



## NMR structure of the chimeric hybrid duplex r(gcaguggc)·r(gcca)d(CTGC) comprising the tRNA-DNA junction formed during initiation of HIV-1 reverse transcription

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Received 28 September 1998; Accepted 21 December 1998

**Key words:** AIDS, DYANA, HIV-1, NMR structure, ribonuclease H, RNA-DNA hybrid, torsion angle dynamics

### Abstract

A high-quality NMR solution structure of the chimeric hybrid duplex r(gcaguggc)·r(gcca)d(CTGC) was determined using the program DYANA with its recently implemented new module FOUND, which performs exhaustive conformational grid searches for dinucleotides. To ensure conservative data interpretation, the use of <sup>1</sup>H-<sup>1</sup>H lower distance limit constraints was avoided. The duplex comprises the tRNA-DNA junction formed during the initiation of HIV-1 reverse transcription. It forms an A-type double helix that exhibits distinct structural deviations from a standard A-conformation. In particular, the minor groove is remarkably narrow, and its width decreases from about 7.5 Å in the RNA/RNA stem to about 4.5 Å in the RNA/DNA segment. This is unexpected, since minor groove widths for A-RNA and RNA/DNA hybrid duplexes of ~11 Å and ~8.5 Å, respectively, were previously reported. The present, new structure supports that reverse transcriptase-associated RNaseH specificity is related primarily to conformational adaptability of the nucleic acid in 'induced-fit'-type interactions, rather than the minor groove width of a predominantly static nucleic acid duplex.

### Introduction

Retroviral reverse transcriptases (RTs) are responsible for the conversion of the single-stranded (ss) genomic (+)RNA into double-stranded (ds) proviral DNA (Baltimore, 1970; Temin and Mizutani, 1970). An RT thus possesses DNA- and RNA-dependent DNA-polymerase activities, as well as a ribonuclease H (RNaseH) activity which degrades the RNA template of DNA/RNA replication intermediates with

high specificity (Telenitsky and Goff, 1997). Like other retroviruses, human immunodeficiency virus type 1 (HIV-1) uses a cellular tRNA, i.e., tRNA<sup>Lys3</sup>, as a primer to initiate synthesis of (–)DNA (Marquet et al., 1995; Mak and Kleiman, 1997). The 3' terminal octadecanucleotide segment of tRNA<sup>Lys3</sup> hybridizes with a complementary primer binding site (PBS) near the 5' end of the genomic RNA to form an RNA/RNA duplex that serves as a substrate for the initiation of reverse transcription.

Although the RT-associated RNaseH domain is in principle also capable of degrading dsRNA (Blain and Goff, 1993; Hostomsky et al., 1994), the PBS and the tRNA primer remain intact during initiation of (–)DNA strand synthesis (Götte et al., 1995). Since the efficiency of nuclease action on dsRNA is very low, cleavages within the initially bound

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RNA/RNA duplex are seen exclusively in stalled RT-tRNA<sup>Lys3</sup>(+)/RNA complexes (Götte et al., 1995). X-ray (Arnott et al., 1986; Horton et al., 1996) and NMR (Fedoroff et al., 1993; Lane et al., 1993) studies revealed that DNA/RNA hybrid duplexes adopt heteronomous conformations, that are intermediate between standard A and B double helices. Such hybrid duplexes are characterized by an intermediate minor groove width, and it has been proposed (Fedoroff et al., 1993; Lane et al., 1993) that the minor groove width is a major structural determinant for RNaseH specificity. In particular, a molecular model of an RNaseH-DNA/RNA hybrid complex derived from the NMR structure of a DNA/RNA hybrid and the X-ray crystal structure of *Escherichia coli* RNaseH, which is a structural homologue (Davies et al., 1991) of RT RNaseH, suggested that several crucial RT-duplex interactions cannot be formed as long as dsRNA presents a wide and shallow minor groove to the enzyme (Fedoroff et al., 1993).

At a later stage of reverse transcription, i.e., during synthesis of the so-called (+)-strand strong stop DNA, RT encounters the 3' end of the tRNA primer, which now serves as a template for synthesis of a DNA copy of the PBS (Telenitsky and Goff, 1997). A specific RNaseH cut, located one nucleotide upstream of the DNA-tRNA junction, then removes the tRNA primer from the (-)DNA strand (Furfine et al., 1991; Smith et al., 1992). The NMR solution structure (Fedoroff et al., 1996) of the chimeric hybrid octamer d(GCAGTGGC)·r(gcca)d(CTGC) (lower case and upper case letters are used to represent ribonucleotides and deoxyribonucleotides, respectively), which includes the aforementioned DNA-tRNA junction, seemed to provide further support for the hypothesis (Fedoroff et al., 1993, 1996, 1997; Lane et al., 1993; Salazar et al., 1994, 1996; Zhu et al., 1995; Horton et al., 1996; Han et al., 1997; Bachelin et al., 1998) that tuning of the minor groove width of the free nucleic acid confers RNaseH specificity. To obtain additional insight into the structural features of oligonucleotide duplexes that govern RNaseH specificity, we determined the NMR solution structure of the chimeric hybrid octamer r(gcaguggc)·r(gcca)d(CTGC) (Figure 1), which comprises the tRNA-DNA junction formed during the initiation of (-)DNA strand synthesis, and represents the first solution structure of a chimeric RNA/RNA-DNA hybrid duplex.

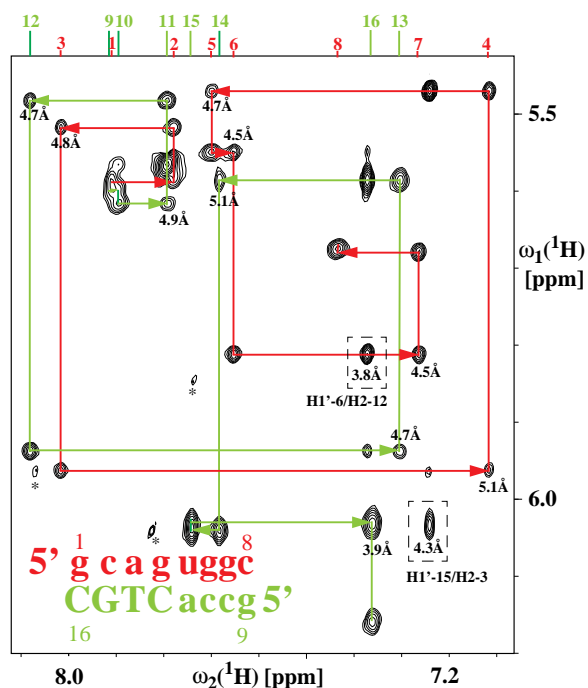


Figure 1. Spectral region of a 2D [<sup>1</sup>H,<sup>1</sup>H]-NOESY spectrum of r(gcaguggc)·r(gcca)d(CTGC) comprising the ω<sub>1</sub> (H1')/ω<sub>2</sub> (H6,H8) connectivities. The spectrum was recorded with τ<sub>m</sub> = 33 ms at a <sup>1</sup>H resonance frequency of 750 MHz (T = 25 °C, duplex concentration 1.5 mM solution, D<sub>2</sub>O buffer: 100 mM NaCl, 20 mM Na<sup>+</sup>-phosphate, 1 mM EDTA, pH 7.0). The chemical shift positions of the base protons in the individual nucleotides are given at the top, and connectivities are indicated with arrows (red: RNA strand; green: chimeric strand). The numbering of the nucleotides is given in the lower left part, and <sup>1</sup>H-<sup>1</sup>H upper distance limit constraints for sequential connectivities are indicated near the corresponding cross peaks. The high accuracy of these input constraints is evidenced by the fact that the actual distances in the 20 energy-refined DYANA conformers (Table 1) are all shorter than these values within a range of 0.5 Å. Two interstrand H2-H1' NOEs, for which the intensities correlate with the minor groove width (Chuprina et al., 1991b), are indicated in boxes and are assigned. Cross peaks arising from impurities are labeled with asterisks.

