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RNase H is responsible for the non-specific inhibition of *in vitro* translation by 2′-O-alkyl chimeric oligonucleotides: high affinity or selectivity, a dilemma to design antisense oligomers

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ABSTRACT

Ribonuclease H (RNase H) which recognizes and cleaves the RNA strand of mismatched RNA–DNA heteroduplexes can induce non-specific effects of antisense oligonucleotides. In a previous paper [Larrouy *et al.* (1992), *Gene*, 121, 189–194], we demonstrated that ODN1, a phosphodiester 15mer targeted to the AUG initiation region of α-globin mRNA, inhibited non-specifically β-globin synthesis in wheat germ extract due to RNase H-mediated cleavage of β-globin mRNA. Specificity was restored by using MP-ODN2, a methylphosphonate-phosphodiester sandwich analogue of ODN1, which limited RNase H activity on non-perfect hybrids. We report here that 2′-O-alkyl RNA-phosphodiester DNA sandwich analogues of ODN1, with the same phosphodiester window as MP-ODN2, are non-specific inhibitors of globin synthesis in wheat germ extract, whatever the substituent (methyl, allyl or butyl) on the 2′-OH. These sandwich oligomers induced the cleavage of non-target β-globin RNA sites, similarly to the unmodified parent oligomer ODN1. This is likely due to the increased affinity of 2′-O-alkyl-ODN2 chimeric oligomers for both fully and partly complementary RNA, compared to MP-ODN2. In contrast, the fully modified 2′-O-methyl analogue of ODN1 was a very effective and highly specific antisense sequence. This was ascribed to its inability (i) to induce RNA cleavage by RNase H and (ii) to physically prevent the elongation of the polypeptide chain.

INTRODUCTION

Several mechanisms have been proposed for oligonucleotide-mediated inhibition of translation or reverse transcription. One of them involves the degradation of the target RNA by RNase H (1,2). Several modifications, introduced into oligomers to reduce their sensitivity to nucleases, impaired their ability to elicit RNase H activity. This is the case with methylphosphonates (3,4), phosphoramidates (5), 2′-O-alkyl RNA (6) and alpha analogues (4,7,8). Composite oligonucleotides made of differently modified (or unmodified) parts were prepared that aimed at combining different properties within a single molecule (9–11). Mixed methylphosphonate-phosphodiester oligonucleotides were synthesized in order to yield antisense sequences resistant to exonucleases and nevertheless able to elicit RNase H activity once bound to their RNA target (10,12). Phosphoramidate-phosphorothioate sandwich oligomers were also used against An2 cyclin mRNA in injected *Xenopus* embryos (13). Successful inhibition of the Ha-*ras* gene has been reported with 2′-O-alkyl RNA phosphodiester chimeras (14). In both An2 cyclin and Ha-*ras* experiments the sandwich antisense sequences were better inhibitors than either the phosphodiester DNA parent oligomer or the homogeneously modified oligonucleotide. This was shown to be related to the nuclease sensitivity of the unmodified oligonucleotides on the one hand and was ascribed to the role likely played by RNase H in the inhibition of translation on the other hand.

RNases H from either prokaryotic or eukaryotic origin can act on mismatched RNA–DNA heteroduplexes. Therefore, RNase H can cleave non-target RNA sites exhibiting partial complementarity to the antisense oligonucleotide (15), leading to non-specific inhibition in some eukaryotic cells (16,17). In order to reduce the cleavage of non-perfect hybrids, we and others previously used methylphosphonate sandwich oligomers which led to an increased specificity of the antisense chimeric oligomer compared to the unmodified one (18–20). We had previously shown that a 15mer, with a central window of five phosphodiesters inserted between two methylphosphonate flanks, targeted to the initiation codon region of the rabbit α-globin mRNA, displayed some selectivity in the inhibition of *in vitro* globin synthesis, despite the presence, on the β-globin mRNA, of nine sites sharing more than 60% homology with the primary α-target site (19).

