak exchange peaks of the imino protons of the non-terminal base pairs in Figure 2A show that, at 4°C, the chemical exchange is slow in the central part of the DNA fragment so that exchange relayed NOE cross-peaks would not be expected to be observable.

## Intermolecular NOE cross-peaks between $H_2O$ and d(GCATTAATGC)$_2$

Figure 4 shows the diagonal cross-sections through the NOE-NOESY and ROE-NOESY spectra of d(GCATTAATGC)$_2$. The positive peaks in the ROE-NOESY cross-section (Fig. 4A) are either residual exchange peaks, like the signal of C10 $NH_2$ at 8.24 p.p.m., or from TOCSY relayed exchange peaks, like the signals of G1 5′,5″H and C10 3′H, which arise during the ROE mixing time from a TOCSY transfer with the rapidly exchanging 5′ and 3′ hydroxyl protons. Chemical exchange peaks tend to be more intense in the NOE-NOESY than in the ROE-NOESY cross-sections, partly because of the longer mixing time $\tau_{m1}$ used in the

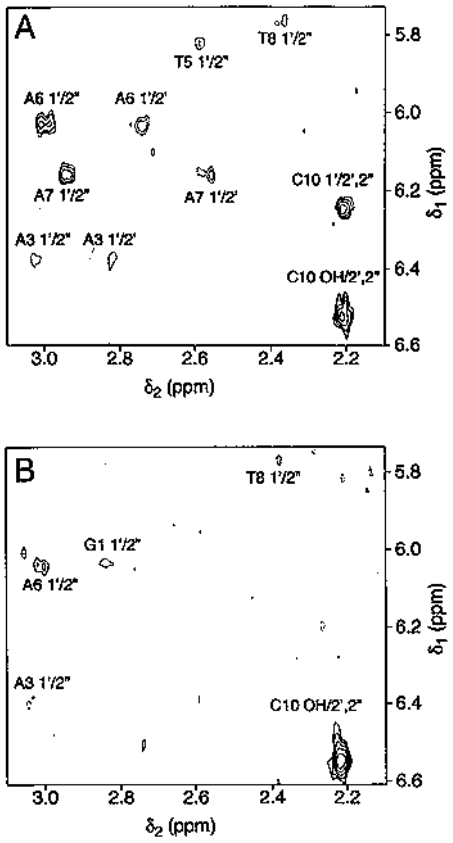


**Figure 3.** Selected spectral region from NOE-NOESY and ROE-NOESY experiments of d(GCATTAATGC)$_2$ showing 1′H–2′H and 1′H–2″H off-diagonal peaks. Negative peaks are plotted with dotted lines. (**A**) Region from the ROE-NOESY spectrum recorded under identical conditions to the NOE-NOESY spectrum of Figure 2B using the pulse sequence of Figure 1B. $\tau_{m1}$ = 50 ms, $\tau_{m2}$ = 200 ms, total experimental time 22 h. (**B**) Same spectral region from the NOE-NOESY spectrum of Figure 2B.

NOE-NOESY experiments and partly because broad exchange peaks relax faster during the ROESY spin-lock. Disregarding the NOEs with protons of the terminal base pairs, which are likely to arise from interactions with rapidly exchanging DNA protons, all negative peaks in the ROE-NOESY spectrum represent direct NOEs with hydration water.

The protons A6 2H, A7 2H and A7 1′H are the only protons which yield positive peaks in the diagonal cross-section through the NOE-NOESY spectrum (Fig. 4B) and which are neither from terminal base pairs nor from labile OH or NH groups. In addition, there is a positive peak with A6 1′H which overlaps with a negative peak from G1 1′H (Fig. 3B) and therefore does not appear in the diagonal cross-section of Figure 4B. The intensity of the off-diagonal peak A7 1′H/2″H is too weak to be seen in Figure 3B, but the A7 1′H resonance appears in the diagonal cross-section of Figure 2B. Only the A6 $NH_2$ protons give a signal at a similar chemical shift, but the very broad line shape of the amino protons excludes the assignment of the relatively narrow peak at 6.13 p.p.m. to any other resonance than A7 1′H.