## Materials and methods

### Sample preparation

r(gcaguggc) and r(gcca)d(CTGC) were synthesized on a 1 μmol scale on an Applied Biosystems 392/8 synthesizer using the standard phosphoramidite method. Crude products were HPLC-purified and analyzed on 20% polyacrylamide-7 M urea gels to confirm the correct length and the sequence using the guanine-specific ribonuclease T1. Fractions containing the RNA and the RNA-DNA strand were separately pooled and lyophilized. After repeated washing with ethanol, the nucleic acids were dissolved in an aque-



plex (1.1 ml) was then lyophilized and dissolved in 550  $\mu$ l of either D<sub>2</sub>O buffer or 90% H<sub>2</sub>O/10% D<sub>2</sub>O buffer, yielding a final duplex concentration of about 1.5 mM (100 mM NaCl, 20 mM Na-phosphate, 1 mM perdeuterated EDTA, pH 7.0).

#### *NMR and CD spectroscopy*

Circular dichroism (CD) spectra recorded for the duplex r(gcaguggc)·r(gcca)d(CTGC) between 20 and 80 °C yielded a melting temperature of about 55 °C, and the spectra recorded between 20 and 35 °C were virtually identical. However, 1D <sup>31</sup>P NMR spectra revealed a change of the <sup>31</sup>P chemical shift of c<sub>2</sub> between 30 and 35 °C, indicating increased fraying of the DNA/RNA duplex segment. Hence, NMR measurements were performed at 25 °C using Bruker AMX500/AMX600 and Varian UNITY750+ NMR spectrometers. (A) 2D <sup>1</sup>H-spectra recorded in D<sub>2</sub>O: 2QF-COSY (Rance et al., 1983) (<sup>1</sup>H-resonance frequency 600 MHz, t<sub>1,max</sub> = 150 ms, t<sub>2,max</sub> = 205 ms, total measurement time 23 h); E. COSY (Griesinger et al., 1985) (600 MHz, 88 ms, 205 ms, 16 h); clean [<sup>1</sup>H, <sup>1</sup>H]-TOCSY (Griesinger et al., 1988) (750 MHz, 133 ms, 128 ms, 20 h;  $\tau_m$  = 30 ms; 600 MHz, 90 ms, 102 ms, 18 h,  $\tau_m$  = 90 ms); [<sup>1</sup>H, <sup>1</sup>H]-NOESY (Anil Kumar et al., 1980) (750 MHz, 86 ms, 128 ms, 82 h,  $\tau_m$  = 33 ms, zero-quantum suppression (Otting et al., 1990) for ribose protons; recycle time 5 s; 600 MHz, 102 ms, 205 ms, 36 h, three experiments with  $\tau_m$  = 50 ms, 150 ms and 300 ms, respectively, recycle time 10 s). 2D <sup>1</sup>H-spectrum recorded in H<sub>2</sub>O: [<sup>1</sup>H, <sup>1</sup>H]-NOESY (750 MHz, 86 ms, 128 ms, 52 h,  $\tau_m$  = 35 ms, water suppression using WATERGATE (Piotto et al., 1992), recycle time 10 s). (B) Heteronuclear 2D experiments recorded in D<sub>2</sub>O: [<sup>31</sup>P, <sup>1</sup>H]-COSY (Sklenář and Bax, 1987) (500 MHz, 60 ms, 51 ms, 12 h); [<sup>13</sup>C, <sup>1</sup>H]-HSQC (Bodenhausen and Ruben, 1980) for the sugars and the base C5H groups (600 MHz, 21 ms, 51 ms, 96 h); [<sup>13</sup>C, <sup>1</sup>H]-HSQC for the other base resonances (600 MHz, 21 ms, 51 ms, 48 h). The spectra were processed and analyzed using the programs PROSA (Güntert et al., 1992) and XEASY (Bartels et al., 1995), respectively.

#### *Resonance assignments and collection of conformational constraints*

Sequential <sup>1</sup>H resonance assignments were obtained using standard protocols based on the observation of <sup>1</sup>H-<sup>1</sup>H NOEs (Figure 1). The spin system identification was achieved using the 2D 2QF-COSY, TOCSY, [<sup>13</sup>C, <sup>1</sup>H]-HSQC (Figure 2) and [<sup>31</sup>P, <sup>1</sup>H]-COSY ex-

periments (Wüthrich, 1986; Varani and Tinoco, 1991). Except for the RNA hydroxyl protons, the amino protons of A and G, and the imino protons of the terminal nucleotides g<sub>1</sub> and g<sub>9</sub>, complete <sup>1</sup>H resonance assignments were obtained. <sup>13</sup>C chemical shifts were obtained for all <sup>1</sup>H-bound carbons, except for C4' of g<sub>1</sub>-g<sub>7</sub> and c<sub>10</sub>-a<sub>12</sub>, and for C2' of g<sub>4</sub> and g<sub>6</sub>. All <sup>31</sup>P chemical shifts except those of g<sub>6</sub> and c<sub>10</sub> were assigned, and the [<sup>31</sup>P, <sup>1</sup>H]-COSY experiment revealed the absence of strongly downfield shifted <sup>31</sup>P resonances. The chemical shifts have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>; accession number: 4247).

<sup>1</sup>H-<sup>1</sup>H upper distance limits (Table 1) were derived from the 750 MHz NOESY spectra using the isolated spin pair approximation (Wüthrich, 1986). Comparison of NOE cross peaks observed in the 750 MHz spectrum recorded with  $\tau_{mix}$  = 33 ms/recycle time 5 s, and in the 600 MHz spectrum recorded with  $\tau_{mix}$  = 50 ms/recycle time 10 s revealed that upper distance limits derived from the 750 MHz spectrum were up to 5% shorter than those derived from the 600 MHz spectrum, which is due to the shorter T<sub>1</sub>-relaxation times of deoxyribose when compared with ribose protons (Wang et al., 1992). Since a recycle time of 10 s would yield a prohibitively long measurement time for a NOESY spectrum recorded with zero-quantum suppression, upper distance limits involving deoxyribose protons were relaxed by 5% to ensure conservative data interpretation. Hydrogen bond constraints (upper and lower distance limits of 2.25 Å and 2.15 Å for the hydrogen-acceptor distance, and of 3.25 Å and 3.15 Å for the donor-acceptor distance, respectively) were inferred from imino proton exchange rates, k<sub>NH</sub>, for the central six base pairs. The rates were estimated from the relative intensities of the diagonal peaks and the cross peaks at  $\omega_1$ (H<sub>2</sub>O) in the NOESY spectrum recorded in H<sub>2</sub>O. k<sub>NH</sub> values are: G<sub>15</sub>  $\approx$  4 s<sup>-1</sup>; T<sub>14</sub>  $\approx$  2 s<sup>-1</sup>; G<sub>4</sub>  $\approx$  3 s<sup>-1</sup>; u<sub>5</sub>  $\approx$  4 s<sup>-1</sup>; G<sub>6</sub>  $\approx$  1 s<sup>-1</sup>; G<sub>7</sub>  $\approx$  1 s<sup>-1</sup>. The values reflect protection factors of about 10<sup>4</sup> relative to the free nucleotides (Wüthrich, 1986), and thus provide experimental evidence for the formation of Watson-Crick base pairs (Guéron and Leroy, 1995). The terminal imino protons could not be observed by 1D <sup>1</sup>H NMR, so that no constraints were introduced for the terminal base pairs (a test calculation revealed that the addition of constraints for these hydrogen bonds did not lead to an increase in residual constraint violations). <sup>3</sup>J<sub>H1'H2'</sub> values of riboses were extracted by inverse Fourier transformation of in-phase multiplets (Szyper-

