

Antisense 2'-O-alkyl oligoribonucleotides are efficient inhibitors of reverse transcription

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ABSTRACT

Reverse transcription is one step of the retroviral development which can be inhibited by antisense oligonucleotides complementary to the RNA template. 2'-O-Alkyl oligoribonucleotides are of interest due to their nuclease resistance, and to the high stability of the hybrids they form with RNA. Oligonucleotides, either fully or partly modified with 2'-O-alkyl residues, were targeted to an RNA template to prevent cDNA synthesis by the Avian Myeloblastosis Virus reverse transcriptase (AMV RT). Fully-modified 2'-O-alkyl 17mers were able to specifically block reverse transcription via an RNase H-independent mechanism, with efficiencies comparable to those observed with phosphodiester (PO) and phosphorothioate oligonucleotides. Sandwich 2'-O-alkyl/PO/2'-O-alkyl oligonucleotides, supposed to combine the properties of 2'-O-alkyl modifications (physical blocking of the RT) to those of the PO window (RNase H-mediated cleavage of the RNA) were quasi-stoichiometric inhibitors when adjacent to the primer, but remained without any effect when non-adjacent. They were not able to compete with the polymerase and inhibited reverse transcription only through RNase H-mediated cleavage of the target.

INTRODUCTION

Oligonucleotides are now widely used either in cultured cells or in intact organisms, to artificially repress the expression of a gene by targeting its messenger or pre-messenger RNA through the so-called antisense strategy (1). One of the limitations to the use of conventional oligonucleotides in such systems is their susceptibility to nucleases. This has prompted chemists to introduce modifications in the structure of these molecules to make them DNase resistant [see (2) for a review]. Among these, phosphorothioate oligonucleotides have received the greatest attention, as they are partially resistant to nucleases and can elicit the degradation of their RNA target by RNase H, a cellular enzyme which specifically digests the RNA moiety of RNA/

DNA hybrids (3). In microinjected *Xenopus* oocytes, the RNase H-mediated cleavage of rabbit β -globin mRNA was demonstrated with a complementary phosphorothioate 17mer, leading to a catalytic action of this oligonucleotide (4). This mechanism is likely involved in the inhibition of mRNA translation in cultured cells by phosphorothioate oligonucleotides, although not demonstrated.

In addition, phosphorothioate oligomers efficiently block the *in vitro* multiplication of HIV (5,6). The step of viral development they are interfering with is not fully elucidated. Indeed, they can potentially inhibit reverse transcription, splicing or translation of viral mRNAs, or other steps by non-antisense mechanisms such as inhibition of the cell penetration by the virus (7) or direct interaction with the reverse transcriptase (RT) (8). We recently demonstrated that phosphorothioate oligonucleotides can inhibit *in vitro* reverse transcription via an antisense mechanism, which involved the cleavage of the RNA template by the RT RNase H activity (9).

Another class of promising chemically-modified oligomers are the 2'-O-alkyl oligoribonucleotides (2'-O-alkyl for short) (10). These nuclease-resistant oligonucleotides are receiving an increasing attention in the frame of the antisense approach, because certain alkyl derivatives exhibit a higher affinity for the RNA target, than the unmodified RNA or DNA oligonucleotides (10,11). However, RNA/2'-O-alkyl duplexes do not form substrates for RNase H. This has been a great advantage for probing RNAs in cell extracts (12), but could be a disadvantage for the antisense strategy, as the RNase H-mediated cleavage of the target (messenger, pre-messenger or viral) RNA seems to play an important role (4,9,13-15).

Physical blocking (i.e. RNase H-independent inhibition) of reverse transcription was observed, either with alpha oligonucleotides (15), or with peptide nucleic acids (PNAs) (16). We therefore decided to check whether RNA/2'-O-alkyl hybrids could interfere with the cDNA synthesis performed by AMV RT. In the present paper, we demonstrate that 2'-O-alkyl oligonucleotides, hybridized to an RNA template, can block the AMV reverse transcriptase. In contrast to α -oligonucleotides, which were inhibitory only if adjacently bound to the primer, antisense 2'-O-alkyl analogues prevented cDNA synthesis whatever their

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binding site on the template, either adjacent to or downstream of the primer.

MATERIALS AND METHODS

Oligonucleotides and oligoribonucleotides

Unmodified oligodeoxynucleotides (see Table 1 for sequences) were synthesized on a Millipore 7500 automated synthesizer by using the conventional phosphoramidite method. They were usually purified in one step by HPLC on a reverse phase column eluted by an acetonitrile gradient (0–48%) in a 100 mM ammonium acetate (pH 7.0) buffer. The alpha oligonucleotide, synthesized as described previously (15), was a gift of Dr N. T. Thuong. The 2'-O-allyl oligonucleotides (Table 1) were synthesized on a solid phase from base-protected 5'-O-dimethoxytrityl-2'-O-allylribonucleoside 3'-O-(2-cyanoethyl *N,N*-diisopropylphosphoramidite)s by using 5-(4-nitrophenyl)-1*H*-tetrazole as activator as previously described (10). The 3'-terminal nucleoside of the oligonucleotides bore a 2'-hydroxyl and a 3'-O-allyl group, to prevent the action of 3'-exonucleases. 2'-O-Alkyl/PO/2'-O-alkyl sandwich oligonucleotides (Table 1) were synthesized from the 3'-end to the 5'-end on an Applied Biosystems synthesizer model 380B using protected 2'-O-alkylribonucleoside-3'-O-phosphoramidite monomers [(12) and references contained therein] as well as standard DNA phosphoramidite monomers. All sandwich oligomers were made using an aminopropyl controlled pore glass support functionalised with 5'-O-dimethoxytrityl-3'-O-allyluridine-2'-O-succinate. Couplings were performed in the presence of tetrazole and an extended coupling time of 15 min was used. After cleavage of base labile protecting groups the crude sandwich oligonucleotides were purified 'trityl-on' by reversed phase HPLC. The trityl group was removed in the usual way and the oligomers were purified once more by reversed phase HPLC.

