Stabilizing effects of the RNA 2'-substituent: crystal structure of an oligodeoxynucleotide duplex containing 2'-O-methylated adenosines

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Background: The stability of hybrids of 2'-O-methyl-ribonucleotides with complementary RNA is considerably higher than that of the corresponding DNA•RNA duplexes. The 2'-O-modified ribonucleotides are thus an attractive class of compounds for antisense applications. Understanding how these substituents stabilize the structure of the hybrid duplex may be important in the design of ribonucleotides with novel properties.

Results: The crystal structure of a dimer of the self-complementary DNA strand d(GCGT)$^{O2'me}$r(A)d(TACGC), which has a 2'-O-methylated ribonucleotide incorporated at position 5, was determined at 2.1 Å resolution. This strand forms a duplex with an overall A-type conformation; the methyl groups of the two modified adenosines point into the relatively wide minor groove. Both 2'-methoxy groups are hydrogen-bonded to solvent molecules. These results allowed us to build a model of a fully 2'-O-methylated RNA double helix.

Conclusions: Insertion of 2'-O-modified RNA residues into a stretch of DNA can nucleate a local A-type conformation, in part because modification with a bulky residue at this position stabilizes a C3'-endo type sugar pucker. The increased stability of fully 2'-O-methylated RNA may result from hydrophobic interactions between substituents in the minor groove. As the 2'-O-methyl groups are directed into the minor groove, it may be worthwhile to introduce tailor-made 2'-O-substituents into RNA; it might be possible to design groups that both stabilize the hybrid duplexes and carry a nuclease function, further improving the efficacy of these modified RNAs in antisense applications.

Introduction

Chemically modified DNA and RNA nucleotides may be useful in a number of ways. For example, modified antisense oligonucleotides may eventually be therapeutically useful, and site-specific chemically modified probes have been used to study nucleic acid-catalyzed processes and protein–nucleic acid interactions. An early modification of RNA was alkylation, in particular methylation at the 2'-hydroxyl group of the ribose moiety [1]. RNAs made from 2'-O-methylated nucleotides are conformationally more rigid and chemically more stable than natural RNA, and the thermal stability of their hybrids with unmodified RNA is superior to that of DNA•RNA hybrids [2,3]. Single 2'-O-methylated residues were used at either splice site of a nuclear pre-messenger RNA substrate to investigate the chemical mechansims of catalysis [4]. The minimum ribonucleotide requirement for catalysis by a RNA hammerhead domain was assessed by incorporation of deoxyribonucleotide residues, as well as 2'-O-methylated analogues [5]. Similarly, 2'-O-methylated nucleotides were introduced into stretches of both the DNA strand and the RNA-substrate strand of homo-DNA•homo-RNA hybrids in order to study the role of the 2'-hydroxyls in the recognition of such hybrids by RNase H and the mechanism of the subsequent cleavage of the RNA strand by the enzyme [6].

The 2'-hydroxyl group of RNA is important for a number of reasons, both for chemical reactivity and for conformational stability. It serves as the nucleophile in the first step of alkaline and ribonuclease-catalyzed RNA hydrolysis [7,8], and in hammerhead ribozyme-mediated cleavage reactions [9]. It has also been proposed that the 2'-OH group can activate the adjacent 3'-oxygen leaving group in cleavage reactions catalyzed by the *Tetrahymena* group I intervening sequence RNA [10]. The 2'-hydroxyl is an important recognition element in the interactions between enzymes and RNA [11,12]. Its importance for the conformation of RNA lies largely in its ability to stabilize the A-conformation of double-stranded RNA by acting as a donor in weak intra-strand hydrogen bonds with adjacent O4'-oxygens [13–17]. Furthermore, it acts both as a donor and an acceptor in a variety of intra- and intermolecular hydrogen bonding interactions that stabilize other secondary structural motifs of RNA, providing one basis for the polymorphic nature of RNA structure [13,18,19]. Moreover, the hydroxyl groups contribute to structure stabilization through bound water molecules, and can function as ligands for coordinating metal cations [20,21].