2′-O-Alkyl oligoribonucleotides display interesting properties for antisense use: they are nuclease resistant and do not elicit RNase H activity. In contrast to methylphosphonate the chemical modification does not introduce a chiral center and therefore 2′-O-alkyl derivatives are stereochemically pure analogues. We extended our previous work to the use of 2′-O-alkyl RNA-phos-

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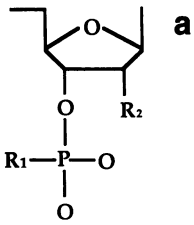

a

	Abbreviation	R_1	R_2
Methylphosphonate	MP	CH_3	H
2'-O-methyl	M	O	O-CH_3
2'-O-allyl	A	O	O-CH_2-CH=CH_2
2'-O-butyl	B	O	O-$(CH_2)_3$-CH_3

b

5'AUG GUG CUG UCU CCC	α-globin mRNA
TAC CAC GAC AGA GGG5'	ODN1
TAC CAC GAC AGA GGG5'	ODN2
ACA CCT TCT TCA ACC AC5'	M-ODN3
5'GG AUG GUG CUG UCU CCC CUU U	RNA [35-55]
5'G GUU AUU GUG CUG UCU CAU CAU CAU U	RNA [390]
5'GGC AAG AAG GUG CUG GCU GCC UAA	RNA [252]

Figure 1. (**a**) Scheme of chemical modifications used in sandwich oligonucleotides. (**b**) Sequences of RNA target and antisense oligonucleotides. The sequence of the initiation region of the rabbit α-globin mRNA is indicated at the top. The sense oligoribonucleotides corresponding to the perfect target [RNA (35–55)] and to the sites of highest homology on the rabbit β-globin mRNA [RNA (390) and RNA(252)] are given below, bold face characters indicate the mismatched positions. The antisense oligonucleotide ODN1 was either unmodified or prepared as the 2'-O-methyl derivative (M-ODN1). ODN2 are sandwich oligomers with methylphosphonate (MP-ODN2), 2'-O-methyl (M-ODN2), 2'-O-allyl (A-ODN2) or 2'-O-butyl (B-ODN2) residues. The underlined regions in ODN2 correspond to the modified nucleotides (either 2'-O-alkyl or 5' methylphosphonate). M-ODN3 is the 2'-O-methyl sequence complementary to nucleotides 113–129 of the β-globin message.

phodiester DNA chimeras. We demonstrate here that selectivity is not restored with such oligonucleotides which bind much more strongly to the RNA than methylphosphonate-phosphodiester oligomers. But the fully 2'-O-methyl derivative was an efficient and specific inhibitor of α-globin synthesis, indicating the key role played by RNase H in non-selective inhibition of translation by antisense oligonucleotides.

MATERIALS AND METHODS

Oligonucleotide synthesis and analysis

Unmodified oligodeoxynucleotides and methylphosphonate sandwich oligomers were synthesized on a Model 7500 Milligen Biosearch automatic synthesizer, using conventional phosphoramidite chemistry. They were purified in one step by HPLC on a reverse phase column eluted with an acetonitrile gradient (10–50%) in 100 mM ammonium acetate buffer pH 7.2. 2'-O-alkyl RNA-phosphodiester DNA sandwich oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380B using protected 2'-O-alkylribonucleoside-3'-O-phosphoramidite monomers (6) 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. All oligonucleotides were analyzed on a 20% polyacrylamide/7 M urea gel, following 5' end-labeling with [γ-^{32}P]ATP (37.5 TBq/mmol; NEN) and T4 polynucleotide kinase, according to standard procedures (21).

Oligoribonucleotides were synthesized by *in vitro* transcription with T7 RNA polymerase, prepared from an overproducing strain according to previously published procedures (22). Oligoribonucleotides were purified by preparative gel electrophoresis on polyacrylamide gels.

Thermal denaturation profiles of oligonucleotide hybrids were monitored at 260 nm on a Uvikon 940 spectrophotometer using a quartz cuvette of 1 cm optical path length. The temperature of the cell-holder was adjusted by circulating fluid with a Hüber Ministat cryothermostat driven by a Hüber PD410 programmer. Oligonucleotide concentrations were determined using molar extinction coefficients according to Fasman (23). RNA and DNA oligomers in a 10 mM cacodylate buffer pH 7.8 containing 50 mM NaCl were pre-heated at 100°C for 5 min. The solution was quenched on ice and magnesium acetate was added up to 1 mM. The final target RNA and antisense oligonucleotide concentrations were 1 and 2 μM, respectively.

