
Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides

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ABSTRACT

We have studied the translation of rabbit globin mRNA in cell free systems (reticulocyte lysate and wheat germ extract) and in microinjected *Xenopus* oocytes in the presence of anti-sense oligodeoxynucleotides. Results obtained with the unmodified all-oxygen compounds were compared with those obtained when phosphorothioate or α -DNA was used. In the wheat germ system a 17-mer sequence targeted to the coding region of β -globin mRNA was specifically inhibitory when either the unmodified phosphodiester oligonucleotide or its phosphorothioate analogue were used. In contrast no effect was observed with the α -oligomer. These results were ascribed to the fact that phosphorothioate oligomers elicit an RNase-H activity comparable to the all-oxygen congeners, while α -DNA/mRNA hybrids were a poor substrate. Microinjected *Xenopus* oocytes followed a similar pattern. The phosphorothioate oligomer was more efficient to prevent translation than the unmodified 17-mer. Inhibition of β -globin synthesis was observed in the nanomolar concentration range. This result can be ascribed to the nuclease resistance of phosphorothioates as compared to natural phosphodiester linkages. α -oligomers were devoid of any inhibitory effect up to 30 μ M. Phosphorothioate oligodeoxyribonucleotides were shown to be non-specific inhibitors of protein translation, at concentrations in the micromolar range, in both cell-free systems and oocytes. Non-specific inhibition of translation was dependent on the length of the phosphorothioate oligomer. These non-specific effects were not observed with the unmodified or the α -oligonucleotides.

INTRODUCTION

The use of antisense oligodeoxynucleotides as specific inhibitors of gene expression has undergone a rapid expansion over the past several years (1,2). Central to this approach is the presumption that messenger RNAs bound as RNA-DNA duplexes either cannot be translated by ribosomes (3-5) or are destroyed by RNase-H (6-10). In order to be effective *in vivo*, synthetic oligonucleotides must share several properties. Among these are :1) chemical stability, 2) water solubility, 3) high thermodynamic stability of the RNA-DNA duplex and 4) nuclease resistance. Unmodified phosphodiester (PO) DNA meets these requirements except for the last one. Thus, a series of modified derivatives have recently been synthesized (11-14). Substitution of sulfur for one of the phosphodiester oxygen atoms yields a molecular species that meets all four criteria (12). These phosphorothioate (PS) oligodeoxynucleotides have indeed recently been shown to inhibit the cytopathic effect of HIV-1 (15; Matsukura et al., unpublished results). In experiments in chronically infected H9 cells, the expression of p24 *gag* protein was shown to fall >90% in the presence of a 10 μ M concentration of a

phosphorothioate sequence complementary to the 5' region of the *rev* (formerly *art/trs*) gene. The normal congener was ineffective as was a methylphosphonate construct. Marcus-Sekura et al. (16) showed that an anti-sense phosphorothioate was an effective inhibitor of chloramphenicol acetyl transferase activity in the standard CAT assay. In a series of experiments conducted in HL60 cells, an anti-sense *c-myc* phosphorothioate oligomer was unable to consistently inhibit cellular proliferation unless supplied in liposomes, whereas in multiple experiments, the normal oligomer inhibited levels of myc protein by >50% at 12 h (17).

Another class of modified oligonucleotide meets the criterion of nuclease resistance. In these compounds the natural β -configuration of the nucleoside is transposed into its α -analogue (13, 14, 18-26). However these α -oligonucleotides were reported to be poor inhibitors of VSV mRNA translation in rabbit reticulocyte lysate (27).

Cell-free systems (28-34) and micro-injected *Xenopus* oocytes (8,35-37) are effective means for evaluating the ability of modified oligonucleotides to act as antisense inhibitors. We present here a comparative study of both unmodified and nuclease resistant oligonucleotides tested for their ability to promote selective arrest of rabbit globin mRNA translation.

MATERIALS AND METHODS

Oligodeoxynucleotides

Phosphodiester oligodeoxynucleotides were synthesized either on a Pharmacia or on an Applied Biosystems Model 380B Synthesizer, and were purified via high-pressure liquid chromatography (Waters) on a PRP-1 column. Phosphorothioates were synthesized and purified via a modification (12) of the procedure of Stec et al. (38). Alpha-oligodeoxynucleotides were synthesized on a Pharmacia automatic synthesizer and purified as previously described (24). The oligomer length homogeneity was periodically evaluated by running samples on 15% polyacrylamide/6M urea gels. After electrophoresis, bands were either stained with ethidium bromide and viewed by UV-light or revealed by autoradiography in the case of ^{32}P -labelled oligonucleotides. All preparations yielded a single species in each lane loaded.

