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Transmission of the conformational information in the antisense/RNA hybrid duplex influences the pattern of the RNase H cleavage reaction†

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Abstract

A set of four hybrid duplexes formed by antisense oligos, each containing one North-form (3′-*endo*) locked 1-(1′,3′-*O*-anhydro-β-D-psicofuranosyl)thymine block **T** at different sites and a matched 15mer RNA, were subjected to the RNase H cleavage reaction, showing how the transmission of the local conformation owing to a single North-locked sugar steers the conformational changes of the four neighbouring nucleotides.

It is known that for eliciting RNase H activity,[1] the antisense oligonucleotide (AON) in the AON/RNA hybrid duplex should retain the B-type DNA conformation with South-type sugar, while the RNA moiety should retain its A-type helix character with North-type sugar.[2] The AONs with one or more conformationally locked nucleoside residues (in the N-[3] or S-[4] form) have been an area of considerable interest.[5–7] The N-form constrained nucleoside containing AONs has been found, however, to be most promising because it drives the AON helix to the A-type resulting in an RNA/RNA type duplex, which accounts for their higher affinity to the target RNA,[5–7] but this leads to the loss of RNase H activity.[3c,6,7] For example, the introduction of the conformationally constrained (N)-methanocarba-thymidine residue in the North-form[3a] increased the thermodynamic stability of the AON/RNA duplex, whereas with (S)-methanocarba-thymidine in the South-form,[4a] a destabilizing effect was observed. Multiple introduction of (N)-methanocarba-thymidines increased the thermodynamic stability of the AON/RNA duplex, but failed to elicit any RNase H activity.[3c]

It is now quite clear[5a,6] that all modifications that lead to a preferential North-type sugar, including its constrained form, in an RNA-type AON result in the loss of RNase H activity, because they resemble an RNA/RNA duplex, except when they appear at the termini or in the middle in the gapmer-AON.[7]

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† Dedicated to Professor C. B. Reese (FRS) on the occasion of his 70th birthday.

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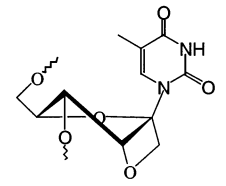
It has been assumed so far that probably three or four North-type conformational repeats are necessary to enhance the thermal stability of RNA-type AON/RNA duplexes.[5a] Clearly, introduction of a few North-type constrained nucleosides in an AON is perhaps necessary to enhance the binding specificity to the target RNA, yet their numbers have to be restricted somehow to be able to elicit an RNase H response. At this time, however, nobody knows where the balance is for accomplishing tighter binding and target specificity to the RNA without losing the conformational tolerance of the RNase H substrate specificity, owing to the *local* structural perturbances in a RNA-type AON/RNA hybrid.

We show here by a combination of CD spectroscopy and RNase H degradation studies on a set of hybrid AON/RNA duplexes, consisting of a series of analogous AONs (**2**)–(**5**), modified by a single bicyclic psiconucleoside with fixed North-sugar conformation, as in **T**, and the complementary target RNA (**6**), that the *local structure perturbance upon introduction of a single North-type constrained **T** spreads up to four nucleotides toward the 5′-end of the modification site*, neither producing any global helical conformational change nor reducing any overall RNase H activity compared with the native hybrid duplex: [(**1**)+(**6**)].

AON (1): 3'-d(CTTCTTTTTTACTTC)-5'
AON (2): 3'-d(CT**T**CTTTTTTACTTC)-5'
AON (3): 3'-d(CTTCT**T**TTTTACTTC)-5'
AON (4): 3'-d(CTTCTTT**T**TTACTTC)-5'
AON (5): 3'-d(CTTCTTTTT**T**ACTTC)-5'

Target **RNA (6)**: 5'-r(GAAGAAAAAAUGAAG)-3'

T =



The bicyclic psiconucleoside, 1-(1′,3′-*O*-anhydro-β-D-psicofuranosyl)thymine[8] moiety, **T**, with the locked 3′-*endo* conformation ($J_{4',5'} = 8.4$ Hz)[9] has been systematically incorporated at different sites in a set of AON sequences [(**2**)–(**5**)] targeted to the coding region [oligo-RNA (**6**)] of the SV-40 large T Antigen.[10] All oligos have been prepared by the phosphoramidite approach[11] on an automated DNA/RNA synthesizer and deprotected at room temperature to give AONs (**2**)–(**5**) (*caution:* opening of the oxetane ring in **T** takes place upon ammonia treatment at 55°C).