When needed, unmodified and 2'-O-alkyl oligomers were 5' end-labeled with [γ - 32 P]ATP and T4 polynucleotide kinase (Boehringer Mannheim). The purity of the oligomers was systematically evaluated by running [32 P]-labeled samples on a 20% polyacrylamide/7 M urea gel.

21mer RNAs (Table 1) used for melting curve analysis and band-shift assays were enzymatically synthesized as described by Milligan *et al.* (17) with T7 RNA polymerase, which was prepared from an over-producing strain (BL21/pAR1219) according to a previously published procedure (18).

Reverse transcription

Two different procedures were used depending on whether a [32 P] 5' end-labeled or a cold primer was used. In the latter case, the polymerization was performed in the presence of [α - 32 P] dCTP (111TBq/mole, Du Pont de Nemours). The standard reaction for cDNA synthesis was performed as follows: a solution containing 100 ng globin mRNAs (corresponding to 0.25 and 0.35 pmole of β - and α -globin mRNAs, respectively, purchased from Gibco-BRL), the desired amount of antisense oligonucleotide and either 5–10 pmoles of a non-labeled primer or 0.5–1 pmole radiolabeled primer (which was in both cases an unmodified phosphodiester oligonucleotide), was pre-incubated for 30 min at 39°C, in a final volume of 7.5 μ l. After adding 2.5 μ l of a solution containing (i) 1 μ l of 10 \times reaction buffer (1 M Tris-HCl pH 8.3, 720 mM KCl, 100 mM MgCl₂ and 100 mM

Table 1.

Name	Sequence	Target
17PO(β 3)	TTGTGTCAAAGCAAGT	β 3-19
17Allyl(β 3)	<u>UUGUGUCAAAAGCAAGU</u>	β 3-19
17Allyl(β 3) <i>mm</i>	<u>UUAUGUCCAAAGCUAGU</u>	β 3-19 (a)
17alpha(β 3)	TGAACGAAAACGTGTGT	β 3-19
15PO(β 34)	CTGTTTTGGGGATT	β 34-48
17PO(β 113)	CACCAACTTCTCCACA	β 113-129
17Allyl(β 113)	<u>CACCAACUUCUCCACU</u>	β 113-129
17Allyl(β 113) <i>mm</i>	<u>CACCAACUCAUUCACU</u>	β 113-129 (a)
12Allyl(β 118)	<u>CACCAACUUCUU</u>	β 118-129
15PO(β 130)	TGCCCAGGGCCTCAC	β 130-144
19PO(β 172)	AAGGACTCGAAGAACCCTCT	β 172-190
20PO(α 7)	TCTCAGTCGGACTGGACCAG	α 7-26
15sAllyl(α 37) (b)	<u>GGGAGACAGCACCACU</u>	α 37-51
15PO(α 52)	GTTGGTCTTGTGACG	α 52-66
15PO(α 70)	TTCCAGGCAGCTCT	α 70-84
Name	Sequence	Sense
21RNA(β 2)	GGCACUUGCUIUUUGACACAAC	β 2-20 sense
21RNA(β 112)	GGAUGUGGAAGAAGUUGGUGG	β 112-130 sense
21RNA(α 37)	AUGGUGCUGUCUCCUUU	α 37-51 sense

Oligonucleotides, written in the 5' (left) to 3' (right) direction, used as primers, antisense or probes, (upper part of the table) and sense 21 mer RNA oligonucleotides of α - or β -globin mRNA region (position indicated in the third column). The names used for oligonucleotides indicate successively the length in nt, the chemical nature (PO: unmodified phosphodiester; Allyl, Butyl and Methyl: 2'-O-allyl, -butyl and -methyl; alpha: alpha configuration of the nucleoside units; RNA: natural ribonucleotide), and in parenthesis, the mRNA target (α - or β -globin) followed by the position of the first targeted nt [+1 is the first nt after the 7-methylguanosine residue (20)]. The 2'-O-alkyl residues are underlined. (a): *mm* design oligomers in which mismatches (indicated in bold) have been introduced. (b): 15sAllyl(α 37) is a 2'-O-allyl/PO/2'-O-allyl sandwich oligonucleotide, the 2'-O-allyl modified nucleotides being underlined. 15sMethyl(α 37) and 15sButyl(α 37) have the same sequence as 15sAllyl(α 37), with either 2'-O-methyl or 2'-O-butyl ribonucleotides in place of the 2'-O-allyl ones.

dithiothreitol), ii) 1 nmole of each of the four dXTPs and, in the case of non-radiolabeled primer, 2 pmole of [32 P] α -dCTP and iii) 1 U AMV RT (0.15 pmole, from Appligène), the reaction was performed for 1 h at 39°C. The reverse transcripts were extracted with chloroform, precipitated with ethanol and loaded on a 10 or 20% polyacrylamide/7 M urea gel (depending on the expected length of the cDNA). The yield of cDNA synthesis was determined by counting gel slices in a β -scintillation counter.