In a B-DNA type structure, the A 2H and the 1′H protons point into the minor groove of a B-DNA structure. The positive NOESY cross-peaks observed with the minor groove resonances of A6 and A7 indicate the presence of hydration water molecules with residence times >0.5 ns. Therefore, a kinetically stabilized spine of hydration exists in the minor groove of the 5′-dTTAA segment. The lower intensities of the water–1′H NOEs compared with the water–2H NOEs correlate with the longer $^1$H–$^1$H distances observed in single crystal structures between the water protons of the spine of hydration and the 1′ protons than between the water protons and the adenine 2 protons (9). Rapid hydration water exchange on a sub-nanosecond timescale is indicated near those protons for which the NOE-NOESY spectrum shows negative NOE peaks with the water signal. This includes all base and methyl protons pointing towards the major groove of the DNA, but also some minor groove protons, like A3 2H, A3 1′H and T8 1′H (Figs 3B and 4B). The kinetically stabilized spine of hydration is thus strictly confined to the central 5′-dTTAA segment.

## Intermolecular NOE cross-peaks between $H_2O$ and decamer 1 and between $H_2O$ and decamer 2

Like in d(GCATTAATGC)$_2$, many of the water–DNA NOE cross-peaks in the NOESY and ROESY spectra of decamer 1 and decamer 2 were obscured by intense exchange cross-peaks (data not shown). Because of the structure stabilizing effect of the hexaethyleneglycol linker in decamer 1 and decamer 2, the exchange peak of the imino proton of G11 was much weaker than the exchange peak of the imino proton of G1. Only small exchange peaks were observed with the imino protons of all other nucleotides.

NOE-NOESY and ROE-NOESY spectra were recorded to unveil the NOEs with the adenine 2 protons in the minor groove. Figure 5 shows the low field regions of the diagonal cross-sections through the NOE-NOESY and ROE-NOESY spectra of decamers 1 (Fig. 5A and B) and 2 (Fig. 5C and D). All adenine 2 protons give intense negative cross-peaks in the ROE-NOESY experiments (Fig. 5A and C), whereas positive, negative and vanishing NOE intensities are observed for the same protons in the diagonal cross-sections through the corresponding NOE-NOESY spectra (Fig. 5B and D). With few exceptions, all A 2H resonances are resolved. The exceptions are the signals of A7 2H and A17 2H in decamer 1, which partially overlap with the resonance of C10 5H, and the A17 2H resonance in decamer 2, which overlaps with the signals of T5 6H, T15 6H and C20 6H.

The intensities of the water–A 2H NOEs in the NOE-NOESY diagonal cross-sections can be assessed qualitatively by comparison with the intensities of the corresponding NOEs in the ROE-NOESY diagonal cross-section. Quite intense positive NOESY peaks are observed with the signals of A6 2H and A16 2H in decamer 1. The peaks with A17 2H and, more so, A7 2H are reduced in intensity by overlap with the negative peak of C10 5H. The NOEs with A7 2H and A17 2H clearly dominate the NOE with C10 5H in the ROE-NOESY diagonal cross-section (Fig. 5A), while they are less dominant in the NOE-NOESY diagonal cross-section (Fig. 5B). Therefore, rigidly confined hydration water prevails in the minor groove at the T-A step composed of the base pairs containing A6 and A16, but the hydration water near the neighboring base pairs with A7 2H and A17 2H must be more mobile. Further increased mobility of the hydration water towards the beginning of the AT-rich segment of decamer 1 is indicated by the negative NOESY cross-peak of A3 2H, which is the first adenine in the AT segment.

No positive NOESY peak is observed with any of the adenine 2 protons in decamer 2, except for a very weak peak with A7 2H (Fig. 5D). The pronounced negative NOESY peak with the A8 2H resonance and the apparent absence of a sizeable positive