The T_ms[12] of the native and modified AON/RNA hybrid duplexes are as follows: 44.5°C for AON (**1**)/RNA (**6**), 37.7°C for AON (**2**)/RNA (**6**), 39.5°C for AON (**3**)/RNA (**6**), 39.7°C for AON (**4**)/RNA (**6**) and 39.3°C for AON (**5**)/RNA (**6**). It can be seen that the T_ms of the modified AONs (**2**)–(**5**)/RNA (**6**) hybrid duplexes are 4.8–6.8°C lower than the native counterpart, (**1**)+(**6**). Interestingly, the T_m differences of AONs (**3**)–(**5**)/RNA (**6**) are within 5±0.2°C around the middle modifications, whereas the modification at the 3′-terminus, AON (**2**), shows a T_m reduction of 6.8°C. The moderate reduction of T_m of the modified hybrids compared with the native hybrid may suggest (along with very comparable rates of the degradation of the target RNA by RNase H in both native and modified hybrids, vide infra) that the 2′,3′-oxymethylene bridged nucleoside moiety, **T**, has most probably introduced only a local conformational heterogeneity[5a] in the AON/RNA hybrid structure in view of the fact that a mismatch in a DNA/RNA duplex normally results in a 12–18°C loss of T_m.[3e]

Fig. 1 shows superimpositions of the CD spectra of all four single modified AONs (**2**)–(**5**)/RNA hybrid duplexes along with the native AON (**1**)/RNA, a typical RNA/RNA and a DNA/DNA duplex. All modified AON/RNA hybrid duplexes AONs (**2**)–(**5**)/RNA exhibited CD spectra intermediate between the native A type RNA/RNA and B type DNA/DNA duplexes, mimicking those of the natural AON (**1**)/RNA hybrid duplex. The spectra of the

modified AONs (**2**)–(**5**)/RNA and the native AON/RNA hybrid duplexes had a positive band at 263–267 nm, a negative band at 240–245 nm and a crossover point at 247–250 nm. These data suggested that the single modification by the conformationally constrained **T** nucleotide in various sites of the AONs (**2**)–(**5**) did not alter the global helical conformation of the resulting hybrid compared with the native DNA/RNA counterpart. This is also an indication of the potential ability of the AONs (**2**)–(**5**)/RNA hybrids to recruit RNase H (see Fig. 1) in a similar manner as that of the native structure.