Cell-free translation systems

Wheat germ extract was purchased either from New England Nuclear or from Genofit (Geneva). Oligomer was added to a translation mixture containing ^{35}S -methionine. Unless otherwise stated experiments in wheat germ extracts were performed under the following conditions: 0.05 μg of rabbit globin mRNA was mixed with the oligonucleotide and added to 30 μl of the translation mixture. The final concentration of total mRNA was 9.3nM (i.e. 3.9nM in β -globin). The reaction was run at 25°C during 30 min. Reactions were generally carried out without premixing RNA with the oligomer. We showed that premixing did not alter the results.

Rabbit reticulocyte lysate was purchased from New England Nuclear. The oligomer, at

the appropriate concentration, was added to the translation mixture (25 μ l) containing 0.1 μ g globin mRNA and ³⁵S-methionine. The samples were then incubated for 90 min at 37°C.

An aliquot of the reaction mixtures was then analyzed either on a 15% polyacrylamide-SDS gel with a 5% stack or on a 12% polyacrylamide gel containing 8 mM Triton X100 and 6M urea. The gels were then fixed in a 40% methanol/7% acetic acid solution for about 1h, soaked in a solution of sodium salicylate ("Fluoro-Hance", Research Products International Corp.) for 30 min, and dried under vacuum prior to autoradiography.

Translation in *Xenopus* oocytes

Stage 6 oocytes (selected via stereotactic microscopy) were obtained from the Laboratoire de Physiologie de la Reproduction (Paris VI University). Specimens were maintained in modified Barth's saline solution (39). 80 nl of a 1/1 (v/v) mixture of globin mRNA (50 μ g/ml) and oligomer, dissolved in sterile distilled water, were injected in *Xenopus* oocytes; 4 to 5 hours after injection, oocytes were incubated in the presence of ³⁵S-methionine for about 15 hours. The samples were then homogenized in 20 μ l (per oocyte) of 20 mM Tris, pH 7.6, 0.1 M NaCl, 1% Triton X100 and 1 mM PMSF (40). Proteins were then analysed by SDS-PAGE electrophoresis on a 13.5% acrylamide gel. Assuming a free diffusion compartment of 0.5 μ l inside the oocyte, the final intracellular concentration of β -globin mRNA was about 16 nM.

Hybridization studies

1 μ g of rabbit globin mRNA was bound to a nitrocellulose filter by heating at 80°C during 2 hours. The filter was incubated in a mixture containing about 10⁷ cpm of ³²P-labelled oligomer and 2 ml of 6xSSC/10xDenhardt's solution (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2; 10xDenhardt's is 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinyl-pyrrolidone). The filters were then placed in a thermostated holder, and were eluted with 6xSSC as the temperature was increased at a rate of 1.2°C/min. Thermal elution profiles were constructed, and the T_c determined to be that temperature at which 50% of the total counts had been eluted (41).

Oligodeoxynucleotide-promoted cleavage of globin mRNA by RNase-H

RNase-H from *E. coli* was obtained from Genofit. Kinetic experiments were run at 37°C in a 20 mM Tris-HCl buffer, pH 7.5 containing 100 mM KCl, 10 mM MgCl₂ and 0.1 mM dithiothreitol. Incubations of 0.3 μ g of rabbit globin mRNA and 30pmoles of oligonucleotide were performed in a total volume of 30 μ l, in the presence of 2.5 units of enzyme. At the appropriate times aliquots of 5 μ l were spotted on a nylon membrane. UV-irradiated membranes were probed with ³²P 5' end-labelled 17PO- β (Figure 1) and autoradiographed.

RESULTS

Sequences studied

The sequences of the oligonucleotides used throughout this study and of the complementary regions on rabbit α - and β -globin mRNAs are given in figure 1. We targeted