In vitro translation

Rabbit globin mRNA (0.05 μg) purchased from Promega, was mixed with the desired oligonucleotide and added to a final volume of 30 μl of wheat germ extract (Promega), according to the supplier's instructions. Each sample contained 0.925 MBq of [^{35}S]methionine (37 TBq/mmol) from NEN. The reaction was run for 1 h at 25°C. ^{35}S-labeled proteins were analysed by electrophoresis on 15% polyacrylamide gels containing 8 mM Triton X100/8 M urea/5% acetic acid, as previously described (24). Globin synthesis was determined either from densitometer scanning of autoradiographs or from counting of gel slices. The percentage of inhibition by antisense oligonucleotides was calculated using the ratio β_i/β_0 and α_i/α_0 where α_i, α_0 and β_i, β_0 refer to the amount of α- and β-globin synthesized in the presence and in the absence of i molecules of oligonucleotide, respectively.

RNA analysis

Following *in vitro* translation under the above conditions, globin RNA was extracted with phenol and precipitated with ethanol according to standard procedures (21) and dissolved in sterile water prior to loading on a 10% polyacrylamide gel containing 7 M urea. After electroblotting the nylon membrane was probed with ^{32}P 5' end-labeled oligonucleotide complementary to nt 3–19 of the β-globin RNA. Hybridization was performed as described previously (19).

Cleavage sites on RNA-oligonucleotide heteroduplexes by the endogenous wheat germ RNase H were determined using *in vitro* transcribed oligoribonucleotides. ^{32}P 5' end-labeled oligoribonucleotide (15 nM) were incubated with oligodeoxynucleotide (1 μM) in wheat germ extract at 25°C for 1 h. Following incubation, RNA was purified as described above and analyzed on a 20% polyacrylamide/7 M urea gel. Digestion by *Escherichia coli* RNase H (1.5 U) was performed by incubating ^{32}P 5' end-labeled oligoribonucleotide/oligonucleotide hybrids, under the concentration conditions used for wheat germ, in 20 mM Tris–HCl buffer, pH 7.5 containing 10 mM $MgCl_2$, 100 mM KCl and 0.1

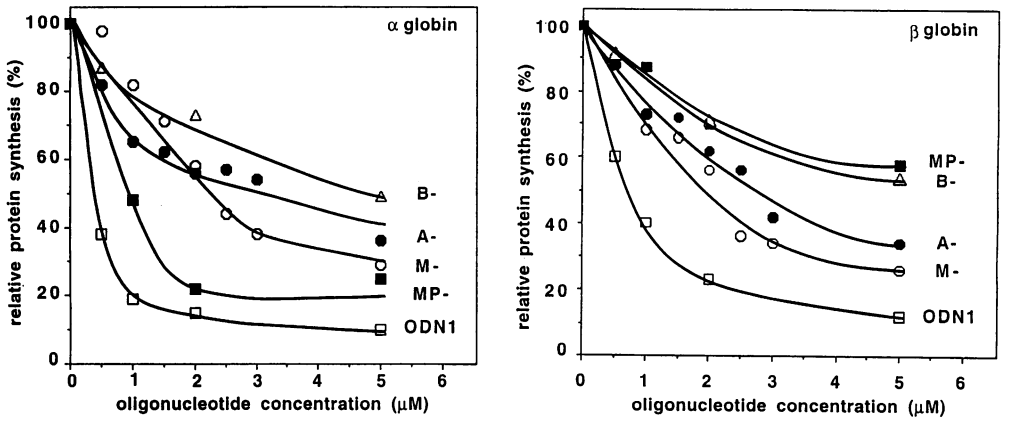

Figure 2. *In vitro* inhibition of rabbit globin synthesis by antisense oligonucleotides. Inhibition of α-(top) and β-globin (bottom) by ODN1 (□) MP-ODN2 (■), M-ODN2 (○), A-ODN2 (●) or B-ODN2 (Δ). The relative protein synthesis was calculated as described in Materials and Methods.

mM DTT. Cleavage products were analyzed on a 20% polyacrylamide/7 M urea gel. Autoradiographs were scanned by densitometry. For each sample, the relative amount of a given band was calculated with respect to all others (intact RNA and cleavage products).