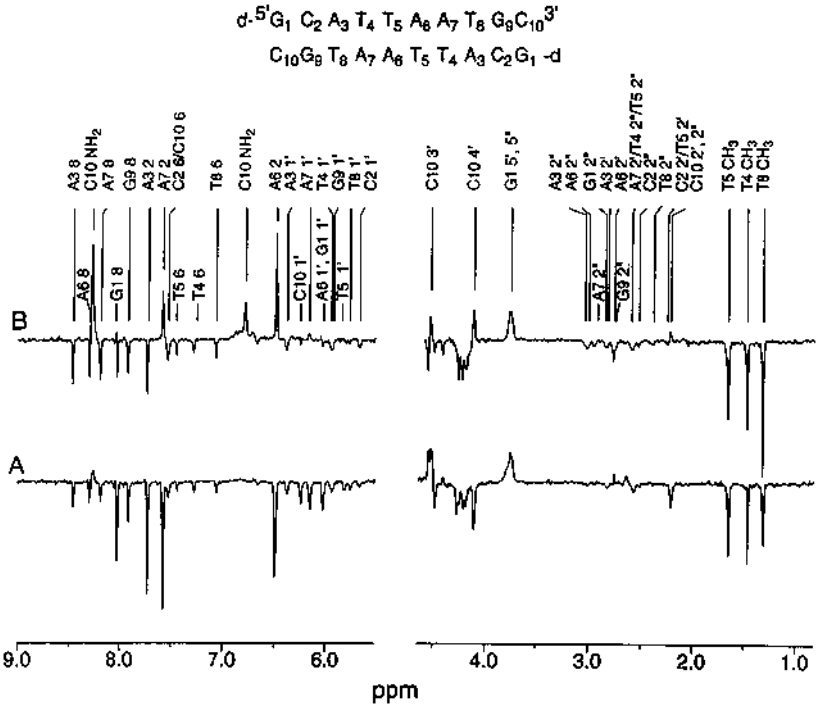


**Figure 4.** Comparison of the diagonal cross-sections through the ROE-NOESY (**A**) and NOE-NOESY spectra (**B**) of Figure 3. The nucleotide sequence and the resonance assignments of the decamer d(GCATTAATGC)$_2$ are indicated at the top. Because of the symmetry of the nucleotide sequence, the same numbering is used for both strands of the DNA.

NOESY peak with the A17 2H resonance indicate that the hydration water in the minor groove is highly mobile towards both ends of the AT-rich segment. The non-negative NOE intensities for the adenine 2 protons in the central part of the sequence of decamer 2 indicate reduced mobility of the hydration water in the minor groove of the 5′-dTTAA segment. Yet, these water molecules are still more mobile than in decamer 1 or in d(GCATTAATGC)$_2$.

Overlap in the diagonal cross-sections through the NOE-NOESY and ROE-NOESY experiments prevented assignment of the water–DNA NOEs with the adenine and thymidine 1′ protons of decamers 1 and 2. Furthermore, the signal-to-noise ratio was insufficient to observe off-diagonal peaks with the 1′ protons. There is, however, no positive NOESY cross-peak intensity in the spectral region of the 1′ protons in the diagonal cross-sections of Figures 5B and D which could not be explained more readily by chemical exchange from amino protons. The absence of positive NOESY peak intensity also for the G 1′H and C 1′H resonances indicates mobility on a sub-nanosecond timescale for the hydration water in the minor groove of the GC tracts of the DNA fragments.

## DISCUSSION

There are not many DNA protons in the minor groove which can be used as probes for the presence and kinetic stability of a spine of hydration. Adenine 2H protons are the only base protons in the minor groove for which water–DNA NOEs can readily be measured. Furthermore, the 1′ protons of the deoxyribose moieties are in the minor groove at a similar distance from the water molecules of the spine of hydration as the A 2H protons. The 1′ protons can be used to report on the spine of hydration in GC-rich DNA segments. While many of the A 2H resonances may be resolved in conventional NOESY and ROESY spectra, intense chemical exchange peaks with hydroxyl and amino protons tend to obscure the NOEs with some of the A 2H and almost all of the 1′H signals. In principle, the required separation of exchange cross-peaks and NOEs could be achieved by homonuclear three-dimensional NOESY-NOESY or ROESY-NOESY experiments. In the present work, more sensitive two-dimensional experiments, NOE-NOESY and ROE-NOESY, were used for this purpose. The sensitivity of these experiments is sufficient to obtain diagonal cross-sections with acceptable signal-to-noise ratios, where the water–DNA NOEs can be observed without overlap from intense exchange peaks. Off-diagonal peaks, which help in the assignment of overlapping water–DNA cross-peaks, are observable at higher sample concentrations.

The sign of the water–DNA NOEs offers a straightforward criterium for fast or slow exchange of the hydration water molecules near the observed DNA protons with respect to a time scale of ~0.5 ns (2). Strictly speaking, the residence times deduced from the sign of the intermolecular NOEs refer only to protons. Yet, the lifetime of water molecules with respect to proton dissociation is by orders of magnitude longer than 1 ns (28), so that the modulation of the intermolecular proton–proton vector must come about by the relative motions of the DNA and entire water molecules (1).