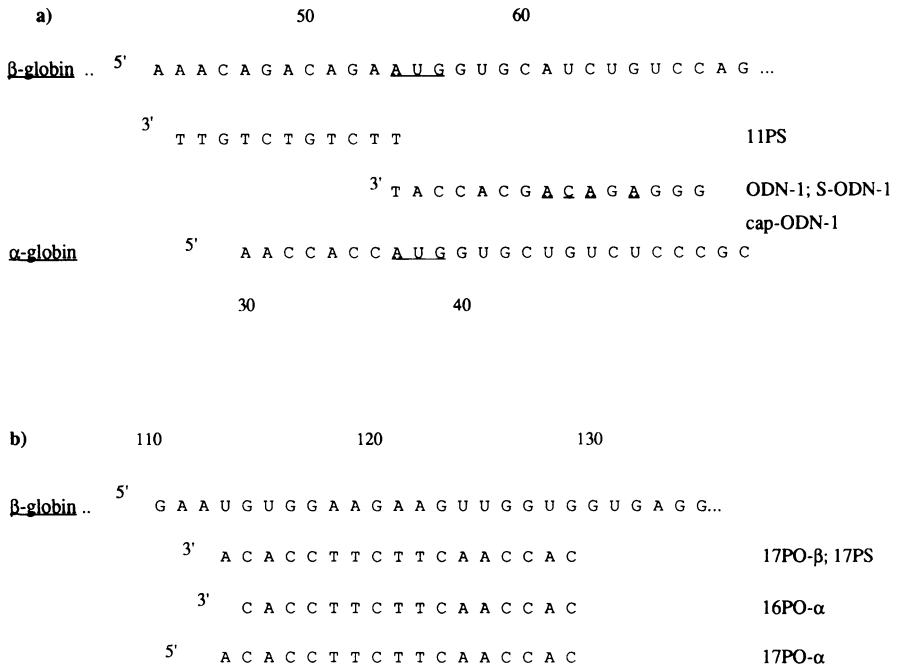


Figure 1 : Nucleotide sequences of oligodeoxynucleotides complementary to a) AUG region of rabbit β-globin and α-globin (51) and b) coding sequence of rabbit β-globin mRNA. The numbering above the RNA sequences refer to the transcription start; the translation initiation codons are underlined. The abbreviations of the antisense oligonucleotides are indicated on the right of the sequences (for details see "Results; Sequences studied"). 11-mers, 17-mers and the 16-mer were complementary to the β-globin mRNA and ODN-1 to the α-globin mRNAs. The latter oligonucleotide can pair with the β-message giving 4 mismatches (underlined letters in the ODN-1 sequence).

two regions of the β-globin mRNA, namely nucleotides 44-54 and 113-129 that were already selected in a previous study (8). The 44-54 sequence is located immediately upstream of the start codon, while the 113-129 sequence is within the coding region of the message. An unmodified phosphodiester (PO) oligonucleotide (17PO-β) and phosphorothioate (PS) analogues (11PS; 17PS), complementary to these two regions, were synthesized. Two α-oligodeoxynucleotides complementary to the coding region were also used. The first one, 16PO-α, was designed to be in an antiparallel orientation with respect to the target sequence. A second one, 17PO-α was synthesized to bind its target in a parallel orientation.

In addition we constructed several oligomers complementary to the α-globin mRNA sequence. ODN-1 is a 15-mer sequence complementary to the α-globin initiation codon and downstream region (Figure 1). This sequence also complements a similar region of the β-globin message with the exception of a 4-base mismatch, assuming the formation of two G-U

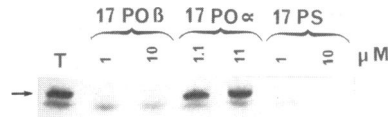


Figure 2 : Effect of various 17-mers on in vitro synthesis of rabbit β -globin. Autoradiograph of a 12% Triton-acetic acid-urea polyacrylamide gel of proteins synthesized in wheat germ extracts as indicated in Materials and Methods in the absence (T) or in the presence of 17PO- β , 17PO- α or 17PS at the indicated concentration. The upper band (arrow) corresponds to β -globin and the lower one to α -globin.

pairs. ODN-1[Sen] is the sense construct. ODN-1 and ODN-1[Sen] were also synthesized in all-phosphorothioate forms (S-ODN-1; S-ODN-1[Sen]). In addition, they were also constructed with two phosphorothioates at both the 3' and 5' ends, and are referred to as cap-ODN-1 and cap-ODN-1[Sen], respectively.

A series of random sequences were also made. ODN-2 (5'-dACTCC-3') is a 5-mer, and ODN-3 (5'-dCCAAACCATG-3') a 10-mer. ODN-2 and -3 were synthesized as phosphorothioate derivatives only. A random 16-mer, termed ODN-4, with base composition equivalent to ODN-1 (5'-dACGCGAGGACCATAGT-3') contained 5 contiguous phosphorothioates at the 5' and 3' ends, separated by 5 phosphodiester linkages. We also synthesized the same 16-mer (5'-dACGCGAGGACCATAGT-3') containing alternating PS and PO linkages (ODN-5). The absence of perfect complementarity between these oligomers and rabbit globin mRNAs was checked by a computer search (CITI 2).

Table 1

Oligos	T _c	WGE	<i>Xenopus oocytes</i>		
			oligo/RNA	Coinjection	RNA/oligo
17PO- β	60	50	>>	30	650
17PO- α	50	>>		>>	
17PS	39	50	30	3	7
11PS	28	3000		8000	

Table 1 : Stability of oligonucleotide/RNA hybrids and inhibition of translation by antisense oligonucleotides. Temperature of half-dissociation (T_c) of filter-bound complexes, determined as described in Material and Methods are given in °C. Concentrations (indicated in nM), leading to a 50% decrease of translation either in wheat germ extracts (WGE) or in oocytes following co-injection or delayed injections (RNA/oligo means RNA first; oligo/RNA means oligo first; see text) were determined from curves shown on figures 3 and 6. The symbol >> indicates that 50% inhibition was not reached at the highest concentration tested.