In one experiment (see Fig. 2), a ladder of cDNA fragments was generated as size markers. Conditions of low processivity of MMLV H⁻ RT (from Gibco-BRL) were determined: 100 ng globin mRNAs (0.25 pmole β -globin mRNA), and 0.1 pmole radiolabeled primer 15PO(β 34) were pre-incubated for 30 min at 39°C. The ladder (*L*) was obtained by adding 0.5 nmole of each of the four dXTPs, 5 U of MMLV H⁻ RT, the reverse transcription buffer (see above), and incubating 1 h at 39°C in a final volume of 10 μ l; the expected aborted products were purified and analysed as described above.

RNA template analysis

Following reverse transcription, RNA was analysed according to the following procedure: 7.5 μ l containing 100 ng globin mRNAs

(corresponding to 0.35 pmole α -globin mRNA), 0.1 pmole of primer [either 15PO(α 52) or 15PO(α 70)] and/or 0.1 pmole sandwich oligonucleotide 15sAllyl(α 37) (Table 1), were pre-incubated for 30 min at 39°C. After addition of 1 μ l reverse transcription buffer $\times 10$ (see above), 1 nmole of each of the four dXTPs (when reverse transcription was required), and 1 U AMV RT, the 10 μ l samples were incubated for 15 min at 39°C. RNA was extracted with chloroform, precipitated by ethanol, loaded on a 10% polyacrylamide gel containing 7 M urea and electroblotted on a nylon membrane (Pall) according to the supplier's instructions. α -globin mRNA cleavage products were detected with a [32 P] 5' end-labeled 20mer [20PO(α 7)].

RNA/oligonucleotide hybrid stability

Melting temperatures (T_m) of the oligonucleotide/RNA duplexes were determined from melting curves: UV absorbance of stoichiometric mixtures (0.5 μ M of each strand) in a 10 mM sodium cacodylate pH 7.0 buffer containing 50 mM NaCl and 1 mM magnesium acetate, was measured at 260 nm on a Uvikon 940 spectrophotometer, while the temperature was increased from 20 to 90°C, at 30°C/h.

Band-shift assays of heteroduplex/RT complexes

Binding of AMV RT to RNA/modified oligonucleotide hybrids was monitored by gel electrophoresis: 0.5 pmole of 5'-end radiolabeled 21RNA(β 2) and 1 pmole of a complementary 17mer were incubated 5 min with 1.5 pmole AMV RT, in 10 μ l of a 100 mM Tris-HCl pH 8.3 buffer containing 10 mM MgCl₂ and 10 mM dithiothreitol. The binary (RNA/oligonucleotide) and ternary (RNA/oligonucleotide/RT) complexes were separated on a 5% polyacrylamide gel, buffered with 25 mM Tris-HCl pH 8 (at 4°C) containing 162 mM glycine. The migration was performed at 7.5 V/cm during 2–3 h, at 4°C.

The affinity of MMLV RT (Gibco-BRL) for RNA/oligomer hybrids was determined according to the same procedure: increasing quantities of enzyme (from 1 to 6 pmoles) were incubated with a mix of 2 pmoles 21RNA(β 2) and 2 pmoles oligonucleotide (either the RNA or the oligonucleotide was 5'-end radiolabeled), in 10 μ l of buffer (see above). Analysis of complexes was carried out as described above. The quantitation of ternary complex was performed by counting gel slices containing i) the remaining free RNA/oligonucleotide hybrid on the one hand; ii) the slow migrating species (ternary complex and smear) on the other hand. Affinity constant K_a was determined from the MMLV RT concentration at which 50% of the RNA/oligonucleotide duplex was converted into RNA/oligonucleotide/RT complex ($K_a = ([\text{MMLV RT}]_0 - 1/2 [\text{RNA/oligonucleotide}]_0)^{-1}$).

RESULTS

A 2'-O-allyl 17mer blocks reverse transcription via an antisense mechanism

Reverse transcription of β -globin mRNA by AMV RT was primed with 17PO(β 113) (the abbreviations used for all synthetic oligonucleotides used throughout are listed in Table 1). This oligonucleotide led to the synthesis of a 129 nt long cDNA (Fig. 1). In the presence of the complementary sequence 17Allyl(β 3), this transcript was less abundant, whereas shorter fragments

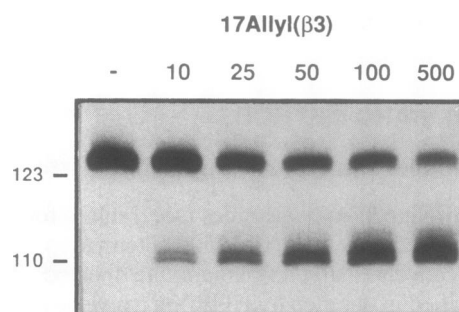


Figure 1. Effect of 17Allyl(β 3) on reverse transcription. cDNA synthesis was primed with the 17PO(β 113) (1 μ M) complementary to nt 113–129 of the β -globin mRNA. Reverse transcription was performed by 1 U AMV RT, in the absence (–) or presence of 17Allyl(β 3), at the concentrations (in nM) indicated at the top of the lanes. On the left, lengths in nt were determined from the 5'-end labeled size markers (pBR322 digested by MSPI, Biolabs). Transcripts were analysed on a 10% polyacrylamide/7 M urea gel.