RESULTS AND DISCUSSION

We targeted the nt 37–51 region of α-globin mRNA by antisense 15-mer oligonucleotides. We generated, from the complementary unmodified phosphodiester sequence ODN1, a series of chemically-modified derivatives ODN2 (Fig. 1). These oligomers, termed sandwich oligonucleotides, were made of three blocks: a central phosphodiester region, five nucleotides long inserted between flanks synthesized from nuclease resistant analogues: methylphosphonate DNA (MP), 2′-O-methyl RNA (M), 2′-O-allyl RNA (A) or 2′-O-butyl RNA (B) giving rise to MP-ODN2, M-ODN2, A-ODN2 and B-ODN2 oligonucleotides respectively (Fig. 1). These modified stretches, either methylphosphonate or 2′-O-alkyl RNA do not elicit RNase H activity. In addition we prepared fully modified 2′-O-methyl oligoribonucleotides: M-ODN1, complementary to the same region (nt 37–51) of the α-globin message and M-ODN3, targeted to nt 113–129 of the β-globin message, that is to the coding region.

Inhibition of *in vitro* translation by a 2′-O-methyl sandwich oligoribonucleotide

In wheat germ extract, the sandwich oligonucleotide M-ODN2 inhibited the synthesis of both α- and β-globin to the same extent, that is it did not display any specificity of translation inhibition (Fig. 2). Therefore, it behaved like the unmodified sequence ODN1 except that the inhibition efficiency of the 2′-O-methyl oligomer was lower than that of the parent unmodified sequence. Inhibition of β-globin synthesis can be ascribed to the presence on the β-message of several sites exhibiting partial homology, with the perfect target on the α-globin mRNA. In particular, two sites, RNA (390) and RNA (252), termed after the position of the 5′ nucleotide bound to the antisense oligonucleotide, exhibit a perfect match at 13 and 12 positions out of 15, respectively, taking into account a G.U pair in the ODN2/RNA (390) duplex (Fig. 1b).

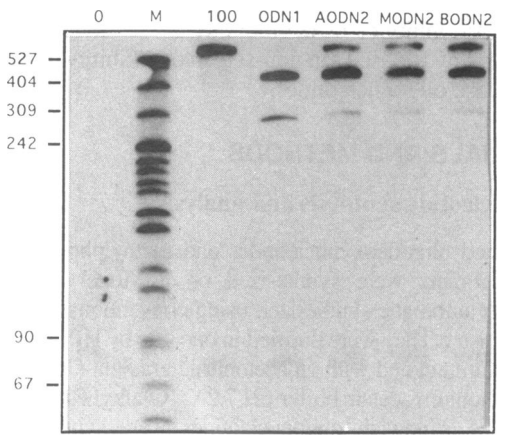

Figure 3. Northern blot analysis of rabbit β-globin mRNA following translation in wheat germ extract. *In vitro* translation was performed in the absence (100) or in the presence of 5 μM of the antisense oligonucleotide indicated at the top the lane. M refers to DNA size markers.

We have shown in a previous study, that non-specific inhibition of *in vitro* translation by the unmodified oligomer ODN1 was due to its binding to these sites, on the β-globin message (19), subsequently leading to the cleavage of β-globin mRNA by RNase H. The association of M-ODN2 with these sites and/or others might also be responsible for the non-specific effect displayed by this oligonucleotide on *in vitro* globin synthesis.

mRNA analysis after translation in the presence of a 2′-O-methyl sandwich oligoribonucleotide

Wheat germ extract is known to contain a class II RNase H activity (25). This enzyme could recognize as a substrate the mRNA region bound to the phosphodiester window of M-ODN2. Indeed, Northern blot analysis of globin mRNA revealed that both α- and β-globin messages were cleaved, following translation in wheat germ extract in the presence of M-ODN2. Two major fragments of β-globin mRNA, ~400 and 250 nucleotides long, were detected as in the case of ODN1 (Fig. 3), likely