It is well established that electronegative substituents such as fluorine shift the conformational equilibrium of the sugar towards C3'-endo [22–24]. This shift is thought to
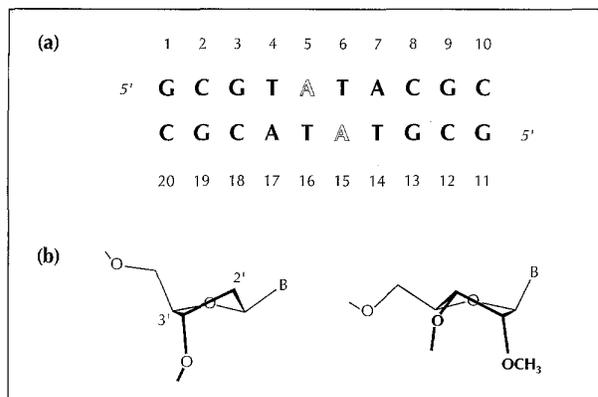
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Fig. 1. (a) Sequence and residue numbering of the self-complementary 2'-O-methylated RNA–DNA chimera. DNA residues are bold and 2'-O-methylated RNA residues are outlined. **(b)** Structures of the sugar moiety in DNA (left) and 2'-O-methylated RNA (right) with the typically adopted conformations C2'-endo (DNA) and C3'-endo (RNA).

stabilize an A-type conformation, and this presumably is one of the reasons for the improved hybridization properties of these as well as of 2'-O-alkylated analogues of RNA [2,3,25]. To date, however, structural details of the interactions of such substituents, particularly of alkyl-substituents, within oligomeric duplexes are lacking.

We have recently shown that chimeric DNA–RNA duplexes with as few as one ribonucleotide residue inserted into a DNA decamer adopt fairly regular A conformations in the solid state [17,19]. A number of hydrogen bonding interactions by the 2'-hydroxyl groups, both intra- and intermolecular, stabilize the A-type conformation and also affect lattice contacts and hydration. A single ribonucleotide residue in a stretch of DNA can thus nucleate an A-type conformation. We have now incorporated 2'-O-methylated residues into a stretch of DNA, crystallized the corresponding decamer and determined its three-dimensional structure by single crystal X-ray crystallography (Fig. 1). Although only partially methylated, the structure allows the analysis of the arrangement of the methoxy substituents in an A-type double helix, and thus in the environment presumably adopted by fully 2'-O-methylated RNA duplexes and hybrids between RNA and 2'-O-methylated strands. The structure of this DNA–RNA chimeric duplex may offer insight into a number of other questions, such as why the stabilizing effects of 2'-O-methylated purine and pyrimidine residues are different, and why there are differences between the conformational influences of 2'-hydroxy and -alkoxy sugar substituents.

Results and discussion
Overall features of the duplex
The chimeric decamer adopts a fairly regular A-type conformation, as indicated by a rather small root mean square deviation of 0.7 Å between its structure and that of an average A-DNA duplex from fiber diffraction data

[26]. The A-conformation very probably results from the incorporated 2'-O-methylated adenosines, as an all-DNA duplex with the same sequence showed B-DNA-like features in the crystal [19]. Thus it seems likely that either unmodified RNA residues or 2'-O-methylated RNA residues incorporated into a stretch of DNA can nucleate an A-type conformation that, at least in the solid state, is maintained for some distance. The van der Waals model of the duplex shows the large inclinations of base-pairs with respect to the helix axis (20° on average), as well as significant propeller twisting between paired bases (−10° on average), which are characteristic of A-DNA (Fig. 2). The values for the helical parameters observed are all within the ranges usually associated with A-type helices. The helix is slightly underwound (11.2 residues per turn) with average values for helical rise and twist of 2.46 Å and 32.8°, respectively. The displacements for base-pairs with respect to the helix axis indicate regular depth (~5 Å) and contour of the major groove. The coil-like arrangement of strands in the duplex renders the major groove narrow and deep, whereas the minor groove is relatively wide and shallow. Thus, the phosphates lie about 5.6 Å apart on average across the major groove. The average minor groove width based on distances between phosphorus atoms is 10.1 Å, whereas the width based on the distances between sugar O4' atoms from opposite strands is 6.1 Å (Fig. 2). Intra-strand distances between phosphates vary between 5.4 Å and 6.6 Å, with an average of 5.9 Å.