Effects of oligonucleotides on translation in cell-free media

Translation in wheat germ extract : Translation of rabbit globin mRNA in cell-free systems gives rise to two bands corresponding to the α - and β -chains which can be separated on Triton-urea-acetic acid polyacrylamide gels. In a first set of experiments we compared the effects on globin synthesis of three 17-mers, 17PO- β , 17PO- α and 17PS, targeted to the coding region of β -globin mRNA (Figure 1). The results shown in figure 2 indicate that 17PO- β is a specific anti-sense inhibitor in a wheat germ system: a fifty per cent decrease was observed at 50 nM (Table 1) and a total inhibition of β -globin synthesis was attained at 1 μ M, whereas α -globin synthesis was not affected, in good agreement with a previous report (8). In contrast even at 11 μ M no effect was observed in the presence of 17PO- α . At low concentration (below ca. 1 μ M) a specific decrease of β -globin synthesis resulted from addition of 17PS to the translation mixture, 50% inhibition being observed at 50 nM as in the case of 17PO- β (Table 1). However, by 1-2 μ M, the synthesis of α -globin was also decreasing and at 10 μ M 17PS both α - and β -globin mRNA synthesis were 100% inhibited (Figure 2). As this oligonucleotide is not complementary to any region of α -globin mRNA (no match above than 70% homology) this should be ascribed to a non-specific effect on translation. Thus, in a defined concentration range (<500nM), the anti-sense 17PS inhibits the synthesis only of its directed target, i.e., β -globin; and the sense analogue, in that same concentration range, has little if any effect on the synthesis of either α - or β -globin (data not shown).

A similar conclusion regarding antisense specificity can be drawn from experiments with 11PS, a phosphorothioate oligomer complementary to the region immediately upstream of the AUG codon of β -globin (Figure 1), homologous to an unmodified 11-mer (11PO) that we used in a previous study (8). Specific inhibition of β -globin synthesis (at low concentration) and non-specific inhibition of α -globin mRNA translation (at high concentration) were observed but 11PS was a less efficient inhibitor than 11PO. It should also be noted that higher concentrations of 11PS (>5 μ M) than those of 17PS were required to observe non-specific effects (Figure 3). This can be related to a length effect (see below).

We also tested ODN-1, a 15-mer complementary to the α -message (Figure 1), in the wheat germ system; 100% inhibition of translation was achieved at 1 μ M (Figure 4) but no inhibition was observed with the sense congener (data not shown). On the other hand, both sense and anti-sense S-ODN-1 constructs were potent inhibitors of translation at 5 μ M, with control levels being reached at ca. 500 nM (Figure 4). The region from 500 nM to 5 μ M was not examined in greater detail in this study. Translation of Brome Mosaic Virus mRNA was also inhibited, in the wheat germ system, in the presence of S-ODN-1[Sen], to which it has no sequence homology above 70% (Figure 4).

We evaluated the dependence of non-specific translation inhibition on phosphorothioate oligomer length. The random 5-mer ODN-2 was not inhibitory to globin mRNA translation up to 25 μ M and only partially inhibitory at higher concentrations (Figure 4). If the random

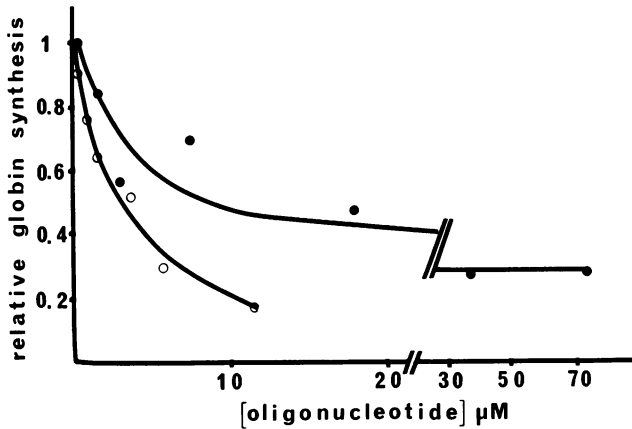


Figure 3 : Effect of 11PS on rabbit β -globin synthesis. Globin mRNA was either translated in wheat germ extracts (O) or in micro-injected *Xenopus* oocytes (●). ^{35}S -labelled proteins were analyzed by gel electrophoresis (see Materials and Methods). β -globin synthesis was determined from densito-meter tracings of autoradiographs, relatively to the synthesis observed in the absence of added oligodeoxynucleotide.