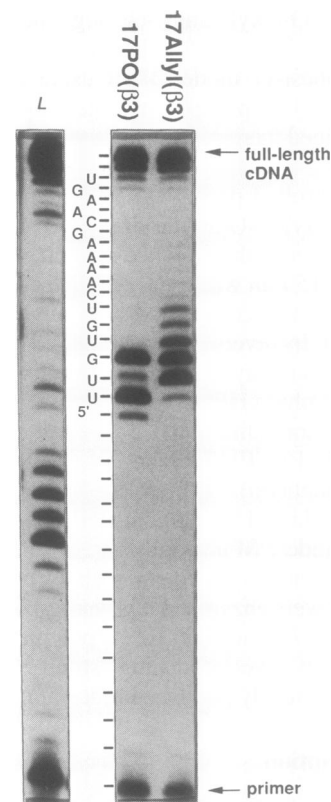


Figure 2. Pattern of AMV RT arrest sites by 17Allyl(β 3) and 17PO(β 3). cDNA synthesis, primed from radiolabeled 15PO(β 34) (100 nM), was performed by 1 U of AMV RT, in the presence of 100 nM 17Allyl(β 3) or 17PO(β 3). L is the ladder of aborted cDNA transcripts, generated as described in Materials and Methods. The sequence of 17Allyl(β 3) is written to the left of the lanes [17PO(β 3) has the same sequence, with T substituted for U]. The fragments were separated on a 20% polyacrylamide/7 M urea gel.

appeared, so that the overall cDNA synthesis remained constant. The length of these short fragments, about 110–115 nt long, suggested that reverse transcription was blocked by the RNA/17Allyl(β 3) hybrid, i.e. through an antisense mechanism.

The inhibition efficiency was estimated by comparing the amount of the full length cDNA transcript (129 nt long fragment) in the presence and in the absence of 17Allyl(β 3). $C_{1/2}$, defined as the antisense oligonucleotide concentration leading to 50% inhibition of the full-length transcript, was about 40 nM. As a comparison, the same sequences either unmodified [17PO(β 3)] or phosphorothioate [17PS(β 3)], had $C_{1/2}$ of 25 and 100 nM, respectively, and the alpha analog 17alpha(β 3) did not lead to any arrest at 1 μ M (9,15). However, the high inhibition efficiency displayed by the unmodified and the phosphorothioate oligomers was related, first to their ability to induce the degradation of their target by RNase H, second to the non-specific inhibition of cDNA synthesis by 17PS(β 3) (9). Despite the great inhibitory efficiency shown by 17Allyl(β 3) at low concentration (40% abortive cDNA at the stoichiometry with the RNA target: 25 nM), this oligonucleotide was not able to totally block cDNA synthesis: at 500 nM, there was 30% full-length transcript left, probably due to the reversibility of the blocking. The stability of RNA/modified oligomer hybrid was in good agreement with a physical blocking of reverse transcription by 17Allyl(β 3): the T_m of the duplex formed with the latter oligonucleotide (66°C) was significantly higher than that formed with 17PO(β 3) (43°C), 17PS(β 3) (35°C) or 17alpha(β 3) (45°C).

The sites of arrested cDNA synthesis were determined using 15PO(β 34) as a primer which led to a better separation of the cDNA fragments. In the presence of 17Allyl(β 3), reverse transcriptase stopped at multiple positions. At least six different products could be seen, corresponding to an arrest at each of the six first base pairs of the RNA/17Allyl(β 3) hybrid (Fig. 2). In comparison, the homologous unmodified oligomer 17PO(β 3) produced only two major arrest sites, after the first and third base pairs (from the 5' end of the oligonucleotide) of the RNA/17PO(β 3) hybrid (Fig. 2), resulting from an RNase H-mediated cleavage of the template (15). As 2'-O-alkyl analogues cannot elicit RNase H activity (14), this was in agreement with a partial denaturation of the RNA/17Allyl(β 3) hybrid by the polymerizing RT (see below).

Other 2'-O-allyl antisense oligonucleotides are less efficient inhibitors

In order to check if the previous results were related to the peculiar location of the antisense oligonucleotide target in the close vicinity of the 5' end of the RNA template, we studied the effect of 17Allyl(β 113) on reverse transcription. Addition of 17Allyl(β 113) decreased the synthesis of the 190 nt transcript primed by 19PO(β 172): 50% reduction of the full length transcript was reached between 150 and 200 nM 17Allyl(β 113) (Fig. 3). In addition, shorter fragments were detected, whose lengths (60–70 nt) indicated an hybrid-arrest of polymerization. Therefore, this sequence was effective but was a slightly less efficient inhibitor than 17Allyl(β 3).

Previous experiments had demonstrated that an alpha oligonucleotide targeted to the same site (nt 113–129) was able to block reverse transcription only when cDNA synthesis was primed by an oligomer immediately adjacent to the antisense one (15). In contrast, 17Allyl(β 113) inhibited reverse transcription with a similar efficiency ($C_{1/2}$ of about 200 nM) whatever the primer used, either adjacent to [15PO(β 130)] or remote from [19PO(β 172)] (not shown). Therefore, the end to end cooperative interaction between the primer and the antisense sequences did

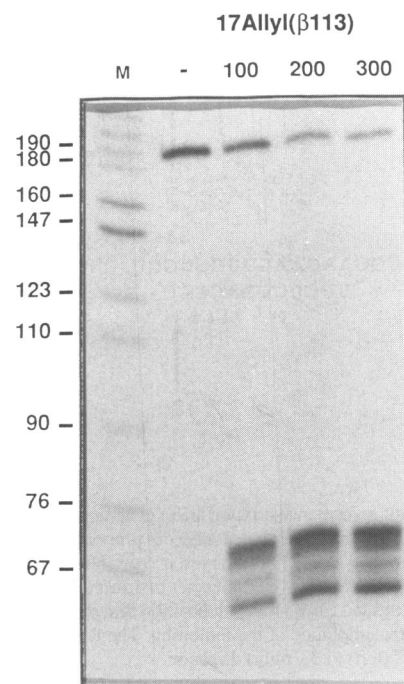


Figure 3. Reverse transcription inhibition by 17Allyl(β 113). The radiolabeled primer 19PO(β 172) (50 nM) was elongated by 1 U AMV RT, in the absence (–) or in the presence of 17Allyl(β 113) at the concentrations (nM) indicated at the top of the lanes. M corresponds to the size markers (in nt). The transcripts were analysed on a 10% polyacrylamide/7 M urea gel.

not increase significantly the inhibitory efficiency of cDNA synthesis by 2'-O-alkyl oligonucleotides, in contrast to the situation displayed by alpha derivatives.