All the sugars in the decamer duplex, except for the deoxyribose of the terminal residue G(11) (C2'-exo), adopt the standard C3'-endo pucker. The backbone torsion angles for most residues fall into the ranges commonly observed for the A-type genus, namely (from α to ζ): $-sc$, ap, $+sc$, $+sc$, ap, $-sc$. The only exception is residue rA(5), which has an α of 115° and a γ of −167°; the other torsion angles also display some smaller differences from the average values. Variation of torsions α and γ usually coincides with an increased base-separation distance, consistent with a maximal helical rise of 3.05 Å at the rA(5) site. Alteration of torsions α and γ as a consequence of a crankshaft-type motion around β leads to a local transition from a folded to an extended backbone with an elongation of the intra-strand distance between phosphates to the 5'- and 3'-sides of residue rA(5) (see also [27]). This conformational variation does not greatly affect the distances between phosphates across strands. The minor groove of the decamer duplex is slightly wider in the center and narrower at both ends, however. The widening of the minor groove in the center of the duplex probably results from the packing arrangement in the lattice of the chimera, in which termini of duplexes are stacked into the minor grooves of neighboring double helices.

2'-O-methylated residues
The 2'-substituents of both modified adenosines point into the minor groove. Whereas the O2'-oxygen atoms are embedded into the sugar–phosphate framework

rimming the groove, the methyl groups are directed away from the sugars, causing a local narrowing of the groove (Figs. 2 and 3). Both methoxy groups adopt *anti* orientations. The distance between the methyl carbon atoms across the minor groove is 8.8 Å (subtracting 4 Å, the assumed sum of van der Waals radii for methyl groups). The distance between the 2'-oxygen atoms of the two 2'-*O*-methylated RNA residues is 14.5 Å.

The arrangement of the methoxy groups is very similar in the two 2'-*O*-methylated adenosines. The O2'-C2M (methyl carbon) bonds are oriented antiperiplanar relative to the C2'-C3' bonds. The C3'-C2'-O2'-C2M torsion angle is 173° for residue rA(5) and 175° for residue rA(15). The glycosidic angles (χ, defined as O4'-C1'-N9-C4 for purine nucleosides) for residues rA(5) and rA(15) are −160° and −159°, respectively. The improper torsions O4'-C1'-O2'-C2M are 136° for both 2'-*O*-methylated adenosines. This puts the methyl carbons more or less within the plane defined by the adenine base atoms. The angle between the vector defined by atoms O2' and C2M and the adenine base plane is 22° for residue rA(5), and 21° for residue rA(15) (Fig. 3a).

In the crystal structure of 2'-*O*-methyladenosine, the torsion angles H2'-C2'-O2'-C2M are 23° and 28° for the two independent molecules [28]. These angles deviate significantly from the nearly staggered conformation found in the present crystal structure. Unlike in the free ribonucleoside, the arrangement of methoxy groups in the hybrid duplex is significantly restricted by the presence of the neighboring nucleotides in the strand. Thus, the methoxy groups of both residues are in van der Waals contact with intranucleotide base atoms and oxygen O4' of the adjacent nucleotide in a 3'-direction. In residue rA(5), N3 lies at a distance of 3.48 Å, whereas O4' of residue T(6) is located at a distance of 3.57 Å from C2M. Similarly, in residue rA(15), N3 lies at a distance of 3.57 Å, whereas O4' of the adjacent residue, T(16), is located at a distance of 3.47 Å from C2M. Staggered arrangements around the C2'-O2' bonds prevent repulsive contacts between the methyl carbons and the adjacent sugar moiety and permit them to nestle against the edges of adenine bases in the minor groove (Fig. 3b). Partial destabilization caused by the loss of hydration of O2' through shielding of potential hydrogen-bond accepting functions, such as N3 in the minor groove, may be compensated for by close lipophilic contacts between the methyl group and the base.

Hydration of the methoxy groups

The 2'-oxygens of both modified adenosines form hydrogen bonds with solvent molecules. The arrangement of water molecules around the 2'-substituent and the base in the minor groove for these residues is shown in Fig. 4a and b. In the case of rA(5) the long distance between W47 and O2' indicates a very weak hydrogen bond. This water molecule participates in a hydrogen bond with a

phosphate group from an adjacent duplex (W47⋯O-P = 3.18 Å), and the negatively charged phosphate may pull it away from the methoxy oxygen. Three water molecules are located near the methoxy oxygen of residue rA(15), and the oxygen is strongly hydrogen bonded to water molecule W29 (Fig. 4b). This water molecule does not form any contacts with other DNA atoms. Two more water molecules are located further away from the 2'-oxygen, and they also form strong hydrogen bonds with acceptors from neighboring duplexes.