10-mer, (ODN-3), was used in the translation assay, control levels of protein synthesis were not achieved at concentrations higher than $3 \mu\text{M}$. ODN-3 has no complementary sequence matching better than 70%, neither in α - nor in β -globin mRNA. In the case of ODN-1 several sequences were found that could form four base pairs. Such hybrids are not expected to be stable under our conditions. A 28-mer, S-dC₂₈, was also examined in the wheat germ system. This oligomer is capable of inhibiting the cytopathic effect of the HIV virus in newly infected H9 cells, at concentrations in the low micromolar range (15). S-dC₂₈ was the most potent inhibitor tested in this system: translation of globin mRNA was entirely inhibited above 500 nM.

As the non-specific inhibitory properties of phosphorothioate oligodeoxynucleotides appeared to be highly length dependent, we wanted to determine if a molecule containing blocks of contiguous phosphorothioates (where each block was itself too short to be an inhibitor) could act in summation to produce an effective translation inhibitor. Because we knew that a 5-mer phosphorothioate had a limited inhibitory effect on translation, we synthesized a random 16-mer ODN-4, with base composition essentially equivalent to ODN-1, containing 5 contiguous phosphorothioates at the 5' and 3' ends separated by 5 phosphodiester linkages. When tested in the wheat germ system, 100% inhibition of translation was seen at concentrations higher than $6.25 \mu\text{M}$ (Figure 4). Thus, this molecule does not behave as if it were two separated 5-mers, but rather as if it were a 10-mer (compare to ODN-3).

The non-specific inhibition of translation is not related only to the length of the

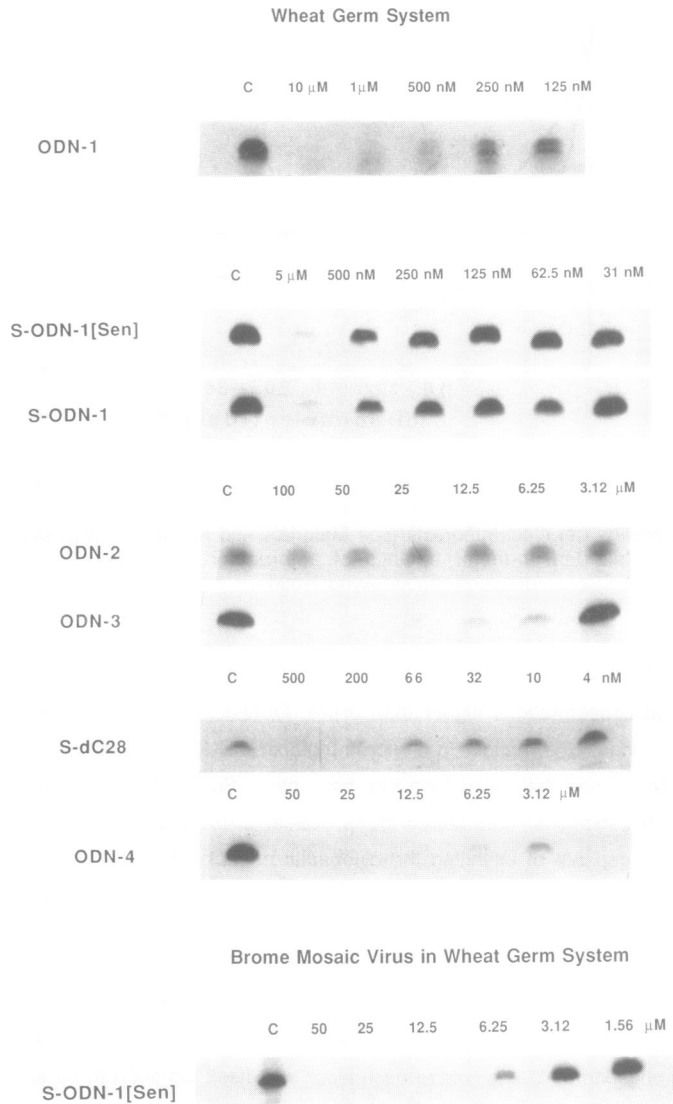


Figure 4 : Translation of rabbit globin mRNA and Brome Mosaic Virus mRNA in the wheat germ system in the presence of various oligomers: ODN-1 is a 15-mer complementary to α -globin mRNA; S-ODN-1 is the phosphorothioate analogue; ODN-1[Sen] and S-ODN-1[Sen] are the sens oligomers; cap-ODN-1 contains two phosphorothioate linkages at the 5'- and at the 3'-ends; ODN-2 and ODN-3 are random phosphorothioate 5-mer and 10-mer, respectively; ODN-4 is a random 15-mer composed of two blocks of 5 phosphorothioates separated by 5 phosphodiester (for more details see "Results; Sequences studied"). C=control (no added oligomer). Numerals above each lane are the concentration of added oligomer (in μ M or in nM). Incubation time was 120 min. at 22°C.

oligomer: cap-ODN-1 (antisense) also inhibited protein translation (100% below 3 μM) while the sense congener exhibited dose dependent inhibition above 3 μM (data not shown). This non-specific inhibition was unexpected as the molecule contained only two phosphorothioates at each end. In contrast, a concentration of 100 μM ODN-5, a random 16-mer with essentially the same base composition as ODN-1 and containing eight alternating phosphorothioates, was required for 100% inhibition of translation (data not shown).