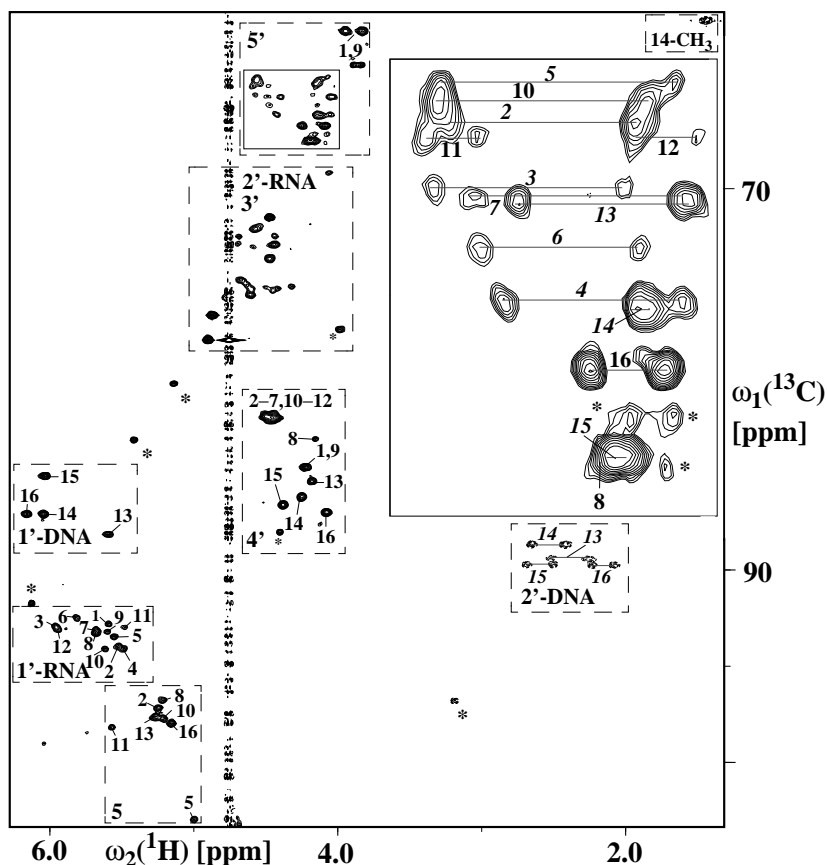


Figure 2. 2D [ $^{13}\text{C}, ^1\text{H}$ ]-HSQC spectrum recorded for the sugar moieties of r(gcaguggc)-r(gcca)d(CTGC). Solid and dashed contour lines represent positive and negative peaks, respectively; the resonances of  $\text{T}_{14}\text{-CH}_3$  and  $2'$  of the DNA have been folded along  $\omega_1$ . The dashed rectangles identify spectral regions of specified carbon types, and resonance assignments are identified with the sequence location of the corresponding nucleotide according to the numbering shown in Figure 1. For clarity, the assignments in the region comprising  $2'$ -RNA and  $3'$  resonances have been omitted. The solid rectangle in the  $5'$ -region identifies the spectral region shown in the insert in the upper right. Cross peaks of stereospecifically assigned methylene protons are denoted in italics (for deoxyriboses,  $\text{H}_2'$  resonates upfield from  $\text{H}_2''$  and, except for  $\text{G}_{15}$ ,  $\text{H}_5''$  resonates upfield from  $\text{H}_5'$ ). The chemical shifts are relative to internal 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt ( $^1\text{H}$  resonance frequency: 600 MHz; same sample as in Figure 1;  $T = 25^\circ\text{C}$ ). Cross peaks arising from impurities are labeled with asterisks.

ski et al., 1992) from the NOESY spectrum recorded with  $\tau_m = 300$  ms.  $^3J_{\text{H}_1'\text{H}_2'}$  and  $^3J_{\text{H}_1'\text{H}_2''}$  values of the deoxyribose rings were measured by E.COSY. For  $\text{g}_1\text{-c}_8$  and  $\text{g}_9\text{-C}_{13}$ ,  $^3J_{\text{H}_1'\text{H}_2'} < 2$  Hz yielded the constraint  $\nu_1 \leq -20^\circ$ , corresponding to a range of  $[-96^\circ, 24^\circ]$  for the pseudorotation angle. For  $\text{T}_{14}\text{-G}_{15}$ , the  $^3J_{\text{H}_1'\text{H}_2'}$  values ( $\text{T}_{14}$ : 6.8 Hz;  $\text{G}_{15}$ : 8.0 Hz), the sum of couplings to  $\text{H}_3'$  ( $\text{T}_{14}$ : 17 Hz;  $\text{G}_{15}$ : 13 Hz) and the  $\text{H}_1'\text{-H}_4'$  NOE upper distance limits ( $\text{T}_{14}$ : 3.7 Å;  $\text{G}_{15}$ : 3.6 Å) reflect conformational averaging, i.e., rapid re-puckering, for the deoxyribose rings, and therefore no angle constraints were derived from  $^3J_{\text{H}_1'\text{H}_2'}$  for these deoxyribonucleotides. Figure 3 affords the dihedral angle constraints that were derived for the dihedral angles  $\beta$ ,  $\gamma$  and  $\epsilon$ . For  $\text{c}_2\text{-c}_8$ ,  $\text{a}_{12}$ , and  $\text{T}_{14}\text{-C}_{16}$ ,

which exhibited neither a  $\text{P-H}_5'$  nor a  $\text{P-H}_5''$  correlation in [ $^{31}\text{P}, ^1\text{H}$ ]-COSY (indicating  $^3J_{\text{PH}_5'}$  and  $^3J_{\text{PH}_5''} < 5$  Hz), the  $\beta$ -angle was constrained (Wijmenga et al., 1993) to  $180^\circ \pm 20^\circ$ . A very weak P-H correlation was observed for  $\text{C}_{13}$ , and the  $\beta$ -constraint was relaxed to  $180^\circ \pm 40^\circ$ . For  $\text{c}_{10}$  and  $\text{c}_{11}$ , the position of one of the potential P-H correlations coincided with other P-H3' or P-H4' peaks, so that only the information that either  $^3J_{\text{PH}_5'}$  or  $^3J_{\text{PH}_5''} < 5$  Hz could be translated into a  $\beta$ -constraint ( $180^\circ \pm 20^\circ$  or  $45^\circ \pm 25^\circ$  or  $-45^\circ \pm 25^\circ$ ). All  $\beta$ -constraints are in agreement with the  $\text{H}_5'$  and  $\text{H}_5''$  NOESY line widths (Kim et al., 1992). Angle constraints for  $\gamma$  ( $\text{c}_2\text{-g}_7$ ,  $\text{c}_{10}$ ,  $\text{c}_{11}$ :  $60^\circ \pm 30^\circ$ ;  $\text{a}_{12}$ :  $60^\circ \pm 40^\circ$  or  $-120^\circ \pm 40^\circ$ ;  $\text{C}_{13}$ :  $60^\circ \pm 40^\circ$  or  $-110^\circ \pm 20^\circ$ ;  $\text{T}_{14}$ ,  $\text{G}_{15}$ :  $60^\circ \pm 60^\circ$  or  $-120^\circ \pm 50^\circ$ ;  $\text{C}_{16}$ :  $60^\circ \pm$