Methylation of the RNA 2'-hydroxyl group prevents it from acting as a donor in hydrogen bonding interactions; however, it can still act as an acceptor in various types of hydrogen bonds. In RNA duplexes the 2'-hydroxyl group often acts as a donor in hydrogen bonds with the adjacent furanose oxygen in a 3'-direction. In addition, water-mediated hydrogen bonds can form between the hydroxyl group and the 3'-phosphate oxygens and the 5'-oxygen of the next residue, respectively, as well as between the O2' and base functions such as purine-N3 or pyrimidine-O2 [14]. Of all these interactions, only
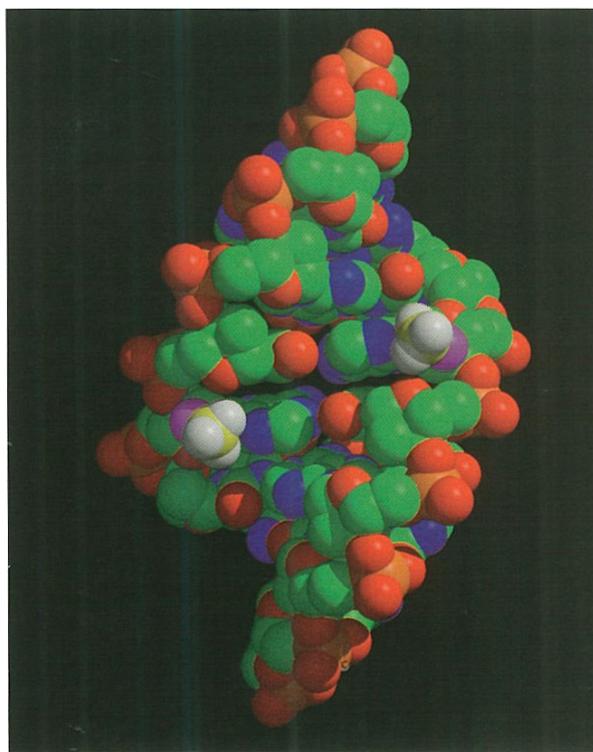

Fig. 2. Van der Waals representation of the decamer duplex [d(GCGT)$^{O2'me}$r(A) d(TACGC)]$_2$. The view is into the minor groove, and the 3'-terminal cytidine residues C10 and C20 are located at the upper left and the lower right, respectively. The drawing shows the orientation of the methoxy groups with respect to the backbone and the minor groove. Their presence results in a marked local narrowing of the relatively wide groove in the A-type duplex. Carbon atoms are green, nitrogen blue, oxygen red, and phosphorus orange. The 2' substituents are highlighted in white (hydrogen atoms), yellow (carbon) and purple (oxygen). The standard van der Waals radii were slightly reduced for the sake of clarity.
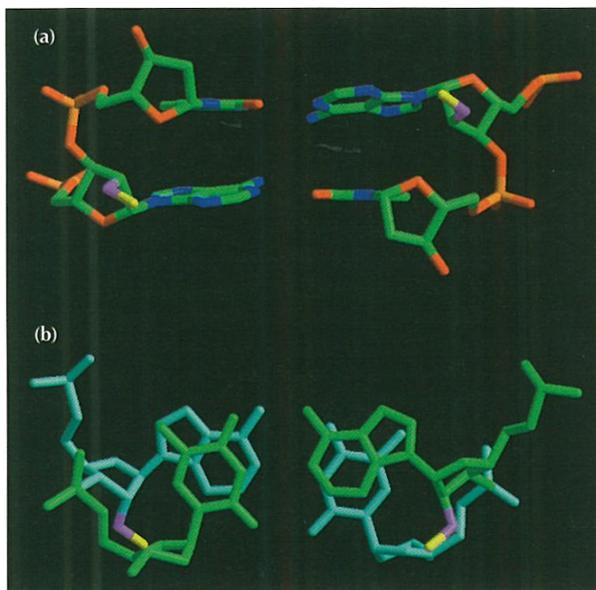
Fig. 3. (a) Projection of the base-pair step $^{O2'me}r(A5) \bullet d(T16)$-$d(T6) \bullet ^{O2'me}r(A15)$ roughly along the planes of base-pairs, looking into the minor groove. The methoxy groups of the RNA residues adopt an *anti* arrangement with respect to the ribose moieties, leading to an approximate *trans* orientation of the O2'-C2M and C2'-C3' bonds. The distance between the two methyl carbon atoms across the minor groove is 8.8 Å (subtracting 4 Å, representing the sum of the van der Waals radii for methyl groups). Note that the distance between hypothetical methoxy substituents along the narrowest dimension of the minor groove in a double-stranded 2'-O-methylated RNA molecule will be considerably shorter. Carbon atoms are green, nitrogen blue, oxygen red, and phosphorus orange. The 2'-substituents are highlighted in yellow (carbon) and purple (oxygen). **(b)** Projection of the same base-pair step roughly along the perpendicular to the top base-pair $^{O2'me}r(A5) \bullet T(16)$. The two methoxy groups are approximately parallel with respect to adenine base planes. For residue $^{O2'me}r(A5)$, the distance C2M–C4 is 4.03 Å, and for residue $^{O2'me}r(A15)$ it is 4.12 Å. For purine residues, methylation at the 2'-oxygen of ribose may thus result in a weak stabilization through lipophilic contacts to bases in the minor groove. The top base-pair residues are drawn in green and the more distant base-pair residues are light blue. The 2'-substituents are highlighted as in (a).