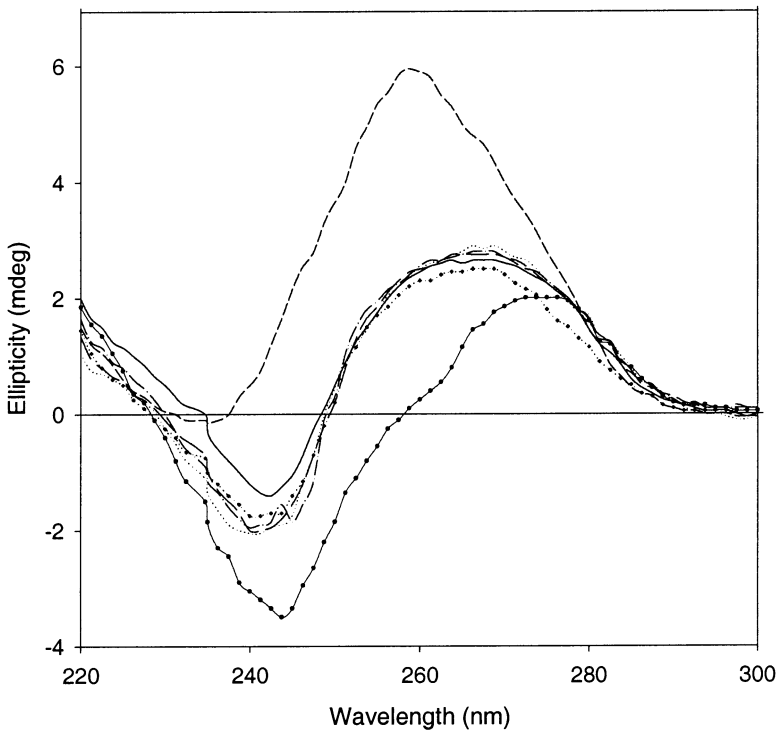


Figure 1. CD spectra of the duplexes formed by the AONs (**1**)–(**5**) and RNA target (**6**): (·····) (**1**), (—) (**2**), (-----) (**3**), (··+··+··) (**4**) and (–·–·–·–) (**5**). For comparison, typical B- and A-type spectra are presented: (–•–•–•–) DNA/DNA duplex formed by AON (**1**) and complementary DNA: 5′-d(GAAGAAA-AAATGAA)-3′, (----) RNA/RNA duplex formed by self-complementary 17mer RNA: 5′-r(UAACAUGUUUGGACUCU)$_2$-3′

To check the ability to elicit RNase H, the 5′-^{32}P labeled 15mer RNA target (**6**) was hybridized with complementary AONs (**2**)–(**6**) and incubated with *Escherichia coli* RNase H1 at 21°C, aliquots were taken after 30, 60 and 120 min, analysed by PAGE, and the extent of the hydrolysis was estimated from the residual full length RNA left after a given incubation time. The cleavage sites and the products were identified by comparison with the partial snake venom phosphodiesterase digest ladder of the target RNA. Results are shown in Figs. 2 and 3: after RNase digestion for 60 min, the PAGE showed that all modified AONs (**2**)–(**5**) were hydrolysed at similar rates compared with the native counterpart (**1**), and the target RNA digestion was complete after 120 min for the native AON/RNA, as well as for the modified AON/RNA hybrids, despite the fact that AONs (**2**)–(**5**)/RNA (**6**) had lower T_ms than the native counterpart, (**1**)+(**6**).

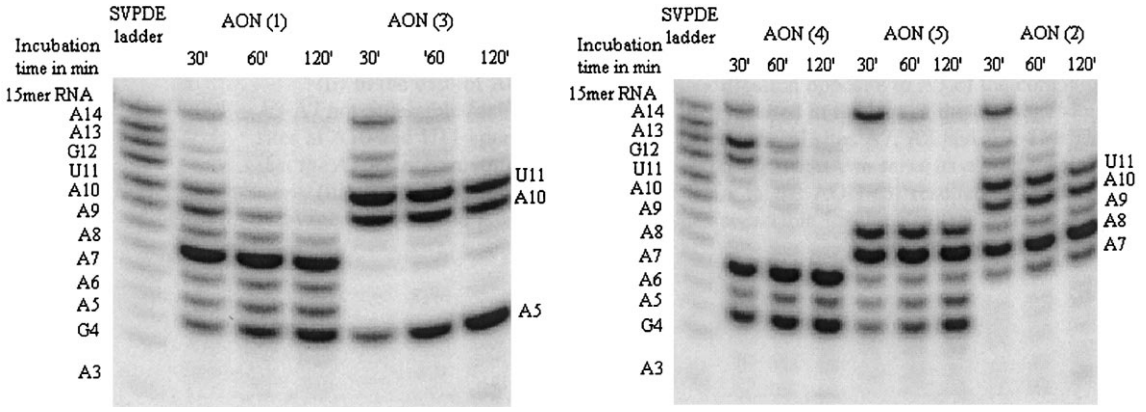


Figure 2. The PAGE analysis of RNase H hydrolysis of the hybrid duplexes, AON (**1**)–(**5**)/RNA (**6**), after 30, 60 and 120 min of incubation. The exact footprint and the assignment of the RNA cleavage sites in the hybrid duplexes can be deduced by the partial digest of the target RNA by Snake venom phosphodiesterase (SVPDE)