$60^\circ$  or  $-110^\circ \pm 20^\circ$ ) and for  $\epsilon$  ( $c_2$ - $g_7$ :  $-40^\circ \pm 30^\circ$ ;  $g_9$ ,  $a_{12}$ :  $-110^\circ \pm 150^\circ$ ;  $c_{10}$ ,  $c_{11}$ :  $-40^\circ \pm 30^\circ$  or  $165^\circ \pm 30^\circ$ ;  $C_{13}$ - $G_{15}$ :  $-120^\circ \pm 130^\circ$ ) were derived from  $H4'$  and  $H3'$  resonance line widths, respectively (Kim et al., 1992).

#### Structure calculation

The intranucleotide and sequential upper distance limits and the dihedral angle constraints were translated into dihedral angle constraints using the FOUND module (Güntert et al., 1998) of DYANA (Güntert et al., 1997), which performs a grid search for allowed conformations in the space spanned by the nine torsion angles describing a dinucleotide segment. The FOUND calculation also provided stereospecific assignments for  $H2'/H2''$  of the deoxyribose rings, and for  $H5'/H5''$  of  $c_2$ ,  $g_4$  and  $g_7$ . Additional stereospecific  $H5'/H5''$  assignments for  $g_6$  and for  $C_{13}$ - $G_{15}$  were obtained from initial structure calculations using the program GLOMSA (Güntert et al., 1991). The stereospecific  $H5'/H5''$  assignments of the RNA strand coincide in all instances with the previously observed correlation between the chemical shift differences  $\delta(H5')-\delta(H5'')$  and the  $C5'$  chemical shift in dsRNA (Marino et al., 1994; Klinck et al., 1997). We therefore used the chemical shifts (Figure 2) to obtain additional  $H5'/H5''$  stereospecific assignments for  $a_3$  and  $u_5$ . The input for the final DYANA structure calculation, which also included constraints to close the sugar rings ( $C4'-O4'$ : 1.41 Å,  $C4'-C1'$ : 2.40 Å,  $C5'-O4'$ : 2.39 Å,  $H4'-O4'$ : 2.12 Å), is listed in Table 1. The calculation was started with 100 randomized structures and the resulting 20 DYANA conformers with the lowest target function values were subjected to restrained energy minimization using the AMBER force field (Weiner et al., 1986) as implemented in the program OPAL (Luginbühl et al., 1996). Minimization parameters included a water shell of 15 Å thickness, a constant dielectric, the TIP3P water model and a cut-off of 15 Å. Upper distance limits and dihedral angle constraints were enforced as described in Fernandez et al. (1997). No hydrogen bond constraints were used during energy minimization. Coordinates and NMR constraints of  $r(gcaguggc)\cdot r(gcca)d(CTGC)$  have been deposited in the Brookhaven Protein Data Bank, accession number 1byx. Figures 4 and 5 were generated using the program MOLMOL (Koradi et al., 1996).

## Results and discussion

### NMR structure of $r(gcaguggc)\cdot r(gcca)d(CTGC)$

In view of the limited proton chemical shift dispersion of the ribose moieties we recorded 2D [ $^{13}C$ ,  $^1H$ ]-HSQC spectra at natural  $^{13}C$  abundance (Figure 2) to obtain nearly complete  $^1H$  resonance assignments. This then provided the starting point for the collection of a large number of rather accurate  $^1H$ - $^1H$  upper distance constraints from NOESY spectra recorded with very short mixing times at a  $^1H$  resonance frequency of 750 MHz (Figure 1). In addition, vicinal spin-spin couplings were experimentally derived that constrain the allowed value ranges for the torsion angles  $\beta$ ,  $\gamma$  and  $\epsilon$  (Figure 3, red color) and the sugar ring pucker (asterisk for  $\delta$  in Figure 3). These experimental data on the torsion angles, combined with upper distance limits from intranucleotide and sequential NOEs, were used as input for grid searches over the dinucleotide conformational spaces, using the newly implemented FOUND module of DYANA (Güntert et al., 1998). The calculations for each dinucleotide segment followed closely the example given by Güntert et al. (1998), where smaller fragments were considered before performing the final nine-dimensional grid search for segments comprising two nucleotides  $i$  and  $i+1$ . The nine variables were the torsion angles  $\epsilon$  and  $\zeta$  of nucleotide  $i$ ,  $\alpha$ ,  $\beta$  and  $\gamma$  of nucleotide  $i+1$ , and the sugar pucker and  $\chi^1$  angles of both nucleotides. Except for the few interstrand NOEs (Table 1), all NOE distance constraints are considered in this approach. FOUND thus makes use of more than 90% of the measured NOE distance constraints to derive torsion angle constraints for use as input for the final DYANA structure calculation. In fact, we obtained quite tight constraints on the value ranges for all torsion angles, including the backbone dihedral angles  $\alpha$  and  $\zeta$  for which no scalar coupling constraints can be derived (Figure 3, green color). This allowed, for the first time, to follow a conservative structure determination protocol (Wüthrich, 1986) for an oligonucleotide double helix using exclusively upper limit NOE distance constraints and scalar couplings. The statistical parameters for the resulting 20 energy-refined conformers, calculated to represent the NMR structure, demonstrate that this approach allowed determination of a high-quality structure for this oligonucleotide duplex (Table 1, Figure 4). To assess the possible impact of the additional use of  $^1H$ - $^1H$  lower distance limit constraints supplementing those derived from the van der Waals radii, 198 well-resolved cross