Translation in rabbit reticulocyte lysate : When ODN-1 was used in the reticulocyte lysate system, concentration dependent inhibition of globin synthesis was observed, with 100% inhibition (α - plus β -) seen at 100 μM , while no inhibition was seen for the sense analog (5'-dATGGTGCTGTCTCCC-3') up to 100 μM (Figure 5). This is approximately a 100-fold decrease in sensitivity as compared to the wheat germ extract, and may reflect low levels of RNase-H activity present in reticulocyte lysate (7). However, when phosphorothioates of identical sequence were used under similar reaction conditions, both the sense and the antisense 15-mers were 100% inhibitory (α - plus β -globin) above 6.25 μM (Figure 5). Note that the sense construct appears to be even more inhibitory than the anti-sense species below 6.25 μM . This observation precludes the existence, under these reaction conditions, of even a narrow concentration range, or "window", of anti-sense specificity in the rabbit reticulocyte system.

The effect of S-oligomer length on inhibition of translation was also evaluated in the reticulocyte lysate system. ODN-2, a random 5-mer, was not inhibitory at any concentration tested (up to 100 μM), while ODN-3, the random 10-mer, was inhibitory (α - plus β -globin) at concentrations above 25 μM . S-dC₂₈ and its phosphodiester congener O-dC₂₈ were tested. They both bind to the reverse transcriptase of HIV-1 (50) but O-dC₂₈ has a lower affinity than S-dC₂₈. In the reticulocyte lysate system, O-dC₂₈ did not inhibit protein translation at 100 μM , while S-dC₂₈ was virtually completely inhibitory at concentrations higher than 3 μM (Figure 5).

Variants of ODN-1, which contains two phosphorothioates only at the 3' and 5' end, were also examined in the reticulocyte lysate system. For the antisense construct (cap-ODN-1), a dose dependent inhibition of protein translation was observed, with 100% inhibition (α - plus β -globin) seen at 100 μM (Figure 5). Control levels of translation were achieved below 25 μM . In experiments with the sense construct (cap-ODN-1[Sen]), no inhibition was seen until a concentration of 100 μM was obtained. Thus, in this system, there appears to be a window of antisense specificity in the 25-100 μM concentration range.

Xenopus oocytes :

Following micro-injection, β -globin mRNA is efficiently translated in *Xenopus* oocytes (the synthesis of the α -polypeptide requires the presence of hemin). Figure 6 displays the results of the gel electrophoresis of oocyte proteins after co-injection of rabbit globin mRNA with two different concentrations of both normal (17PO- β), α (17PO- α) and phosphorothioate (17PS) oligomers. Results similar to those in the wheat germ system were obtained. 17PO- α