Following comparison (Figs. 2 and 3) of the cleavage sites of the AON/RNA hybrids compared with the native counterpart sheds light on how the local structure differences dictate the substrate specificity, recognition and cleavage properties by RNase H:

1. In the native hybrid duplex, [(**1**)+(**6**)], the major cleavage sites (after 120 min) were found to be at the 3′-phosphate of A5 and A8 (shown by an arrow in Fig. 3), and the minor cleavage sites were at the 3′-phosphate of A6 and A7. However, after a digestion for 30 min, longer 9-11mer RNA species were detected as a result of the cleavage at A9, A10 and U11, which were later degraded further to shorter fragments. *This shows that the whole region from A5 to U11 in the RNA of the native duplex was accessible for RNase H promoted cleavage.*
2. In the case of AON [(**2**)+(**6**)], modification opposite to A3 of the complementary RNA made the A3–A7 region inaccessible for RNase H cleavage, which resulted in the loss of the cleavage sites at A5 and A6. Instead, major sites at A10 and U11 appeared in addition to the preserved sites A7, A8 and A9. *This shift of the cleavage sites shows that the A3–A7 region, although not accessible to the cleavage, can serve as a binding site for the enzyme.*
3. A modification opposite to A6 of RNA in the AON (**3**) resulted in complete loss of RNase H promoted cleavage in the A6–A10 region and the only sites accessible for the cleavage were at A5, A10 and U11 situated on the edges of the A6–A10 region. *This shows that there are at least two binding sites available in this hybrid in order to cleave the RNA both at the A5 site as well as A10/U11 sites.*
4. This kind of RNase H recognition of the structural perturbances was also found in the cleavage behaviour of AON (**4**)/RNA hybrid (modification opposite to A8 site in RNA), where further shift of the cleavage sites was observed. The cleavage sites after 120 min were found to be at A5, A6 and A7. However, after a digestion for 30 min, the cleavages at G12 and A13 were revealed, showing that *the A13–G15 region is accessible for the cleavage reaction, which again proves that the A8–G12 region affected by the modification is not accessible for cleavage, but can serve as a binding site for the enzyme.*
5. As expected, the AON modification opposite to A10 of the complementary RNA, as in AON (**5**), resulted in the absence of any cleavage sites in the region between A10 and G15, while regaining all the cleavage sites from A5 to A9 present in the native hybrid duplex. Interestingly, AON (**5**) *retains the major cleavage sites of the native duplex while five basepairs from A10 to A14 were insensitive to RNase H promoted cleavage.*