the one between the O2' and the adjacent O4' requires the presence of a hydroxyl group with donor functions. Upon 2'-O-methylation, water-mediated hydrogen bonds between the O2' and backbone oxygens or the 3'-phosphate group are still feasible, whereas hydrogen bonding to base functions through a water molecule in the minor groove is degenerate because of the bulky methyl group in the vicinity of the base (Figs. 2, 3 and 4). The methyl group and the adjacent purine base atoms form a small lipophilic patch on the border of the minor groove that will lead to the exclusion of water molecules at this site. A more subtle difference between the hydration of hydroxyl and methoxy substituents may result from the different preferred distribution of donors around the oxygen lone pairs [29], and one can expect the arrangement of water molecules around the hydroxyl group to be geometrically less restricted.

Although a water-mediated interaction between O2' and adenine N3 is prevented by the arrangement of the methyl group in the 2'-O-methylated adenosines, the N3 atoms of residues rA(5) and rA(15) are both hydrogen-bonded to water molecules (Fig. 4a,b). Besides contacting O2' of rA(5), W63 forms an additional hydrogen bond with N3 of the adjacent residue A(17) from the opposite strand (distance 3.06 Å). Such water bridges between base functions across strands in the minor groove of DNA duplexes are not unusual [30]. In the case of residue rA(15), W21 is engaged in two additional hydrogen bonds with atoms N2 and N3 of residue G(1) in the minor groove of an adjacent duplex (distances 3.29 Å and 3.13 Å). An analysis of the distribution of water molecules around DNA bases in A- and B-type duplexes indicates that the positions of water molecules hydrogen-bonded to N3 are not restricted to the direction of the nitrogen lone-pair vector within the base plane, but are slightly shifted to the center of the groove, away from C4 and N9 [31]. This is similar to the arrangement of water molecules around the bases of residues rA(5) and rA(15) (Fig. 4a,b). Thus, adenine hydration may not be affected by the presence of the methoxy group in the minor groove.

Comparison of purines and pyrimidines

Pyrimidine O2 and purine N3 atoms are hydrated to a similar extent in the minor grooves of A- and B-form duplexes [32]. However, analysis of the spatial distribution of water molecules around O2 of pyrimidine residues in the minor groove of A- and B-form duplexes reveals that, rather than lying along the direction of the sp^2-hybridized lone-pair vector in the plane of the base, in the majority of cases water molecules are shifted towards the side of N1, closer to the glycosidic bond [31]. Such an arrangement would result in a conflict between the water molecule and the methyl group (residue T(16), Fig. 4c). Hydration of pyrimidines in the minor groove may thus be more seriously affected than that of purines by the presence of the methoxy substituent. Thus, we speculate that the effect of 2'-O-methylated pyrimidines on duplex stability will be different from that of 2'-O-methylated purines.