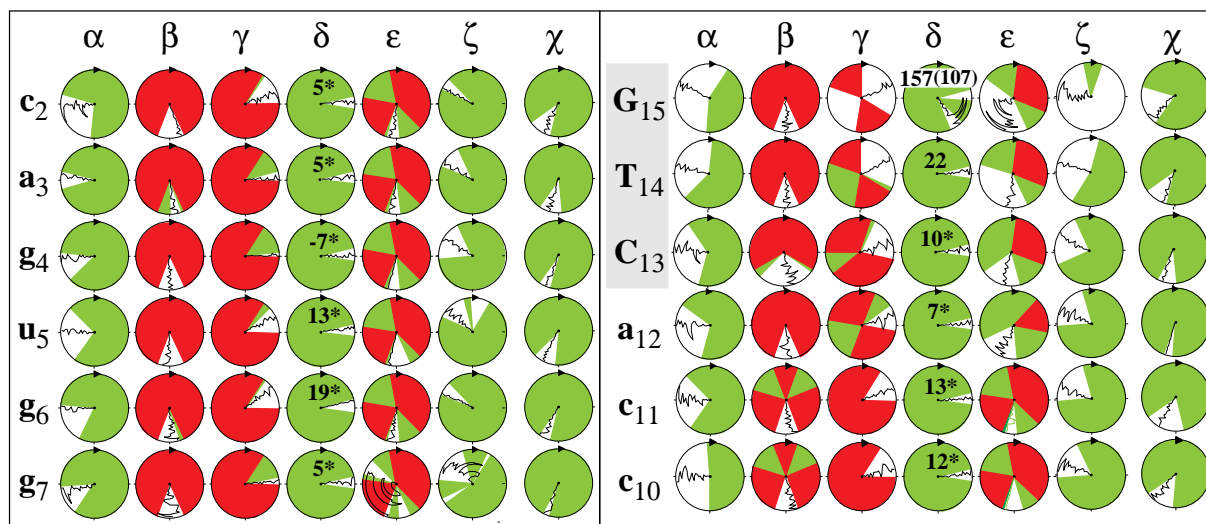


Figure 3. Backbone and glycosidic dihedral angles of the six non-terminal base pairs of r(gcaguggc)·r(gcca)d(CTGC). The dihedral angles were taken from the 20 best energy-refined DYANA conformers (Table 1) which were ordered according to increasing residual DYANA target function values before energy minimization. The 20 values for each angle are plotted in this order from the center to the periphery, which gives rise to the black lines in the circles. Sections colored in red in the circles of  $\beta$ ,  $\gamma$  and  $\epsilon$  represent the ranges that were excluded by the experimentally determined scalar couplings (see Materials and methods). The green sections represent the dihedral angle ranges that were excluded based on the calculations with the FOUND module of DYANA, using the local NOE distance constraints and the experimental spin-spin coupling constants as input (Table 1). Angles corresponding to the RNA strand and the chimeric strand are shown in the left and right boxes, respectively. The pseudorotation phase angles of the sugar rings are indicated within the circles of  $\delta$  and were calculated from the corresponding  $\nu_1$  values assuming a maximal pucker amplitude of  $40^\circ$ . An asterisk indicates that  $\nu_1$  was constrained due to  $^3J_{H1/H2'} < 2$  Hz (see Materials and methods). For G<sub>15</sub>, two conformers exhibited a pseudorotation angle of about  $107^\circ$  (given in parentheses). Dihedral angle values for standard A-RNA (Kennard and Hunter, 1991), and A- and B-DNA (Dickerson, 1992) are:  $\alpha$  (A-RNA:  $-68^\circ$ , A-DNA:  $-73^\circ$ , B-DNA:  $-65^\circ$ ),  $\beta$  ( $178^\circ$ ,  $173^\circ$ ,  $167^\circ$ ),  $\gamma$  ( $54^\circ$ ,  $64^\circ$ ,  $51^\circ$ ),  $\delta$  ( $82^\circ$ ,  $78^\circ$ ,  $129^\circ$ ),  $\epsilon$  ( $-153^\circ$ ,  $-151^\circ$ ,  $-157^\circ$ ),  $\zeta$  ( $-71^\circ$ ,  $-77^\circ$ ,  $-80^\circ$ ),  $\chi$  ( $-158^\circ$ ,  $-165^\circ$ ,  $-103^\circ$ ). Note that  $\delta$  and  $\chi$  show the largest change when comparing A- and B-type duplexes.

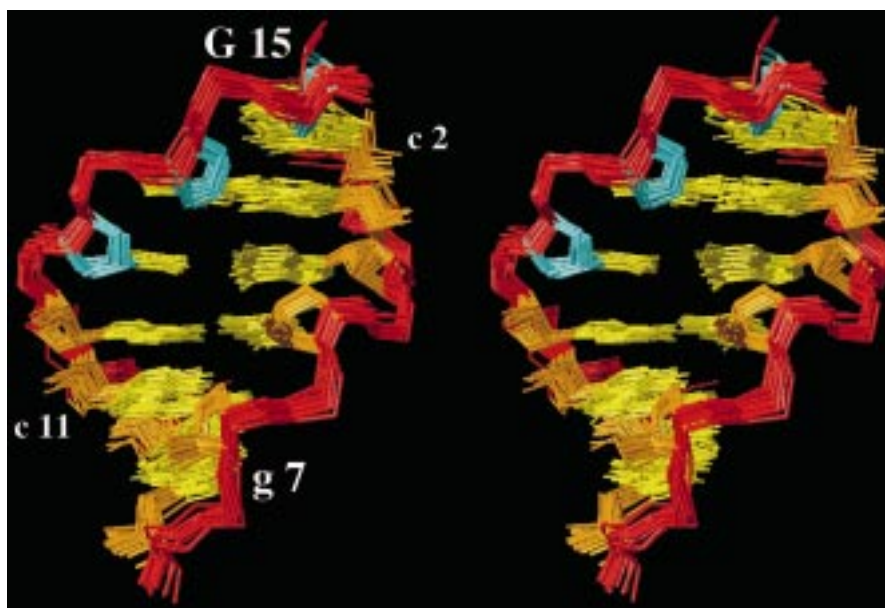


Figure 4. Stereoview into the minor groove of the central hexanucleotide segment of r(gcaguggc)·r(gcca)d(CTGC). The 3'-end of the chimeric strand shown on the left is located at the top, and selected nucleotides are indicated (the numbering of the nucleotides is shown in Figure 1). The 20 best energy-refined DYANA conformers (Table 1) were superimposed for pairwise minimal rmsd of the heavy atoms of this segment. Color code: phosphodiester backbone, red; deoxyribose rings, cyan; ribose rings, orange; bases, yellow.

peaks of the NOESY spectrum recorded in D<sub>2</sub>O were translated into lower limits according to the relation  $lower-limit = 0.6 * upper-limit + 0.5 \text{ \AA}$ , where only lower limits shorter than 3.5 Å were retained. Sixteen additional lower limits of 3.5 Å were derived from the absence of NOE cross peaks. This procedure yielded a total of 191 lower limits which were combined with the constraints listed above. The resulting residual DYANA target function value remained constant ( $0.84 \pm 0.03 \text{ \AA}^2$ ), and the average rmsd values calculated for the 20 best energy-minimized DYANA conformers decreased by only about 15% (0.47 Å for nucleotides 2–7 and 10–15; 0.33 Å for 3–6 and 11–14; 0.94 Å for 1–8 and 9–16).

Considering the increased opening rate of the terminal base pairs (see Materials and methods), the following structure analysis is focussed on the six non-terminal base pairs (Figure 1). Figure 3 affords a graphical representation of the backbone and glycosidic dihedral angle values in the 20 individual, energy-refined DYANA conformers. The comparison with standard A- and B-DNA helices (Table 2 and Figure 3) reveals that the structure of r(gcaguggc)-r(gcca)d(CTGC) belongs to the 'A-family' (Figure 5A) but exhibits the following clearcut differences relative to a standard A-duplex. (i) The sugar puckers of all riboses and the deoxyribose of C<sub>13</sub>, which is located at the RNA–DNA junction, are in an N conformation (C2'-*exo* to C3'-*endo*). In contrast, the <sup>3</sup>J<sub>HH</sub> couplings and H1'-H4' NOE upper distance limits (see Materials and methods) indicate rapid repuckering for the deoxyribose rings of T<sub>14</sub> and G<sub>15</sub>. In the framework of a two-state model (Hartmann and Lavery, 1996) assuming rapid interconversion between C2'-*endo* and C3'-*endo*, these data yield ~50% and ~70% S conformation for T<sub>14</sub> and G<sub>15</sub>, respectively. Hence, in the C<sub>13</sub>-T<sub>14</sub>-G<sub>15</sub> segment we observe an increasing fraction of S conformation towards the 3' end of the chimeric strand. The fact that the population of the S conformer is lower than typically found in B-DNA duplexes (~80–90%) indicates increased flexibility for the segment of T<sub>14</sub>-G<sub>15</sub> when compared with dsDNA. Consistently, the corresponding imino proton exchange rates are faster than those within the RNA segment (see Materials and methods). Reduced populations of S conformer have previously also been reported for the DNA strand in a DNA/RNA hybrid duplex (González et al., 1994), and have been implicated by a recent molecular dynamics simulation of oligonucleotide duplexes (Cheatham et al., 1996). Furthermore, all  $\chi$ -angles observed for the non-terminal