The shorter oligonucleotide 12Allyl(β 118) which had the same 5' end as 17Allyl(β 113), yielded a poor inhibition: at 500 nM, there was no significant decrease of the cDNA synthesis when reverse transcription was initiated from a non-adjacent primer [19PO(β 172)]; however, fragments about 60 nt long, were detected over 200 nM 12Allyl(β 118), suggesting that the RT had been blocked at the level of the hybrid (not shown). Melting temperatures [73 and 57°C for 17Allyl(β 113) and 12Allyl(β 118), respectively], suggested that the inhibition efficiency was correlated with the hybrid stability, i.e. with the length of the antisense oligonucleotide.

The pattern of pause sites induced by 17Allyl(β 113) was significantly different from that obtained with 17Allyl(β 3): bands corresponded to the first nine base pairs, with a majority (60%) after the first base pair (Fig. 4a). It might indicate that, after denaturation of about 10 base pairs, the seven pairs left did not provide a stable enough duplex with the template to prevent denaturation. Indeed, in the presence of 12Allyl(β 118), the major pause site was after the first base pair, with minor arrest at the three following base pairs (Fig. 4b), suggesting in this case also that a 7–8 base pair hybrid could not prevent scanning of the template by the polymerizing RT. The arrest profile by 17Allyl(β 113) was slightly different when either an adjacent [15PO(β 130)] or a non-adjacent primer [19PO(β 172)] was used (Fig. 3): the first band of arrest (presumably after the first base

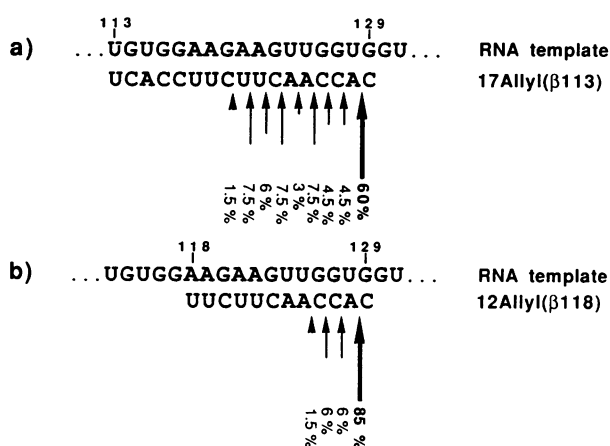


Figure 4. Pattern of reverse transcription arrest generated by 17Allyl(β113) (a) or 12Allyl(β118) (b). The radiolabeled adjacent primer [15PO(β130), 50 nM] was elongated by 1 U AMV RT, in the presence of 500 nM 17Allyl(β113) (a) or 12Allyl(β118) (b). The relative efficiencies of arrest (compared to the global arrest sites) are indicated (in %) for each position designed by an arrow, whose size is related to the amplitude of the inhibition. The RNA template sequence is written in the 5' (left) to 3' (right) direction.

pair) represented no more than 30% of the global arrest, compared to 60%.

Sequence specificity of cDNA synthesis inhibition

As 2'-O-allyl oligonucleotides have a high affinity for their RNA target, these molecules could induce reverse transcription arrest by binding to sites exhibiting partial complementarity. In order to check this point, we prepared 17Allyl(β3)*mm*, an oligomer derived from 17Allyl(β3), leading to three mismatches when bound to region 3–19 of the β-globin mRNA (see Table 1); this oligonucleotide, which formed a duplex characterized by a T_m of 34°C, did not lead to any arrest at concentrations up to 500 nM (not shown). On the contrary, 17Allyl(β113)*mm* derived from 17Allyl(β113) by introduction of two adjacent mismatches in the center of the hybrid, was able to block reverse transcription: cDNA fragments were detected corresponding to cDNA synthesis arrest at the position of the imperfect hybrid, 50% inhibition of the full-length cDNA being achieved in the presence of about 500 nM 17Allyl(β113)*mm* (not shown). The T_m of this mismatched hybrid was 58°C, i.e. 15°C below that of the perfect duplex. In contrast, the ΔT_m between the perfect and mismatched duplexes formed with the sequence 3–19 was 32°C, i.e. substantially larger. Therefore, the amplitude of the non-specific effect was correlated with the stability of the mismatched duplexes.

Affinity of reverse transcriptase for different substrates

Why are 2'-O-alkyl derivatives blockers of reverse transcription, as efficient as phosphodiester oligonucleotides which can induce the degradation of the template by RNase H? Why are 2'-O-allyl oligonucleotides more efficient than alpha oligonucleotides (15)? We checked if RT could stabilize RNA/2'-O-allyl oligonucleotide hybrids, leading to a ternary complex that would prevent the