Model of an all-2'-O-methylated RNA duplex

Knowledge of the arrangement of the 2'-substituents in the base-pair step containing the 2'-O-methylated adenosines should yield a tentative picture of a fully 2'-O-methylated strand in a double helical conformation, as well as of a fully 2'-O-methylated RNA double helix. Using the averaged C3'-C2'-O2'-C2M torsion angles from residues rA(5) and rA(15), methoxy groups were attached to all residues of the decamer duplex using the molecular modeling program MOMO (MacIntosh, version III, ETH-Zürich, Zürich, Switzerland). This approach seems reasonable, because the sugar puckers for all residues are within the C3'-*endo* range, and RNA duplexes are commonly believed to adopt relatively uniform A-type conformations.
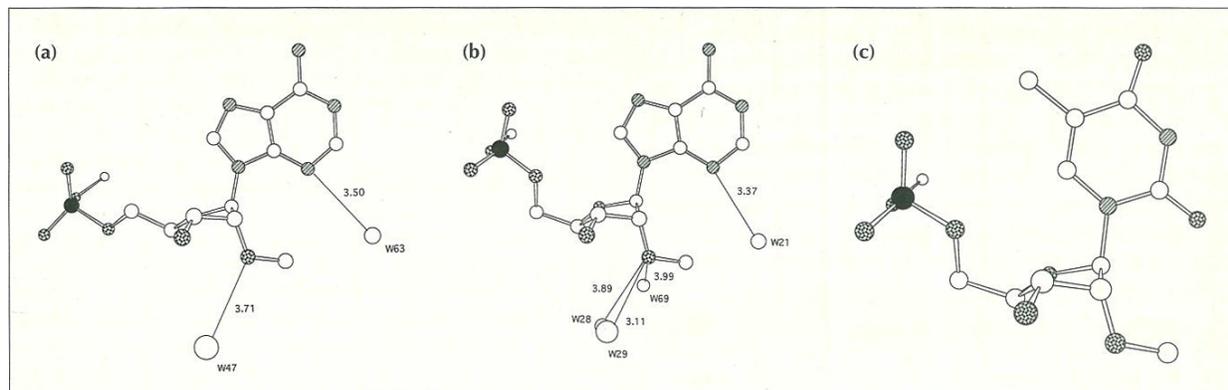
Fig. 4. Hydration of the 2′-*O*-methylated adenosines in the minor groove and comparison of the relative arrangements of 2′-methoxy substituent and base for purine and pyrimidine nucleotides. **(a)** Residue rA(5); **(b)** residue rA(15); **(c)** hypothetical geometry of thymidine T(16) based on the arrangement of the 2′-methoxy substituent observed for adenosines. All views are approximately along the normal to the base plane. Nucleotides are drawn with open bonds, phosphorus atoms are black, oxygen atoms are hatched, nitrogen atoms are dashed, and carbon atoms are empty circles. Water molecules are numbered and are drawn as larger circles. Hydrogen bonds are thin solid lines with distances in Å.

A comparison between the A-form duplex described in this paper (methoxy groups of residues rA(5) and rA(15) removed) and an all-2′-*O*-methylated A-form duplex is depicted in Fig. 5. From the view into the minor groove of the 2′-*O*-methylated duplex, it is apparent that the closest contacts between methyl groups across strands occur in a direction approximately perpendicular to that of the minor groove. The average inter-strand distance between methyl groups normal to the minor groove in the modeled duplex is 6.4 Å with a standard deviation of
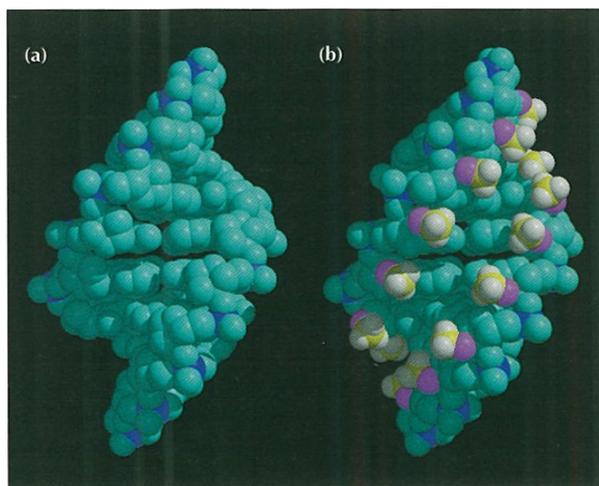