base pairs are close to the standard value measured for A-helices, with the sole exception of G<sub>15</sub> (Figure 3). Overall, the chimeric strand thus shifts from an A-like to a B-like conformation at the dinucleotide segment T<sub>14</sub>-G<sub>15</sub>, as is also evidenced by the intensities of the sequential H1'-H6/H8 NOEs observed for the chimeric strand (Figure 1). Notably, the final distance geometry calculation, which was performed without <sup>3</sup>J<sub>HH</sub>-derived constraints for T<sub>14</sub> and G<sub>15</sub> (see Materials and methods), generated exclusively C3'-*endo* conformations for T<sub>14</sub> while both C2'-*endo* and C3'-*endo* conformations were obtained for G<sub>15</sub> (Figure 3). This finding supports the general notion that proper ring pucker analyses should include measurement of scalar coupling constants (e.g., Wijmenga et al., 1993; Szyperski et al., 1998). (ii) The absence of strongly downfield-shifted <sup>31</sup>P resonances is consistent with canonical B<sub>1</sub> conformations ( $\epsilon^{\text{t}\zeta^-}$ ) (Gorenstein et al., 1988) for all deoxyribonucleotides (Figure 3), and the apparently deviating  $\epsilon^-$  values found for some of the 20 energy-refined conformers at g<sub>7</sub> (Figure 3) are very likely due to the fact that this dihedral angle is least well defined by the experimental constraints. Moreover, there are no crankshaft transitions ( $\alpha^- \gamma^+$  to  $\alpha^{\text{t}} \gamma^{\text{t}}$ ) (Figure 3), which have in other studies been shown to induce bends in dsRNA (Dock-Bregeon et al., 1989). (iii) Large propeller twists are observed for the two base pairs at the RNA–DNA junction (Figure 5B; Table 2), but there is no evidence for bifurcated inter-strand hydrogen bonds (Nelson et al., 1986), and only moderately large buckle and opening angles are found (Table 2). (iv) Except for the last base step, the local slides are closer to an A-form, while the standard value for the twist is intermediate between the standard A- and B-forms (Table 2). (v) The minor groove is narrow, which is directly evidenced by strong interstrand H2-H1' NOEs (Figure 1), and its width decreases from about 7.5 Å in the dsRNA segment to about 5.5 Å at the RNA–DNA junction and 4.5 Å in the hybrid segment (Figure 5C). This shift is unexpected, considering that the groove width of A-RNA is ~11 Å (Kennard and Hunter, 1991), and that of DNA/RNA duplexes is ~8–9 Å (Fedoroff et al., 1993, 1996, 1997; Lane et al., 1993; Salazar et al., 1994, 1996; Zhu et al., 1995; Hartmann and Lavery, 1996). In fact, the minor groove width in this chimeric hybrid duplex is close to that of B-DNA, for which a range of 3.0 Å–8.2 Å has been reported (Dickerson, 1992).



Table 2. Helix parameters<sup>a</sup> for the non-terminal base pairs of (gcaguggc)-(gccCTGC)

Base pair	Base pair parameters		
	Propeller	Buckle	Opening
c <sub>2</sub> -G <sub>15</sub>	-3.6±0.1	-6.5±1.2	1.2±0.1
a <sub>3</sub> -T <sub>14</sub>	-8.6±1.4	2.0±0.6	5.0±0.8
g <sub>4</sub> -C <sub>13</sub>	-24.0±0.7	-1.9±1.4	8.4±0.1
u <sub>5</sub> -a <sub>12</sub>	-25.8±0.5	-2.9±0.5	3.3±0.9
g <sub>6</sub> -c <sub>11</sub>	-11.6±0.6	-14.1±1.4	1.3±0.2
g <sub>7</sub> -c <sub>10</sub>	-14.0±0.5	-5.6±2.2	6.2±0.8
Average	-14.6	-4.9	4.2
A-DNA <sup>b</sup>	-7.5	0.0	0.0
B-DNA <sup>b</sup>	-13.3	0.0	0.0

	Local base step parameters <sup>c</sup>					
	Tilt	Roll	Twist	Shift	Slide	Rise
c <sub>2</sub> -G <sub>15</sub> /a <sub>3</sub> -T <sub>14</sub>	0.2±0.2	-7.6±1.3	28.6±0.2	0.88±0.01	-1.30±0.02	3.65±0.05
a <sub>3</sub> -T <sub>14</sub> /g <sub>4</sub> -C <sub>13</sub>	0.8±0.1	-5.9±0.5	35.2±0.9	-0.04±0.04	-1.45±0.04	3.61±0.03
g <sub>4</sub> -C <sub>13</sub> /u <sub>5</sub> -a <sub>12</sub>	0.6±0.1	-1.4±0.8	34.9±0.6	-0.51±0.06	-1.16±0.01	3.17±0.01
u <sub>5</sub> -a <sub>12</sub> /g <sub>6</sub> -c <sub>11</sub>	0.9±0.3	15.5±0.1	34.3±0.3	0.19±0.01	-1.23±0.01	3.27±0.06
g <sub>6</sub> -c <sub>11</sub> /g <sub>7</sub> -c <sub>10</sub>	1.4±0.1	6.7±0.4	30.6±1.0	0.26±0.01	-0.64±0.03	3.29±0.03
Average	0.8	1.5	32.7	0.16	-1.15	3.40
A-DNA <sup>b</sup>	0.0	1.4	30.7	0.0	-1.92	3.44
B-DNA <sup>b</sup>	0.0	0.9	35.6	0.0	0.08	3.38

<sup>a</sup>Averaged values (angles in degrees and distances in Å) and their standard deviations calculated for 20 energy-refined DYANA-conformers using the program RNA (Babcock et al., 1994).

<sup>b</sup>From Table 2 in Hartmann and Lavery (1996).