unwinding of the duplex by a polymerizing RT molecule. Band-shift assays were used to estimate the relative affinity of RT for 21mers RNA hybridized to complementary phosphodiester, 2'-O-allyl or alpha 17mers targeted to the 3–19 region of the β-globin mRNA. A faint band corresponding to a slow migrating species could be detected upon the addition of AMV RT to RNA/17PO(β3), whereas neither RNA/17Allyl(β3) nor RNA/17alpha(β3) duplexes led to anything equivalent (not shown). This slow migrating species likely corresponded to the ternary RNA/17PO(β3)/AMV RT complex. This indicated a lower affinity of AMV RT for the RNA/2'-O-allyl or RNA/alpha hybrids compared to the RNA/PO hybrid. However, the low concentration of the AMV RT preparation prevented us to increase the amount of added enzyme to get access to quantitative results. For this reason, we undertook such an experiment with MMLV RT, which was also blocked by 2'-O-allyl antisense oligonucleotides (not shown) and was available as a concentrated solution. Increasing amounts of MMLV RT were added to equimolar mixtures of RNA and one of the following oligomers: 17PO(β3), 17Allyl(β3) and 17alpha(β3). Nearly 100% of the RNA was hybridized to the oligomer under these experimental conditions (Fig. 5a). The RNA/17Allyl(β3)/MMLV RT ternary complex was clearly detectable (Fig. 5a), as well as the RNA/17PO(β3)/MMLV RT and RNA/17alpha(β3)/MMLV RT complexes (not shown). Most (about 70%) of RNA/17PO(β3) or RNA/17Allyl(β3) and about 55% of RNA/17alpha(β3) duplexes were associated with the enzyme in the presence of a 2-fold excess of MMLV RT over the hybrid (Fig. 5b). The affinity of MMLV RT for the RNA/2'-O-allyl hybrid ($K_a \sim 10^7 M^{-1}$) was identical to that for the RNA/PO hybrid, and slightly higher than the one for the RNA/alpha duplex ($K_a \sim 4.10^6 M^{-1}$). Therefore, the binding of RT to hetero-duplexes does not account for the difference observed between 2'-O-allyl and alpha oligomers.

2'-O-alkyl/PO/2'-O-alkyl sandwich oligonucleotides inhibit reverse transcription

Taking into account the above results, we hypothesized that a sandwich oligonucleotide, composed of a central part with an unmodified backbone (PO) surrounded by two 2'-O-allyl parts, would generate an efficient inhibitor of reverse transcription: on the one hand, the unmodified part might elicit the cleavage of the template by RNase H (15), whereas on the other hand, the 2'-O-allyl stretches might physically block the enzyme. In addition, the 2'-O-allyl blocks will protect the oligonucleotide against exonucleases (10), which might be of interest for use with cultured cells.

A sandwich 2'-O-allyl/PO/2'-O-allyl 15mer [15sAllyl(α37)], targeted to nt 37–51 of the rabbit α-globin mRNA (Table 1) was prepared: this oligonucleotide was composed of a five unmodified deoxynucleotide window, flanked by six and two 2'-O-allyl nucleotides on the 3' and 5' sides, respectively (the sandwich had two unmodified deoxynucleotides at its 5' end to allow enzymatic radiolabeling). In addition, two similar sandwiches were synthesized with either 2'-O-butyl [15sButyl(α37)] or 2'-O-methyl [15sMethyl(α37)] groups. It is known that increasing the size of the substituent modulates the binding of 2'-O-alkyl oligonucleotides to their target.

The three sandwich oligonucleotides were efficient blockers of reverse transcription when an adjacent primer [15PO(α52)] was used. The efficiency slightly decreased when the size of the

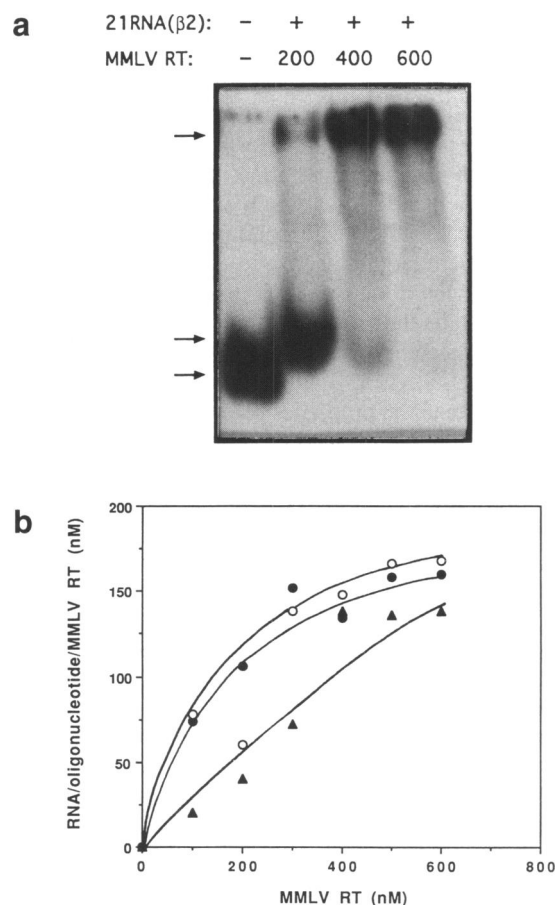


Figure 5. Band-shift assay analysis of RNA/oligonucleotide/MMLV RT ternary complexes. (a) 17Allyl(β 3) (200 nM) was 5'-end radiolabeled, and incubated in the absence (-) or presence (+) of 200 nM 21RNA(β 2), and in the absence (-) or presence (+) of different concentrations (indicated above the lanes in nM) of MMLV RT. The arrows indicate 17Allyl(β 3), the duplex formed by addition of 21RNA(β 2) and the ternary complex, from the bottom to the top, respectively. (b) Binding curves of MMLV RT to radiolabeled 21RNA(β 2) hybridized with either 17PO(β 3) (●), 17Allyl(β 3) (○) or 17alpha(β 3) (▲). The concentration (in nM) of the ternary complexes is reported as a function of the concentration (in nM) of MMLV RT. Each point is the average of 2-4 different experiments.