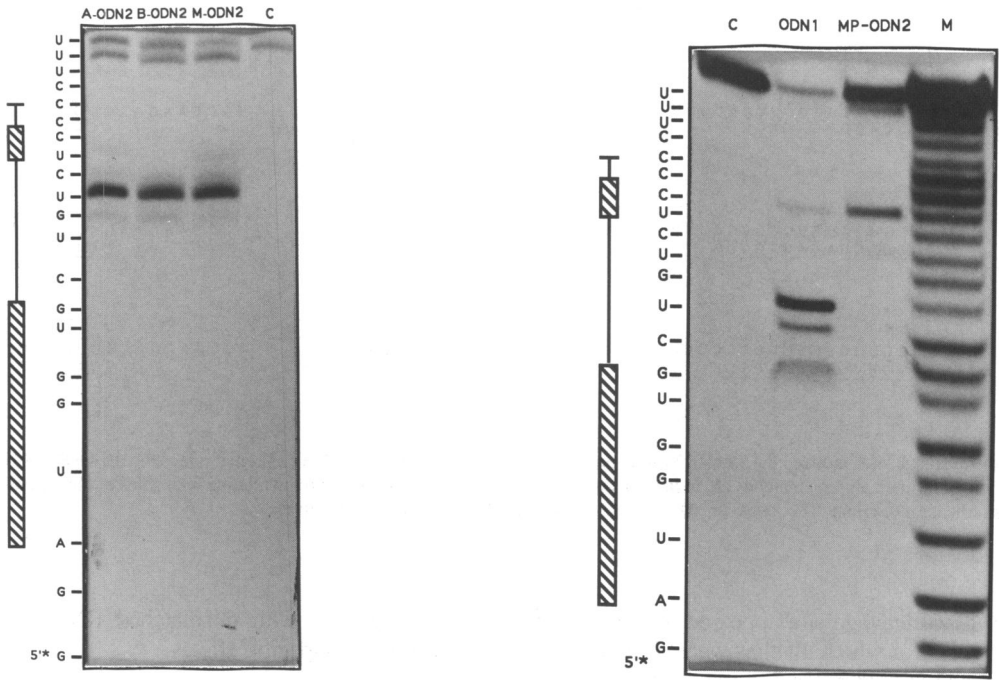

Figure 4. Cleavage analysis of antisense oligonucleotide–RNA heteroduplexes. 5′ End-labeled RNA (35–55) was incubated with the antisense oligonucleotide indicated at the top of the lane in wheat germ extract conditions used for *in vitro* translation. C corresponds to RNA (35–55) incubated in the extract, in the absence of any complementary oligonucleotide.The RNA sequence is given to the left of the panels deduced from the alkaline hydrolysis ladder (M); the boxes indicate the modified residues of the antisense sequence.

induced by the binding of antisense oligomers to the sites located at positions (390) and (252). In addition, a faint band, corresponding to an RNA fragment ~180 nucleotides long, was generated in the presence of M-ODN2 but not in the presence of ODN1 (Fig. 3). A computer search did not reveal any significant homology of this region of rabbit β-globin mRNA with the target.

It should be noted that, after *in vitro* translation intact β-globin mRNA was still observed in the presence of 5 μM M-ODN2 but not in the presence of ODN1. As the stability of the complex formed with 2′-O-methyl sandwich oligoribonucleotide, is higher than that formed with the unmodified sequence (see below), this indicated that the modified oligomer bound to RNA formed a poorer RNase H substrate than ODN1. Therefore, in contrast to the methylphosphonate oligomer MP-ODN2 (see Fig. 2 and ref. 19), the homologous sandwich oligomer M-ODN2 did not restrict RNase H activity at non-target site and, consequently, inhibited non-specifically globin synthesis in wheat germ extract.

Mapping of RNase H cleavage sites on synthetic RNA–DNA duplexes

The results obtained with MP-ODN2 on the one hand and M-ODN2 on the other hand, could be related to a different activity of RNase H on the duplexes that these oligonucleotides formed with RNA. Although the central window is identical in both sandwich oligomers, the terminal segments exhibit very different properties. The neutral internucleoside linkage of methylphosphonates or the presence of a bulky substituent on the 2′ position of 2′-O-alkyl analogues could alter either the recognition of the duplex by RNase H or the location of its catalytic site with respect to the phosphodiester stretch.

We performed a comparative analysis of a 21mer RNA [RNA (35–55)] corresponding to the target site on the α-globin message, associated either to ODN1 or to one of the sandwich oligomer, after incubation in wheat germ extract. The unmodified RNA (35–55)-ODN1 heteroduplex led to three major cleavage sites, the most prominent ones being 5–8 nucleotides away from the 3′ end of the DNA (Fig. 4). These sites were no longer seen for either of the sandwich oligomer. Instead, a strong and almost unique site appeared, shifted 3′ (with respect to the oligonucleotide) compared to the ones induced by ODN1. This position, on the 5′ side of the phosphodiester window of the sandwich oligomers, was not the same for the two types of chimeric oligonucleotides. The location of the cleavage site was close to the methylphosphonate-phosphodiester junction but was two nucleotides away from the window side for the 2′-O-alkyl analogues (Fig. 4). Surprisingly, all sandwich oligomers also induced the release of the 3′ terminal RNA residue which is four nucleotides away from the end of the heteroduplex. Therefore, this shows that the binding of the enzyme to the duplex is altered by the chemical nature of the flanks.