The precise values of the water residence times are, however, difficult to assess from the water–DNA NOEs alone. Only grossly oversimplifying relaxation models are available (2). Based on a model of intermolecular diffusion (29), water–DNA cross-peaks with a positive sign in NOESY and of comparable intensity in ROESY were usually interpreted as indicating hydration water

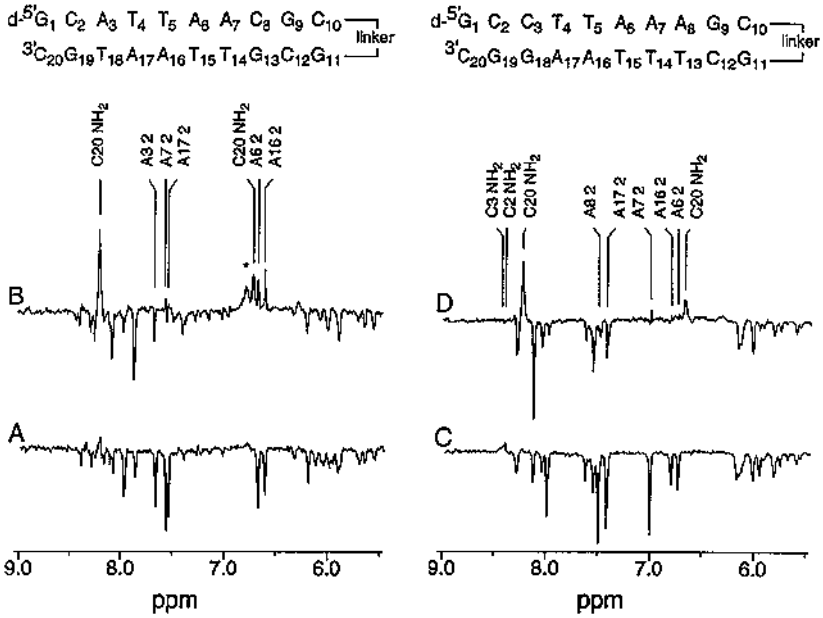


**Figure 5.** Diagonal cross-sections through the ROE-NOESY and NOE-NOESY spectra of decamer 1 [5′-d(GCATTAACGC)-3′–linker–5′-d(GCGTTAATGC)-3′] and decamer 2 [5′-d(GCCTTAAAGC)-3′–linker–5′-d(GCTTTAAGGC)-3′]. The low field region between 5.5 and 9.0 p.p.m. is shown which contains the water–DNA NOE cross-peaks with the base protons and most of the 1′ protons. The assignments are indicated for the water–DNA cross-peaks with the adenine 2 protons and for the most intense residual exchange peaks. The spectra were recorded at 4°C, pH 7.0 using the ROE-NOESY and NOE-NOESY pulse sequences of Figure 1. $\tau_{m1}$ = 50 and 100 ms in the ROE-NOESY and NOE-NOESY spectra respectively, $\tau_{m2}$ = 200 ms, total experimental time ~20 and 22 h per spectrum for decamer 1 and decamer 2 respectively. The nucleotide sequences of the decamers 1 and 2 and the sequence numbering are shown at the top. The linker is hexaethylene glycol. (**A**) Diagonal cross-section of the ROE-NOESY spectrum of decamer 1. (**B**) Diagonal cross-section of the NOE-NOESY spectrum of decamer 1. The asterisk identifies an unassigned chemical exchange peak which was also present in the conventional NOESY and ROESY experiments. (**C**) Diagonal cross-section of the ROE-NOESY spectrum of decamer 2. (**D**) Diagonal cross-section of the NOE-NOESY spectrum of decamer 2.

with residence times >1 ns (1,3–8). NOEs which were present in ROESY but absent in NOESY were interpreted by hydration lifetimes of ~0.5 ns or by significantly increased mobility of the water molecules while they are bound at their hydration sites (4,7). Recent $^{17}O$ and $^{2}H$ relaxation dispersion measurements indicate, however, that even the hydration water molecules with the longest residence times exchange within 2 ns at 4°C (30). To account for these results, we now attribute residence times >0.5 ns to hydration water detected by sizeable positive water–DNA cross-peaks in NOESY. In the limit of very long residence times, rigidly bound water would be expected to result in 2-fold faster cross-relaxation rates in ROESY than in NOESY. This prediction is independent of the model used. Experimentally, the positive NOESY cross-peaks observed in the present study were consistently weaker than their ROESY counterparts, even though 2-fold longer mixing times had been used in NOESY than in ROESY (Figs 3–5). This observation supports the finding that the water molecules of the spine of hydration in the minor groove of B-DNA are characterized by much shorter residence times and/or smaller order parameters than water molecules in the interior of proteins (2,31–33).

The data obtained in the present study show that the residence times of the hydration water molecules in the minor groove depend on the nucleotide sequence in a more complicated way than previously thought. A kinetically more restrained spine of hydration water is indicated for the 5′-dTTAA segment in $d(GCATTAATGC)_2$, whereas no positive water–A 2H NOEs had been observed for the corresponding segment in $d(GTGGTTAACCAC)_2$ (7). Clearly, the sequence 5′-dTTAA alone does not determine the mobility of the hydration water in the minor groove even in the center of the TTAA segment.