2'-O-substituent increased; the $C_{1/2}$ were 10, 15 and 25 nM for 15sMethyl(α 37), 15sAllyl(α 37) and 15sButyl(α 37), respectively (Fig. 6). The inhibition efficiency was directly correlated with the relative affinity of 2'-O-alkyl oligonucleotides for their RNA target: melting temperatures were 72, 70 and 66°C for duplexes formed with methyl, allyl and butyl derivatives, respectively.

In contrast, these sandwiches were not able to block reverse transcription from a non adjacent primer: no inhibition of cDNA synthesis primed with a 15mer complementary to nt 70-84, i.e. twenty nucleotides upstream, was seen even in the presence of 500 nM antisense oligonucleotides (not shown).

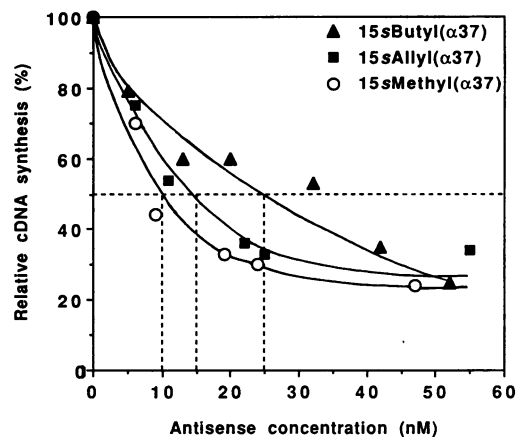


Figure 6. Efficiency of reverse transcription inhibition by 15sAllyl(α 37), 15sButyl(α 37) and 15sMethyl(α 37), when adjacent to the primer. Full-length cDNA synthesis in the presence of different concentrations (nM) of 15sAllyl(α 37) (■), 15sButyl(α 37) (▲) or 15sMethyl(α 37) (○), relative to the one in the absence of antisense oligonucleotide, when the reverse transcription was primed with the adjacent primer 15PO(α 52) (1 μ M), and performed by 1 U AMV RT.

RNase H-mediated cleavage of the template in the presence of the 2'-O-allyl/PO/2'-O-allyl sandwich oligonucleotide

Effects of sandwich oligomers could be due to RNase H-mediated cleavage of the RNA target. In order to check this, the RNA was analysed by Northern blot after incubation with the RT in the presence of the sandwich 15sAllyl(α 37) and/or of adjacent or non-adjacent primers. The probe [20PO(α 7)] was not able to hybridize to the intact α -globin mRNA, and could only detect break-down fragments. We assumed that a secondary structure resulting from long range interactions with the 5' end of the mRNA, which prevented association with the probe, was removed by cleavage; the short distance between the 5' end and the binding site of 15sAllyl(α 37) (36 nt) prevented us from using another probe.

Incubation of the RNA with either the adjacent or the non-adjacent primer in the absence of dXTPs, gave rise to cleavage in the region of primer binding, but 15sAllyl(α 37) alone could not promote the action of RNase H (Fig. 7). The simultaneous addition of 15sAllyl(α 37) and of the non-adjacent primer led to cleavage only at the level of the primer. In contrast, two sites of cleavage were detected in the presence of both 15sAllyl(α 37) and the adjacent primer (Fig. 7). The results were essentially similar when reverse transcription took place (i.e. in the presence of dXTPs), although the pattern was different in the absence of 15sAllyl(α 37) (not shown): the primers [15PO(α 52) and 15PO(α 70)] were elongated, giving rise to minor cleavage at the level of their binding site, and at downstream positions (probably resulting from reverse transcriptase pausing). These results clearly demonstrated that the inhibition of reverse transcription by these sandwich oligomers was related to an RNase H-mediated cleavage of the RNA template.

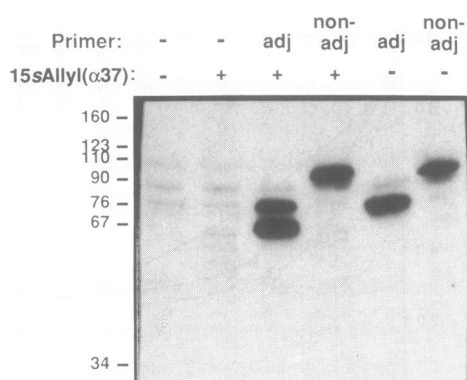


Figure 7. α -Globin mRNA analysis, after incubation with 15sAllyl(α 37) and/or primers. α -Globin mRNA was incubated in the absence (-) or presence (+) of 100 nM 15sAllyl(α 37), and in the absence (-) or presence of 100 nM 15mer primers either adjacent (adj) or non-adjacent (non-adj) to 15sAllyl(α 37). Lengths (in nt) to the left are from DNA size markers.

DISCUSSION

We studied the inhibition of reverse transcription by antisense 2'-O-alkyl oligonucleotides. In previous studies, we had demonstrated that cDNA synthesis could be blocked by phosphodiester or phosphorothioate oligonucleotides bound to the RNA template via the cleavage of the template by the RNase H activity of the RT (9,15). In the present case, RNase H could not generate any cleavage at the level of the RNA/2'-O-alkyl oligoribonucleotide duplex. As we observed a sequence-dependent inhibition of cDNA synthesis by 2'-O-alkyl analogues, we conclude that these oligomers were able to physically block the RT.