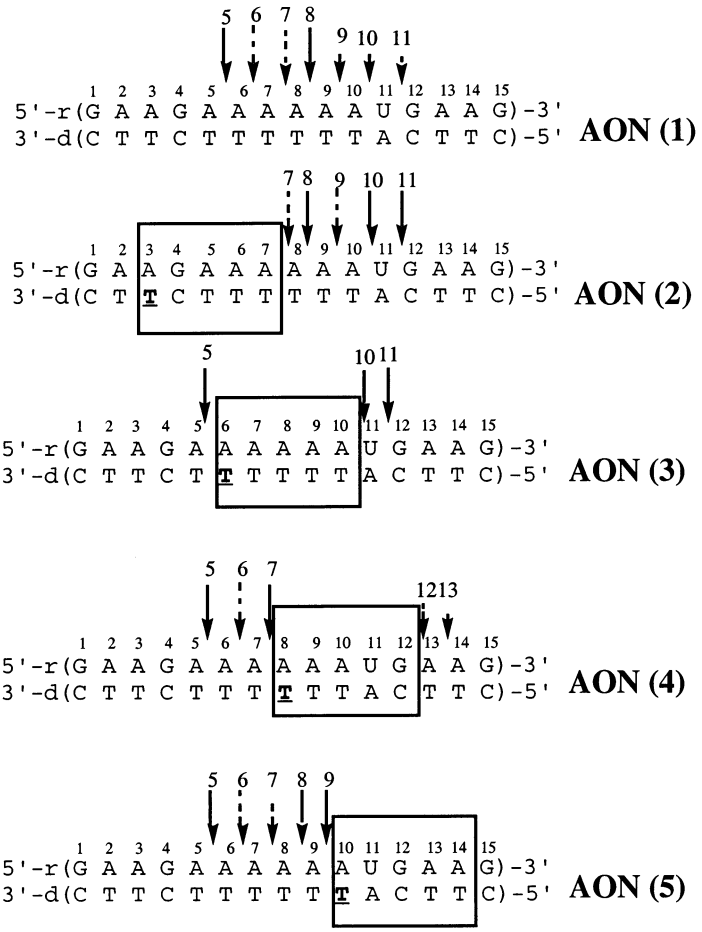


Figure 3. RNase H cleavage pattern of the hybrid duplexes formed by the AONs (**2**)–(**5**) and the RNA target (**6**). Solid and dashed arrows represent the major and the minor cleavage sites, respectively, at complete degradation (after 2 h of incubation). Short arrows demonstrate the sites accessible for cleavage at the initial reaction times (e.g. 30 min of incubation). Boxes represent the parts of the RNA sequence insensitive towards RNase H cleavage as a result of hybrid duplex structure change owing to the AON modification by introduction of N-locked nucleosides, **T**

No detectable difference in the hydrolysis rates between native and modified AON/RNA duplexes shows that the enzyme binds to the region affected by modification almost as well as to the native hybrid, although the former has both globally and locally DNA/RNA type structures and the latter produces a local conformational distortion giving an RNA/RNA type structure. RNase H is however absolutely unable to cleave the RNA in the modified hybrid duplex within this modified region spanning a stretch of five basepairs although it can bind to it, which means that the structural requirement of the enzyme binding and the catalytic cleavage are very different.

The above results suggest the following: (a) the cleavage activity of the enzyme was suppressed within a five basepairs long region towards the 3′-end of the RNA in the modified AON/RNA hybrids, starting from the base opposite to the modified **T** nucleotide in the AON strand, while the neighbouring sites beyond this five basepair region were fully susceptible to the hydrolysis by the enzyme; (b) the binding site and the cleavage site in the substrate–enzyme complex are

different[13] and the binding site is at least five nucleotides away toward the 5′-end of the RNA from the cleavage site; (c) it is also interesting to note that the structural requirements for the substrate binding and substrate cleavage by RNase H appear to be different. RNase H could bind to the A3–A7 region in the AON (**3**)/RNA duplex to produce hydrolysis at A7, but the cleavage at position A5 (which is present in the native duplex) is absent.

One of the interesting aspects of this work is how the *conformational transmission*[9] from the **T** residue to the neighbouring nucleotides in AONs controls the enzyme recognition and function: the structural distortions caused by the constrained North-type sugar conformation of **T** in any of the AONs (**2**)–(**5**) makes the neighbouring four basepairs at the 5′-end of the AON strand (i.e. the 3′-end of the RNA strand) completely inactive to the catalytic cleavage reaction, although good for the enzyme binding, showing that a conformationally constrained nucleotide indeed enforces the neighbouring nucleotide structure locally to adopt to a specific RNA/RNA duplex type conformation (compared with the normal B-DNA/A-RNA type conformation). This work therefore provides a new tool to map the local microscopic conformational change, as well as constituting a new example of how the conformation of a substrate can be understood by exploiting the stereochemical sensitivity of an enzyme. The second implication of this work is that the above information may help to optimize the number of constrained residues that can be incorporated to coax the antisense strand to adopt either A- or B-type geometry in the hybrid duplex, with or without the loss of RNase H recognition and/or cleavage property.

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