We then carried out a systematic investigation of the effect of both chemical modification in the antisense sequence and of mismatches, on the cleavage of heteroduplexes by *E.coli* RNase H. We used unmodified ODN1 or sandwich oligonucleotides in combination with either the perfect target RNA (35–55) or the partially complementary ones [RNA (390) and (252)]. The patterns of cleavage induced on RNA (35–55) were similar to those obtained in the wheat germ extract, except that the terminal cleavage site was not seen with sandwich oligomers MP- or M-ODN2 and that four sites instead of one were detected with MP-ODN2 (Fig. 5). The presence of mismatches led to a

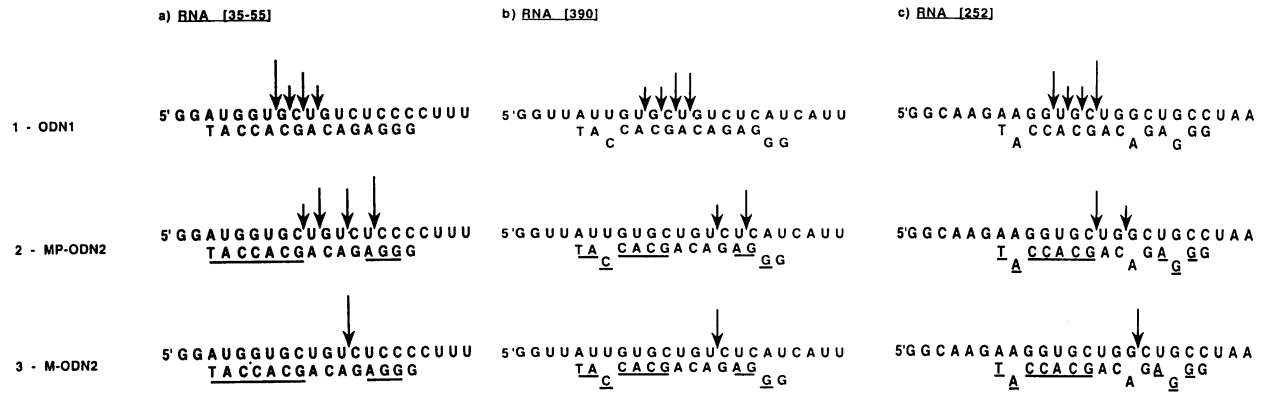

Figure 5. Location of the cleavage sites induced by *E.coli* RNase H on RNA (35–55), RNA (390) or RNA (252) in the presence of ODN1, MP-ODN2 or M-ODN2. The modified residues are underlined and mismatches are indicated. Arrows indicate both the location and the relative intensities of the cleavage sites; long, medium and short tailed arrows indicate more than 30%, between 10 and 30% or less than 10% cleavage, respectively.

moderate effect: no new cleavage site appeared, except for the ODN1/RNA(252) hybrid for which all sites were shifted by one position (in the 3′ direction with respect to the oligonucleotide). With MP-ODN2 only two out of the four sites seen with the perfect target can be seen with either of the mismatched hybrids. Identical results were obtained with M-, A- and B-ODN2 (not shown). The general trend was a reduced yield of breakdown products induced by sandwich oligomers with either RNA (390) or RNA (252) compared to RNA (35–55).