Fig. 5. Comparison of a modeled 2′-*O*-methylated RNA duplex with a model of an A-form DNA duplex. **(a)** Van der Waals model of an A-form DNA duplex; **(b)** van der Waals model of a uniformly 2′-*O*-methylated RNA duplex with sequence GCGUAUACGC, based on the conformation of the two 2′-*O*-methylated adenosine residues observed in the present crystal structure. The two views are into the minor groove, illustrating the narrowing of this groove as a consequence of the 2′-methoxy substituents. 2′-Oxygen atoms are purple; 2′-*O*-methyl carbon atoms are yellow, and methyl hydrogen atoms are white. Carbon, nitrogen and oxygen atoms are cyan, and phosphorus atoms are dark blue.

0.9 Å. This corresponds to a distance of 2.4 Å when the sum of van der Waals radii for the methyl groups (4 Å) is subtracted. The shortest contact, which is 4.6 Å, occurs between residues T(4) and C(19) (see upper right in Fig. 5b). Thus, these methyl groups are nearly in van der Waals contact, assuming a standard A-type geometry with the usual conformational fluctuations. For intra-strand contacts a somewhat similar picture emerges. The average distance between methyl carbon atoms from neighboring residues within strands is 6.6 Å with a standard deviation of 0.4 Å (2.6 Å when taking the sum of van der Waals radii for the methyl groups into account). Here, the closest contact (6.0 Å) occurs between residues G(13) and T(14). Closely spaced methyl groups within and across strands in a stretch of double-stranded 2′-*O*-methylated RNA may partly account for the increased stability of such duplexes compared with RNA. It is also evident that contacts between methyl groups within strands could contribute significantly to the increased stability of hybrid duplexes between all-DNA strands and uniformly 2′-*O*-methylated strands relative to the corresponding DNA duplexes.

Significance

Antisense RNA has been used in several model systems to inhibit the expression of a specific gene product, thus creating a dominant negative mutation in that gene. Analogous technologies may be useful in the treatment of several human diseases, but in many cases successfully inhibiting expression of the target gene product requires the delivery of large amounts of RNA into cells. This requirement is often prohibitive, because unmodified RNA is chemically unstable, and is susceptible to degradation by cellular nucleases. RNAs made from 2′-O-methylated nucleotides, however, are conformationally more rigid and chemically more stable than natural RNA, and are more

resistant to degradation by nucleases than is DNA [33]. Hybrid duplexes of 2'-O-methylated RNA with unmodified RNA are more stable than those of RNA and DNA, suggesting that such modified RNAs may be useful in antisense technology. However, hybrids between 2'-O-methylated RNA and RNA are not cleaved by RNase H [6]. Models of the hybrid–enzyme complex have been used to propose chemical and steric reasons for the resistance of these analog hybrids to enzymatic cleavage [11,34,35].

To better understand the differences between modified and unmodified RNA, and how different substituents may vary in their effects, we determined the structure of a short DNA duplex with two 2'-O-methylated adenosines incorporated into it. The methyl groups in the investigated duplex point into the minor groove and cause a marked local narrowing of that groove. Larger substituents such as long alkyl chains are known to destabilize the duplex structure [3]; this is probably mainly caused by crowding in the minor groove and distortion of surface hydration. Thus, it might be possible to design 2'-O-substituents with more polarizable groups that could hydrogen-bond with minor groove base functions and solvent molecules. This would allow the incorporation of larger substituents without destabilizing the duplex. Such substituents might completely bridge the minor groove, and could even be designed to carry nuclease function.

Our structure may also eventually provide insight into the observation that incorporation of 2'-O-methylated residues at the flanking sequences of a hammerhead-type ribozyme resulted in improved catalytic activity [36]. The 2'-modified nucleotides have proved to be useful tools in the past, for instance in mapping the interactions between ribonuclear proteins and pre-mRNA [37]. Such modifications could also be helpful for RNA structure determinations in solution, as the additional 2'-O-methyl protons may facilitate NMR assignments.