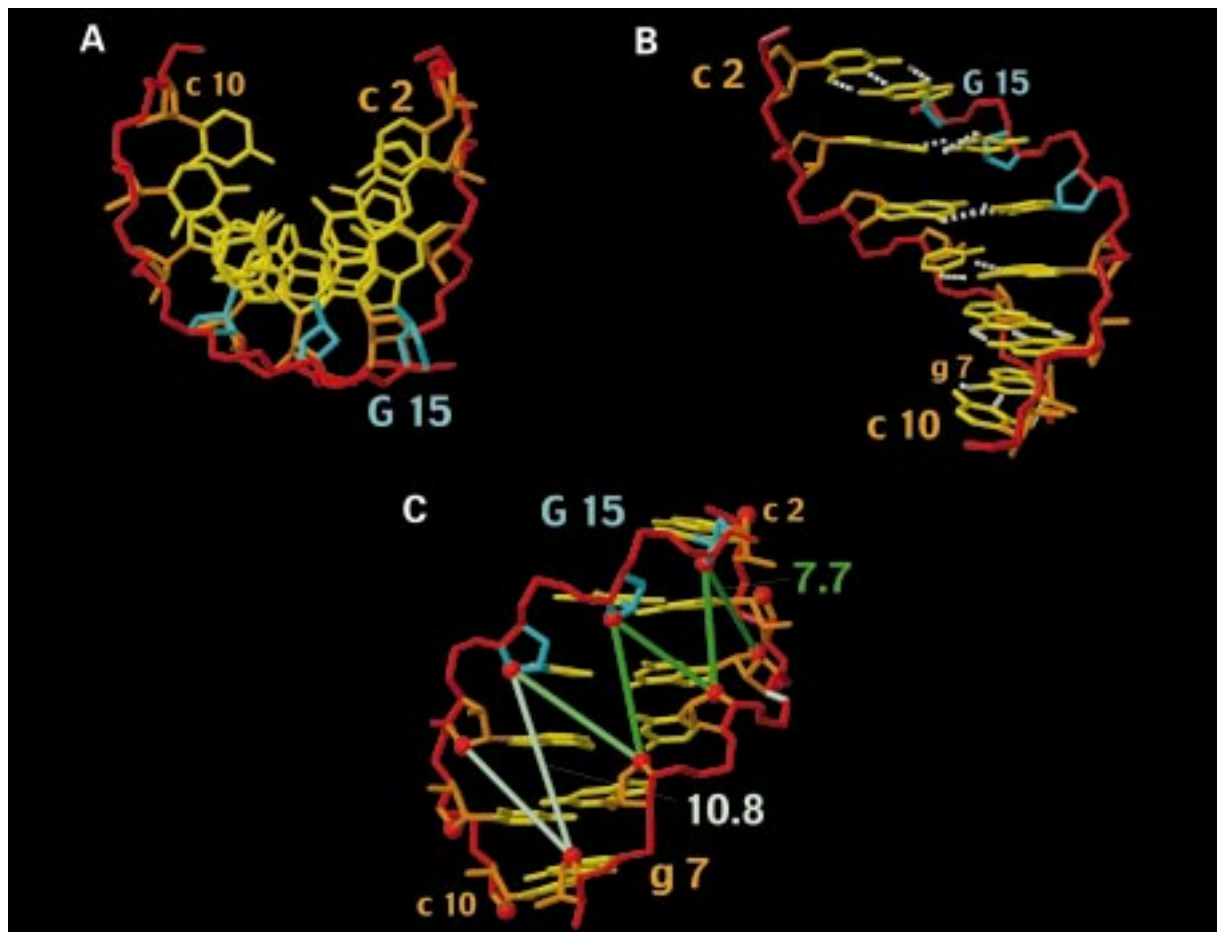
<sup>c</sup>Local parameters as defined in Babcock et al. (1994). For a comparison with global parameters see Hartmann and Lavery (1996).

#### Comparison with the crystal structure of *r(gcaguggc)·r(gcca)d(CTGC)*

A recent X-ray crystal structure of the presently investigated RNA/RNA–DNA hybrid (Mueller et al., 1998) differs markedly from our NMR solution structure. The crystal structure exhibits C3'-endo sugar conformations for all nucleotides, including the four deoxyribonucleotides. The sugar puckers, along with a small rise per base pair, an average twist value of 33° and a uniform minor groove width of 10.1 Å, indicate that the structure very closely resembles an ideal A-helix in the crystal. In contrast, the NMR structure shows a shift from an A-like to a B-like conformation in the chimeric strand, and a concomitant decrease in the minor groove width. Thus, unlike the crystallographic data, the NMR solution structure points to structural differences between the RNA/RNA and DNA/RNA segments.

Discrepancies between corresponding X-ray crystal and NMR solution structures are well documented for hybrid oligonucleotide duplexes. Crystal structures

of duplexes comprising a chimeric DNA–RNA strand and a complementary DNA strand exhibit nearly ideal A-conformations, irrespective of the number of ribonucleotides and their sequence locations in the chimeric chain (Egli et al., 1992, 1993). Remarkably, a single ribonucleotide has been shown to lock an entire duplex in an A-like conformation (Egli et al., 1993; Ban et al., 1994). In sharp contrast, NMR studies of DNA/DNA–RNA duplexes revealed that these adopt neither A- nor B-type conformations in solution (Fedoroff et al., 1993; Lane et al., 1993). For the crystal structure of the presently studied RNA/DNA–RNA duplex, Mueller et al. (1998) suggested that the RNA strand may induce the formation of the uniform A-conformation. The solution structure disagrees with this suggestion, and there are indications that apparent discrepancies between NMR solution and X-ray crystal structures are presumably attributable to crystal packing forces and dehydration during crystallization (Salazar et al., 1994; Wahl and Sundaralingam, 1995; Fedoroff et al., 1996).



**Figure 5.** Structure of  $r(gcaguggc) \cdot r(gcca)d(CTGC)$  represented by the energy-refined DYANA conformer with the lowest residual target function value. (A) View along the helix axis obtained after rotating the conformer from its orientation in Figure 4 by  $90^\circ$  about a horizontal axis in the projection plane. The large displacement of the bases from the helix axis is typical for an A-type duplex. The same color code has been chosen as in Figure 4. (B) View into the major groove obtained after rotating the conformer from its orientation in Figure 4 by  $180^\circ$  about a vertical axis in the plane. Hydrogen bonds are represented by white dashed lines to indicate the large propeller twists (Table 2) observed for the base pairs at the RNA–DNA junction ( $g_4 \equiv C_{13}$  and  $u_5 \equiv a_{12}$ ). (C) Same view into the minor groove as in Figure 4.  $O4'-O4'$  distances across the minor groove are indicated by green bars. The gradual change from light green to dark green indicates the narrowing of the minor groove when crossing the DNA–RNA junction. From top to bottom, the following  $O4'-O4'$  distance ranges (in Å) are observed for the 20 energy-refined DYANA conformers:  $G_{15}-g_4$ , 7.0–8.4;  $G_{15}-u_5$ , 6.7–8.1;  $T_{14}-u_5$ , 6.4–7.6;  $T_{14}-g_6$ , 7.0–8.4;  $c_{13}-g_6$ , 7.7–9.1;  $C_{13}-g_7$ , 10.0–11.5;  $a_{12}-g_7$ , 9.3–10.7. The averaged  $O4'-O4'$  distances are given for  $C_{13}-g_7$  (10.8) and  $G_{15}-g_4$  (7.7). The minor groove width can be estimated by subtraction of  $2.8 \text{ \AA}$  from these values (e.g. Han et al., 1997), i.e. the width decreases from about  $7.5 \text{ \AA}$  to  $4.5 \text{ \AA}$  when moving from the dsRNA to the hybrid segment. The  $O3'-P$  bond between  $g_4$  and  $u_5$ , which is primarily cleaved by RNaseH during the initiation, is located on the right and is indicated in white.