All DNA fragments studied here showed negative NOE-NOESY peaks for the A 2H resonances of the adenines next to GC base pairs, i.e. any kinetic stabilization of the minor groove hydration seems to be confined to the central part of the AT base pair segments. This situation differs from 5′-dAATT-containing DNA fragments, where stable spines of hydration are also observed when the 5′-dAATT segment is immediately preceded and followed by GC base pairs (2–4,7). Based on the observation of negative NOE-NOESY peaks with the G 1′H and C 1′H resonances, the minor groove of GC base pairs does not contain kinetically stabilized hydration water.

The sequences of decamers 1 and 2 were chosen to find out how the water residence times in the minor groove of a 5′-dTTAA segment are influenced by different nucleotides following the 5′-dTTAA segment. More rigid minor groove hydration is indicated by positive NOESY peaks with all but one A 2H resonance for decamer 1, which contains the sequences 5′-dTTAAC and 5′-dTTAAT (Fig. 5B). For decamer 2, which contains the sequences 5′-dTTAAA and 5′-dTTAAG, the NOE-NOESY experiment yielded positive cross-peak intensity only for the central adenine in the 5′-dTTAAA sequence (Fig. 5D).

The picture is complicated by long range sequence effects, which are clearly manifested in a comparison between the water–DNA NOEs of $d(GCATTAATGC)_2$ and decamer 1. Both nucleotide sequences are identical except for the base pair at position 8. Yet, the NOESY cross-peak intensity with the A 2H at position 4 [A7 in $d(GCATTAATGC)_2$ and A17 in decamer 1

respectively] is quite different (Figs 4 and 5B). Apparently, these water–DNA NOEs report on conformational differences caused by the base pair substitution four positions further towards the 3′-end. The presence of conformational differences in the minor groove is confirmed by the observation of, for example, a well-observable interstrand NOE between T5 1′H and A6 2H in d(GCATTAATGC)$_2$ at 15°C which was clearly absent in the NOESY spectrum of decamer 1 recorded under the same conditions (data not shown). Furthermore, the $^1$H chemical shifts are identical within experimental error for the first 4 bp, but chemical shift differences of 0.08–0.16 p.p.m. were observed for the A 2H and 6NH$_2$ and the T 1′H and 2′H, 2″H resonances of the base pairs at position 5. This indicates that the conformational differences start at base pair 5 and that the water molecule detected by the NOE with the A 2H of base pair 4 is located between base pairs 4 and 5, in agreement with single crystal X-ray data, where the innermost water molecules of the spine of hydration tend to be between neighboring base pairs (34–36). Because the NOEs with the A 2H at position 4 are significantly weaker than the corresponding ROEs in both DNA fragments, this hydration water must be quite mobile on a sub-nanosecond timescale. In this time regime, a change in residence time by a few hundred picoseconds is sufficient to explain the observed differences in NOE intensities (1). Therefore, the water–DNA NOEs can report on small conformational differences in the minor groove with a sensitivity comparable with that of chemical shifts.

Although it is plausible that hydration water in the minor groove is immobilized most when the minor groove is narrow, it is practically impossible to determine the width of the minor groove with good accuracy by high resolution NMR experiments (37). In addition, the number of single crystal structures solved by X-ray crystallography is too small to predict the width of the minor grooves of the DNA fragments studied here. X-ray analyses showed a well-defined spine of hydration associated with a narrow minor groove in d(CCATTAATGG)$_2$, but not in d(CGATTAATCG)$_2$ (10,11). The differences in minor groove width for the central 5′-dTTAA segments were ascribed to crystal packing forces (10). Still, the spine of hydration in d(CCATTAATGG)$_2$ correlates closely with the present observation of relatively long hydration lifetimes in the minor groove of d(GCATTAATGC)$_2$. Assuming a close correlation between minor groove width and residence times of the hydration water in the minor groove, our data would indicate wide minor grooves in GC-rich nucleotide sequences and narrow minor grooves in 5′-dAATT segments, while the width of the minor groove in 5′-dTTAA segments would depend on the flanking nucleotide sequences. However, a much larger number of nucleotide sequences needs to be studied to confirm this putative connection between hydration lifetimes, minor groove width and nucleotide sequence, since the hydration lifetimes, like the minor groove widths, result from the cumulative effect of interactions between more than just four or five sequentially neighboring base pairs.

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