Materials and methods
Synthesis, purification and crystallization
2'-O-methylated RNA A-amidite was synthesized and purified according to standard procedures [33]. The chimeric decamer was synthesized in a 15 μmol scale on a DNA synthesizer (Applied Biosystems Inc. 394A-08), using commercially available DNA amidites. After detritylation, the oligomer was deprotected in dilute ammonia at 55 °C overnight. The crude decamer was purified by HPLC using a preparative RP-C4 column (Rainin-Dynamax 300A, 12 μm particle size) in 0.1 M triethylammonia acetate (TEAA) pH 7.0; an acetonitrile gradient was used to elute the oligonucleotide. The column was kept at 45 °C. The final purity of the oligonucleotide was > 95 %. After solvent evaporation

and microfiltration, the concentration of the decamer was adjusted to 14 mM. Crystals were grown at room temperature in sitting drops, using the vapor diffusion technique. The initial concentrations in the drop were 2.6 mM DNA (single stranded), 29 mM sodium cacodylate (pH 6.5), 7 mM magnesium chloride, and 9 mM spermine tetrahydrochloride. The reservoir was 20 ml 40 % (vol/vol) 2-methyl-2,4-pentanediol (MPD). Crystals began to appear in only one droplet after a period of several months, following an initial precipitation; however, crystal growth was reproducible under conditions identical to those established in this one case. The biggest crystal grew to a size of about 1.0 x 0.25 x 0.25 mm and was used for precession photography and data collection. The space group is orthorhombic $P2_12_12_1$ with cell constants $a = 25.13$ Å, $b = 45.65$ Å and $c = 47.55$ Å.

Data collection, structure determination and refinement
Data were collected at room temperature on a Nicolet-Siemens multiwire area detector, mounted on an Elliott–Marconi GX21 rotating anode generator, equipped with a graphite monochromator (Cu K_{α}, focus 0.3 mm). The generator was operated at 34 kV and 90 mA. The crystal–detector distance was 12 cm, the framesize was 0.25° and the exposure time per frame was 120 s. The detector was swung out to a maximum of 24°. A total of 9 702 reflections were collected up to a resolution of 2 Å. Data were merged to 3 576 unique reflections with an R-factor of 3.7 %. These constitute 90 % of the 3 990 theoretically measurable reflections within this resolution range. The completeness of the data is >98 % (2.5 Å, resolution in parentheses), >91 % (2.2 Å), >81 % (2.2–2.1 Å) and >56 % (2.1–2.0 Å). Data were corrected for Lorentz and polarization effects, but no absorption correction was applied. The structure was solved with the molecular replacement method, both with an A-type duplex model [19] using program ULTIMA [38], and with a standard A-DNA duplex [26] using program AMORE (The CCP4 Project, SERC Daresbury Laboratory, Warrington, UK). Including data in an initial resolution range of between 25 and 8 Å, the model was properly oriented, and the resolution of the included data was then increased in 1 Å-steps up to a resolution of 2 Å. The R-factor after several rounds of rigid body refinement was 41 %. The DNA duplex was subjected to Konnert–Hendrickson-type restraint least-squares refinement with program PROLSQ [39], as modified for nucleic acids [40]. The relatively tight initial restraints were loosened as more reflections were included and at a resolution of 2.4 Å and an R-factor of 29 %, sum $(2F_o-F_c)$ and difference (F_o-F_c) electron density maps were calculated with program FFT (The CCP4 Project, SERC Daresbury Laboratory, Warrington, UK), and displayed on a Silicon Graphics Indigo 2 graphics computer with program Chain (version 5.4 [41]). These showed superimposed sum and difference electron density around the ribose moieties of both modified adenosine residues. Methoxy groups were then incorporated and refinement was continued for several rounds. A value of 1.41 Å was used for O2'-CH$_3$ (C2M) bond lengths and the methoxy groups were further restrained by definition of a 1–3 type distance of 2.38 Å between C2' and the methyl carbon atom. These numbers were taken from the crystal structure of 2'-O-methyladenosine [28]. Water molecules were placed into regions of well-defined superimposed sum and difference densities, followed by several cycles of restraint least-squares refinement. The final R-factor is 19.3 % using 3 188 reflections with $F_o > 2\sigma(F_o)$ between 10 and 2 Å resolution. The asymmetric unit contains 408 DNA atoms and 53 water molecules. The

maximum deviations for bond lengths are smaller than 0.02 Å and maximum deviations for bond angles are smaller than 3°.

Coordinates have been deposited with the Brookhaven Protein Data Bank, entry identification code 168D.

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