#### *Implications for the specificity of RT-associated RNaseH*

Structure determinations of DNA/RNA hybrid duplexes in solution (Lane et al., 1993; Fedoroff et al., 1993) as well as modeling studies (Nakamura et al., 1991; Fedoroff et al., 1993) have previously led to the hypothesis that the minor groove width of a duplex represents a key structural feature for specific interactions between RNaseH and its nucleic acid sub-

strates. In particular, it has been suggested that a minor groove width of about  $8\text{--}9 \text{ \AA}$  should be optimal (Fedoroff et al., 1993) for efficient recognition (Salazar et al., 1994, 1996; Zhu et al., 1995; Fedoroff et al., 1996, 1997; Horton et al., 1996; Han et al., 1997; Bachelin et al., 1998). The presently described solution structure of  $r(gcaguggc) \cdot r(gcca)d(CTGC)$ , which is the first structure determination of an RNA/RNA–DNA hybrid duplex in solution, now indicates alterna-

tive interaction mechanisms. Firstly, the minor groove diameter (Figure 5C) is only about 4.5 Å to 5.5 Å in the hybrid segment and at the RNA–DNA junction, respectively, where the RNA strand is cut by the RT-associated RNaseH during initiation of HIV-1 reverse transcription. Secondly, the minor groove width in the dsRNA segment is closer to the putative optimum, but cleavages within the dsRNA are exclusively seen in stalled RT–tRNA<sup>Lys3</sup>(+)/RNA complexes (Götte et al., 1995).

There are other recent studies that also indicate that the minor groove width may not necessarily be a key structural feature determining RNaseH specificity. Firstly, two structural studies have been pursued to shed light on the observation that the DNA/RNA hybrid corresponding to the polyribopurine tract (PPT) of the HIV genome is resistant to RNaseH digestion, where the two groups arrived at contradictory conclusions: one suggests that it is widening of the minor groove (Fedoroff et al., 1997), and the other claims that it is narrowing of the minor groove (Han et al., 1997) that can explain the resistance to RNase digestion. Secondly, no apparent relationship between the minor groove width and *E. coli* RNaseH cleavage sites could be identified in two recently published NMR solution structures of chimeric hybrid duplexes (Nishizaki et al., 1995).

It has also been proposed that not only the minor groove width but also bending of the double helix (Fedoroff et al., 1996) may confer RT RNaseH specificity at RNA–DNA junctions. In this respect it is of interest to compare the solution structure of d(GCAGTGGC)·r(gcca)d(CTGC) (Fedoroff et al., 1996), which includes the DNA–tRNA junction that is cleaved during the primer removal reaction, with the presently studied duplex r(gcaguggc)·r(gcca)d(CTGC). The two duplexes contain the same chimeric strand, which is annealed either with a DNA or an RNA strand. For d(GCAGTGGC)·r(gcca)d(CTGC), a wider minor groove width is observed in the hybrid segment when compared with the dsDNA segment. Hence, assuming that the RT RNaseH domain follows the polymerase during DNA synthesis as a part of an ‘elongation complex’ (Lanchy et al., 1996), inverse shifts in groove widths would have to be accommodated, i.e., the RNaseH domain would encounter a narrowing of the groove during the initiation of reverse transcription but a widening during the primer removal reaction. Considering that the minor groove widths at the respective cleavage sites are also different, the comparison of the

two structures indicates that the minor groove width may not, after all, be a decisive factor in determining RT RNaseH specificity. On the other hand, a large variation of the minor groove width along the sequence of the presently studied duplex (Figure 5C) suggests (Chuprina et al., 1991a) that it may be bent [A six base-pair segment is too short to reliably identify a bend. However, analysis with the program CURVES (Ravishankar et al., 1988) indicates a bend for all 20 energy-refined DYANA conformers]. A bend has been described for d(GCAGTGGC)·r(gcca)d(CTGC), and it is thus tempting to speculate (Fedoroff et al., 1996) that the preformation of a bend in the free duplex would lower the cost for the site-specific strand cleavages documented for RT RNaseH.

The available data thus show that the three-dimensional structure of an oligonucleotide duplex alone is not sufficient for predicting its susceptibility to RNaseH digestion, indicating that the protein–nucleic acid interaction may involve substantial conformational rearrangements of the nucleic acid, a view initially set forth by Nakamura et al. (1991). Accordingly, it is the increased malleability of the hybrid segment mediated, for example, by the inherently higher flexibility of deoxyribose versus ribose moieties, which allows for the adaptation of the hybrid duplex to the enzyme. Protein binding-induced changes in the sugar puckers of a DNA duplex retaining its B-conformation upon complex formation have very recently been demonstrated for an *Antennapedia* homeodomain–DNA system (Szyperski et al., 1998). This indicates that the specificity of RT RNaseH might be partly based on the conformational adaptability of deoxyriboses. Such an ‘induced-fit’ scenario is also indicated by the investigation of the backbone dynamics of *E. coli* ribonuclease HI (Mandel et al., 1995), which revealed increased flexibility for the polypeptide segments comprising the active site residues, and by a kinetic analysis of *E. coli* RNaseH (Kanaya et al., 1995).

Induced-fit interactions, facilitated by the inherent flexibility of DNA residues, might also explain the 50-fold increased rate for nucleotide incorporation which is observed once the RT has passed the initially bound RNA/RNA duplex and synthesizes the hybrid instead (Lanchy et al., 1996). In fact, the very recently determined X-ray crystal structure of the active DNA polymerase from *Bacillus stearothermophilus* showed that the sugar puckers of the terminal base pair change conformation upon incorporation of the subsequent nucleotide triphosphate (Kiefer et al., 1998).

With regard to RT, such a mechanism has not been proven yet, but the crystal structure of an RT-dsDNA complex (Jacobo-Molina et al., 1993) indicates that the enzyme enforces an A-conformation for the terminal six base pairs that are located in the vicinity of the polymerase active site. It has been suggested that the A-form is induced by dehydration of the nucleic acid in the polymerase active site (Ding et al., 1994), and an underwound A-form DNA has actually been identified in the active DNA polymerase from *Bacillus stearothermophilus*. In contrast, the oligonucleotide is comparably solvent-exposed at the RNaseH active site. Hence, induced-fit interactions between RT RNaseH and oligonucleotide substrates might additionally be modulated by the hydration of the oligonucleotide as it is manifested in solution (Liepinsh et al., 1992), i.e., RT RNaseH might distinguish dsRNA, RNA-DNA junctions and hybrid duplexes according to their distinct hydration patterns. Consistent with this hypothesis, it has recently been shown that ribose 2' OH groups serve as a scaffold for a water network in the minor groove that may be involved in protein-dsRNA interactions (Egli et al., 1996). Moreover, it has recently been suggested that the interaction of the minor groove of a given primer/template substrate with a structural motif dubbed the 'minor groove binding track' located near the polymerase active site of HIV-1 RT plays an important role for initiation of reverse transcription (Bebenek et al., 1997). Considering the remarkably narrow minor groove of the presently studied duplex, this interaction might likewise depend on an 'induced fit' being important for the transition from initiation to elongation of reverse transcription (Isel et al., 1996).

Finally, the action of RNaseH plays a key role for the antisense strategy for medical and biochemical applications (De Mesmaeker et al., 1995). Hence, the insights into RNaseH-oligonucleotide duplex interactions inferred from the present structural study might well support future rational design of antisense oligonucleotides.

### Acknowledgements

Financial support was obtained from the Schweizerischer Nationalfonds (project 31.32033.91), and the Bundesamt für Bildung und Wissenschaft (Nr. 97.0593) as a part of an EU Biomed concerted action (Nr. BMH4-CT 97-2641).

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