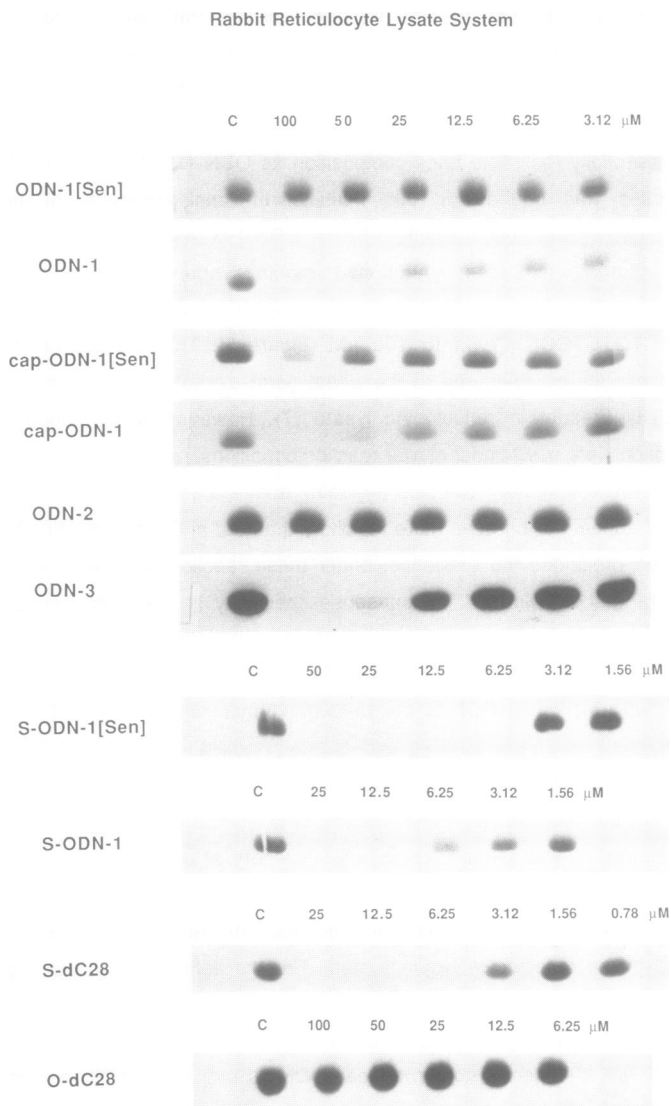


Figure 5 : Translation of rabbit globin mRNA in the reticulocyte lysate system in the presence of various oligomers (see legend of figure 4). C=control (no added oligomer). Numerals above each lane are the concentration of added oligomer (in μM or in nM). Incubation time was 90 min. at 37°C .

did not inhibit β -globin synthesis at either concentration ($3.2 \mu\text{M}$ or $16 \mu\text{M}$, lanes 3 and 6). Both 17PO- β and 17PS were partially inhibitory at low concentration (lanes 7 and 8). At higher concentration ($16 \mu\text{M}$) 17PO- β selectively inhibited the production of β -globin. In

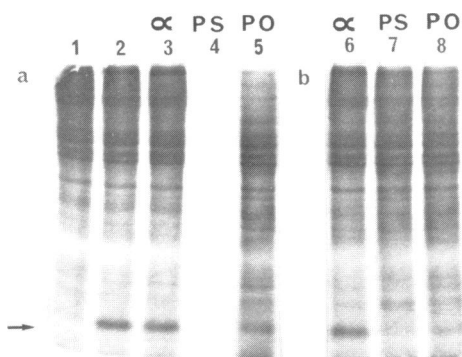


Figure 6 : Effect of various 17-mers on the synthesis of rabbit β -globin in micro-injected *Xenopus* oocytes. Autoradiograph of a 12.5% polyacrylamide-SDS gel of proteins synthesized in oocytes injected with rabbit globin mRNA in the absence (lane 2) or in the presence of 17PO- α (lanes 3 and 6), 17PS (lanes 4 and 7), or 17PO- β (lanes 5 and 8), at a concentration of 16 μ M (panel a lanes 2-5) or 3.2 μ M (panel b, lanes 6-8). Lane 1 corresponds to non injected oocytes. The arrow indicates the position of β -globin.

contrast, global protein synthesis was completely prevented by 17PS at this latter concentration (lane 4). This result was reminiscent to the non-specific effect induced by this oligomer in the wheat germ extract.

When the oligomers were co-injected with the message, the ability of 17PS to specifically inhibit β -globin production was greater than that of its oxygen analogue at similar concentrations (Figure 7 and Table 1). This may be due in part to the decreased sensitivity of this compound to nucleases (12). The effect of nuclease resistance on translation inhibition was better seen when a delay was introduced between injections of the oligomer and of the globin mRNA. Specific inhibition of β -globin synthesis still occurred when 17PS was injected 6 hours prior to the message although it was less efficient than upon co-injection. The concentrations leading to 50% reduction were 30 and 3 nM respectively (Figure 7b and Table 1). In contrast no effect was detected when 17PO- β was injected 6 hours prior to mRNAs even when the oligomer concentration was as high as 3 μ M (Figure 7a).

In order to test a more physiological situation in which the mRNA was already engaged in translation (as is the case for endogenous RNAs from the oocyte), 17-mers were injected 6 hours after globin mRNA. Previous experiments have shown that within 6 hours after microinjection, globin mRNA is recruited into polysomes and efficiently translated. Specific inhibition of β -globin synthesis was observed in the nanomolar range with 17PS whereas 100 fold higher concentrations of 17PO- β were required for half-inhibition (Figure 7 and Table 1).

In fair agreement with what was observed in the wheat germ extract, 11PS was much less inhibitory (3 orders of magnitude) in oocytes compared to 17PS. Concentrations in the μ M