Stability of synthetic heteroduplexes

Methylphosphonate oligonucleotides bind weakly to complementary RNA sequences, compared to unmodified oligonucleotides, whereas 2′-O-methyl derivatives bind more strongly (6,26,27). We compared the binding of sandwich oligomers or of ODN1, to the perfect RNA target (35–55) and to a mismatched one corresponding to the (390) site of the β-globin mRNA. The *T*m values deduced from the UV-monitored melting curves obtained for the different mixtures are presented in Table 1. As expected, the MP-ODN2/RNA (35–55) duplex had a *T*m ~15°C lower than the ODN1/RNA (35–55) complex under our conditions. In contrast, the fully-paired hybrid formed with M-ODN2 melted at a higher temperature ($\Delta T = +12.5°C$). The same order was observed for the stability of complexes formed with the mismatched RNA: *T*m decreased in the order M-ODN2>ODN1>MP-ODN2. In addition, MP-ODN2 led to broader transitions compared to others, indicating a lower cooperativity (not shown). This behaviour likely accounts for the differences observed between M-ODN2 and MP-ODN2. The high affinity displayed by 2′-O-methyl analogues leads to exceedingly stable duplexes, with mismatched RNA sequences, ultimately leading to non-specific effects.

Effects of other 2′-O-alkyl sandwiches

Can we achieve selective inhibition of translation with sandwich antisense oligonucleotides, made with 2′-O-alkyl segments? In order to minimize RNase H activity on mismatched hybrids we should decrease the binding constant of the antisense sequence. This could be done in two ways: (i) by reducing the length of the oligomer; or (ii) by increasing the size of the 2′-O substituent. In our case, the sequence of the (390) RNA site on the β-globin message shows the limit of the first possibility: as mismatches are close to the ends of the duplexes, shortening the antisense oligonucleotide will lead to a perfect hybrid with β-globin mRNA as well as with α-mRNA.

Therefore, we prepared sandwich antisense oligonucleotides with bulkier groups on the 2′ position as this leads to a decreased affinity for the complementary sequence (6). Indeed 2′-O-allyl (A) and 2′-O-butyl (B) analogues of ODN2 gave lower *T*m values than M-ODN2 with both the complementary sequence RNA (35–55) and with the mismatched one (Table 1). Nevertheless, the *T*m of the B-ODN2/RNA (390) duplex was still higher than the one obtained with MP-ODN2. This agrees with the fact that the trend observed with M-ODN2 was also seen with either A-ODN2 or B-ODN2: these sandwich oligomers induced non specific inhibition of β-globin synthesis (Fig. 2) and the cleavage of β-globin mRNA (Fig. 3). Moreover the same cleavage pattern was observed with short synthetic RNA (Fig. 4). Therefore in our case we were not able to achieve selective translation inhibition with chimeric oligonucleotides containing 2′-O-alkyl RNA. We then investigated the effect of the fully modified 2′-O-methyl oligomer, as no RNase H contribution to the antisense effect is expected in this case.

Table 1. Melting temperatures for antisense oligonucleotide–RNA heteroduplexes

	ODN1	MP-ODN2	B-ODN2	A-ODN2	M-ODN2	M-ODN1
RNA (35–55)	59.5	44.3	66.9	70.5	72.0	85.5
RNA (390)	41.5	36.5	44.4	49.0	50.9	63.7

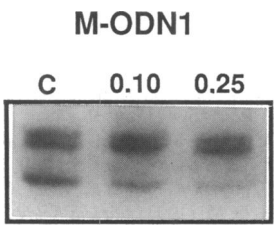

Figure 6. *In vitro* inhibition of α-globin synthesis by a 2′-O-methyl antisense oligonucleotide. Translation of rabbit globin mRNA was performed either in the absence (0) or in the presence of 0.1 or 0.25 μM of M-ODN1, the 2′-O-methyl oligoribonucleotide complementary to nt 37–51 of the α-globin message. Experimental conditions were as described in Figure 2.

Inhibition of *in vitro* translation by 2′-O-methyl oligoribonucleotides

The elongating ribosome is endowed with an unwinding activity. Consequently, antisense oligonucleotides targeted to the coding region of mRNA do not inhibit translation unless they induce permanent damage (crosslink or RNase H-mediated cleavage for instance). Indeed, M-ODN3, complementary to nt 113–129 of the β-globin message, that is to the coding region, did not induce any effect either on β-globin or on α-globin synthesis up to 5 μM (not shown). This is in fair agreement with previously published results for alpha (4,7) or 2′-O-methyl analogues (28). These derivatives do not elicit RNase H activity and cannot physically arrest the elongating ribosome.