However, the pattern of bands resulting from blocking was unexpected, since the ten first base pairs of a 17 bp duplex encountered by the polymerizing RT were arrest sites (Figs 2 and 4a). Such results were not observed with any of the other modified oligonucleotides which blocked reverse transcription by an RNase H-independent mechanism. An 'all or nothing' mechanism was seen with alpha oligomers, which means that when the RT had been able to unwind the first encountered base pair of the RNA/alpha hybrid, it could denature all the following base pairs without pausing (15). Our results with 2'-O-allyl oligoribonucleotides could result from different mechanisms of inhibition. Two hypothesis were considered: i) first, the blocking species was the ternary complex made of a RT molecule sitting on the RNA/2'-O-allyl hybrid; indeed, a 2'-O-allyl oligoribonucleotide with a single unmodified ribo- or deoxyribonucleotide at the 3' end could prime reverse transcription, demonstrating that the RT could bind to a RNA/2'-O-allyl duplex (19). We supposed that the polymerizing enzyme could unwind the RNA/2'-O-allyl duplex up to the region covered by a non-polymerizing RT molecule. However, the slight difference in affinity of MMLV RT for RNA/17Allyl(β 3) hybrid ($K_a = 10^7 \text{ M}^{-1}$) and for RNA/17alpha(β 3) ($K_a = 4.10^6 \text{ M}^{-1}$) is not correlated with the inhibition of reverse transcription by MMLV RT [$C_{1/2} = 100 \text{ nM}$ with 17Allyl(β 3) whereas no blocking is detected with 17alpha(β 3) at least below $1 \mu\text{M}$, data not shown]. Moreover, the affinity of AMV RT for the RNA/17Allyl(β 3) hybrid is below that for the natural hybrid. ii) second, the low efficiency of the

'unwinding activity' associated to the RT on 2'-O-allyl/RNA duplexes could generate pause sites. It should be mentioned that 2'-O-allyl oligomers bind more strongly to the RNA template than alpha or unmodified analogues. This would result in an increased probability for the RT to dissociate. This hypothesis was supported by an experiment performed with the MMLV RT, which gave a similar pattern of arrest bands (not shown); for incubation in which one dXTP was in a 50-fold excess over the three others, the pause sites corresponding to the dXTP in excess disappeared or were largely decreased (not shown). This result supports the 'unwind or dissociate' mechanism. The pause sites could also depend on the sequence of the hybrid (local structure, relative base pair stability).

Sandwich 2'-O-alkyl/PO/2'-O-alkyl oligonucleotides were supposed to combine the properties of both types of backbones: high affinity for the RNA template, exonuclease resistance and ability to form RNase H substrates once bound to the RNA template. The three oligomers [15sAllyl(α 37), 15sButyl(α 37) and 17sMethyl(α 37)] were strong inhibitors when the reverse transcription was primed adjacently: 15sMethyl(α 37) and 15sAllyl(α 37) acted at a quasi-stoichiometric concentration, indicating both a high binding constant and a high inhibition efficiency. In this case, one took advantage of the two mechanisms as sandwich oligonucleotides promoted the cleavage of the target by RNase H.

In contrast, when combined with a remote primer, sandwich oligonucleotides were without any effect either on the reverse transcription, or on the template integrity, although 2'-O-alkyl sandwich/RNA hybrids had melting temperatures in the same range as those formed by the fully-modified 2'-O-allyl oligomers 17Allyl(β 3), 17Allyl(β 113) and 12Allyl(β 118), which were efficient blockers when used with a non-adjacent primer. Therefore, the stability of the RNA/antisense oligomer was not, in the present case, the key parameter for blocking. The RT RNase H did not act on the RNA/sandwich oligonucleotide duplex in the absence of an adjacent oligomer, i.e. on an isolated sandwich/RNA hybrid. This did not reflect a general property of RNases H toward these duplexes as they were recognized as substrates by the wheat germ RNase H activity (Larrouy *et al.*, to be published). Moreover, it is known that three to five contiguous deoxynucleotides allow the bacterial and mammalian RNases H to cleave (11,14) (Frank *et al.*, unpublished results). Therefore, we conclude that the unmodified deoxy gaps of the sandwich molecules were not properly located to direct the RNase H action of the AMV RT. Moreover, the last two deoxynucleotides present at the 5' end of the sandwiches did not lead to the cleavage of their RNA strand counterpart, likely due to the too small size of this deoxy portion.

Surprisingly, sandwich oligomers failed to physically block the enzyme, in contrast to the fully modified oligonucleotides 17Allyl(β 3), 17Allyl(β 113) and 12Allyl(β 118) (Figs 1 and 3). This could perhaps be ascribed to the composition of the sandwiches, in which only two 2'-O-alkyl residues were incorporated in the 5' half (Table 1): this could not be enough to prevent unwinding of the duplex and to induce the RT to pause.

We previously investigated different chemical modifications in antisense oligomers designed to interfere with *in vitro* reverse transcription. On the one hand, unmodified and phosphorothioate oligonucleotides were efficient inhibitors, and acted through RNase H-mediated degradation of their target (9,15). On the other hand, alpha- and methylphosphonate oligomers could not

elicit the action of RNase H. Alpha-oligonucleotides were poor inhibitors; in addition, they had to be targeted adjacently to the primer to prevent cDNA synthesis (15). We could not detect any inhibitory effect of reverse transcription by methylphosphonate analogues (15). In contrast to what could have been expected taking into account these previous experiments, 2'-O-alkyl oligonucleotides inhibited reverse transcription as efficiently as did unmodified and phosphorothioate oligomers, even though RNA/2'-O-alkyl hybrids are not susceptible to RNase H. Moreover, we demonstrated that one or even two mismatches in the RNA/2'-O-alkyl duplex could not totally prevent the formation of the complex, allowing cDNA synthesis arrest, even if mutations appeared in the retroviral genome at the binding site selected for antisense oligonucleotides.

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