It should be noticed that the (252) and (390) sites, partially complementary to ODN1, are located in the open reading frame of the β-message. Therefore, if RNase H is the major source of the non-specific inhibition of β-globin, in wheat germ extract, by the M-ODN2 sandwich oligomer, a fully modified 2′-O-methyl oligoribonucleotide should display a selective antisense effect towards α-globin. As a matter of fact, the 15mer M-ODN1, complementary to nt 37–51 of the α-globin mRNA induced a dose-dependent reduction of α-globin synthesis whereas no effect was seen on β-globin synthesis (Fig. 6). This is related to the RNase H-independent mechanism previously described for α-oligomers: antisense oligonucleotides targeted to the 5′ leader region, can physically impede the initiation complex to scan the mRNA (24). Therefore, the high affinity of the 2′-O-modified oligomer for its RNA target allows efficient inhibition of protein synthesis in good agreement with a recent report (28).

CONCLUSION

One of the major interests of antisense oligonucleotides is the potential selectivity which derives from the specificity of interaction between the antisense strand and the target sequence. Antisense oligonucleotides 15 nt long are expected to lead to specific inhibitory effects (29). However, RNase H which cleaves the RNA target of RNA–DNA heteroduplexes, can act on mismatched hybrids (15,30). This might be a source for non-specific effects resulting from the destruction of RNA sequences partially complementary to the antisense oligomer.

The design of sandwich molecules made of a central part allowing RNase H activity inserted between two methylphosphonate fragments, not only improved the metabolic stability of the oligonucleotides compared to unmodified ones (31), but led to increased specificity due to the restricted RNase H sensitivity of mismatched heteroduplexes (18,19). Due to the problem raised by stereo-isomers in methylphosphonate derivatives it was of interest to check whether similar properties could be displayed by other kinds of sandwich molecules.

We synthesized 2′-O-alkyl sandwich oligomers with a phosphodiester window of five nucleotides which corresponds to the lower limit to observe RNase H activity (14). We demonstrated here that the substitution of 2′-O-methyl ribonucleotides for methylphosphonate residues in a sandwich 15mer drastically changed the behaviour of chimeric antisense sequences with respect to the inhibition of translation in wheat germ extract. The methylphosphonate sandwich sequence MP-ODN2, complementary to the rabbit α-globin mRNA, displayed a higher specificity than the unmodified sequence. In contrast, none of the 2′-O-alkyl homologues of MP-ODN2 was a selective inhibitor of translation: whatever the 2′-O substituent (methyl, allyl or butyl) both α- and β-globin synthesis were decreased to the same extent. Such sandwich oligomers retained the capacity shown by the unmodified oligonucleotide, to induce the cleavage of partially complementary sites on the β-globin mRNA. The major difference between sandwich oligonucleotide–RNA hybrids resided in the stability of the various heteroduplexes. The *T*m for the duplex formed by the methylphosphonate chimeric oligomer MP-ODN2 was much lower than that obtained with the unmodified homologous antisense sequence ODN1, in agreement with previous results (32). In contrast, all 2′-O-alkyl derivatives exhibited significantly higher *T*m values. Therefore, the specificity of inhibition seems directly related to the stability of the antisense oligonucleotide-RNA hybrid and to the activity of RNase H on mismatched hybrids.

The role played by RNase H in non-specific inhibition of *in vitro* translation is further supported by the fact that fully modified 2′-O-methyl oligomer leads to a selective decrease of protein synthesis despite that it binds stronger to RNA than the sandwich oligomer. Such derivatives can prevent translation if targeted to the 5′ leader region but not to the coding region. Lastly it should be pointed out that despite their increased affinity for the RNA target, 2′-O-alkyl sandwich oligonucleotides inhibited less efficiently *in vitro* α-globin synthesis than MP-ODN2. This is likely related to a reduced ability of 2′-O-alkyl sandwich oligomers to elicit RNase H.

This work shows that exceedingly stable duplexes will give rise to non-specific effects when RNase H contributes to antisense inhibition. This will occur when long antisense sequences are used; similarly, conjugation of stabilizing ligands such as intercalating agents (29,33), will increase the affinity for both the perfect and partially complementary target sequences. The ideal antisense oligonucleotide should be chosen to saturate the target site and to simultaneously lead to negligible amounts of mismatched hybrids. Consequently the oligonucleotide should be designed with respect to the biological milieu in which it will be used. Moreover, derivatives displaying very high affinity might never allow to achieve specificity as the length of the oligomer showing the appropriate binding constant might be below the limit required to ensure the uniqueness of the target sequence in the genome of interest.

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