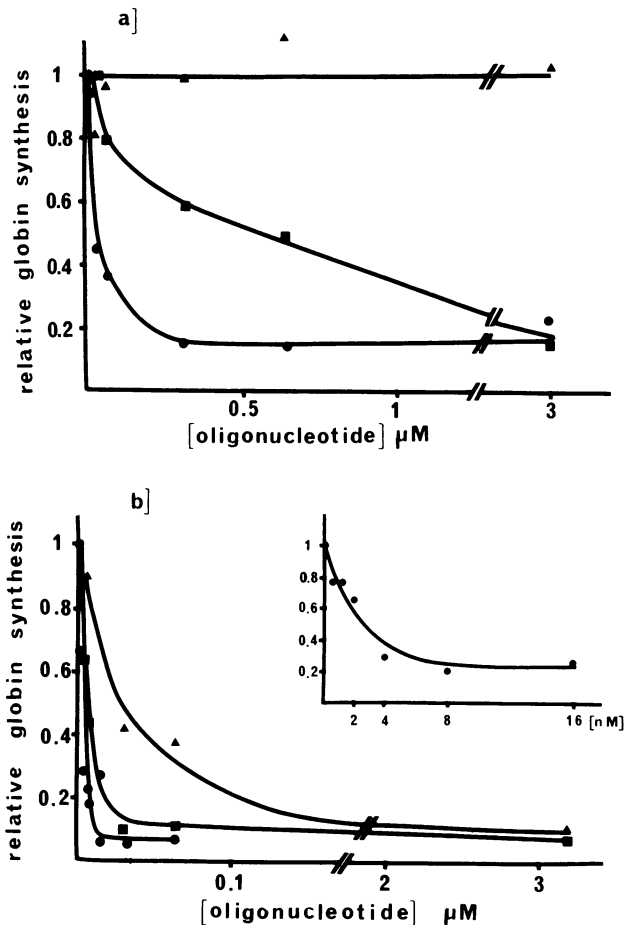


Figure 7 : Effect of 17PO-β (a) and 17PS (b) on the synthesis of rabbit β-globin in micro-injected *Xenopus* oocytes. Globin synthesis was determined from densitometer tracings of autoradiographs, relatively to the synthesis observed in the absence of added oligodeoxynucleotide. Oligonucleotides injected 6 hours prior to (▲), 6 hours after (■) or coinjected with the mRNA (●).

range had to be used to observe 50% inhibition (Figure 3). This is probably due to the weak affinity of this oligomer for its target as indicated by a low value of the melting temperature of the DNA-RNA duplex (Table 1). It is worth mentioning that the unmodified 11PO had no effect at any concentration up to 20 μM (8).

The differences in inhibition efficiencies between the various oligomers could be related to their affinity for their target or to the sensitivity to RNase-H of the hybrid they formed with β-globin mRNA. In particular it was of interest to understand why the α-oligomer had no

effect on translation. To delineate this we first investigated the binding of 17-mers to the mRNA and then the sensitivity of oligonucleotide-mRNA hybrids to RNase-H.

Thermal stability

We compared the affinity of rabbit globin mRNA for various oligonucleotides, namely 17PO- β , 17PS, 17PO- α and 16PO- α using hybridization experiments (see Materials and Methods). The two α -oligomers have the same target but whereas 16PO- α was designed to bind RNA in an antiparallel orientation, 17PO- α was synthesized to bind in a parallel orientation (Figure 1). We did not detect non-specific interactions between oligomers and the filters. All oligonucleotides but one, 16PO- α , gave a signal from thermal elution of filter-bound complexes (data not shown). Binding of 17PO- α to the immobilized mRNA indicated that this α -oligodeoxynucleotide formed a double-stranded structure with RNA in which the two chains run parallel to each other, in good agreement with a previous report (27). Assuming ΔH values are similar for all three oligonucleotides, relative affinities of 17-mer analogues for globin mRNA can be deduced from the relative temperatures T_c of half-dissociation of the complexes, given in Table 1. Even though T_c obtained with 17PO- α was lower than that of its β -homolog it was still higher than that of 17PS. Binding of 17PO- α occurred specifically to its target region of β -globin mRNA as demonstrated by a competition experiment : translation inhibition of β -globin mRNA by 17PO- β was reversed by addition of an excess of 17PO- α both in wheat germ extracts and in *Xenopus* oocytes, indicating that the two oligomers competed for binding to the same RNA sequence (data not shown). Therefore the failure to inhibit rabbit β -globin mRNA translation with the α -derivative cannot be ascribed to a weak stability of the α -oligonucleotide/mRNA hybrids.

RNase-H activity on oligodeoxynucleotide-RNA hybrids

It was shown that RNase-H, which cleaves the RNA part of RNA-DNA hybrids, amplified the antisense effect produced by oligodeoxynucleotides both in wheat germ extracts and in *Xenopus* oocytes (6-9). We therefore investigated the activity of RNase-H on RNA associated with various complementary 17-mers (17PO- β , 17PO- α and 17PS). Rabbit globin mRNA was incubated, in the presence of the oligomers, with *E.coli* RNase-H. Aliquots of the mixtures were withdrawn at various times, spotted onto nylon membranes and probed with ^{32}P end-labelled 17PO- β . As shown on figure 8 no RNase activity was detected during the time course of the experiment in the absence of added oligonucleotide. On the other hand the presence of the various 17-mers did not prevent binding of the probe. Under our experimental conditions about 85% of the β -globin mRNA was cleaved by RNase-H after a 2 h incubation in the presence of 17PO- β . Under the same conditions, 50% mRNA remained intact in the presence of 17PS, whereas no degradation was detected with 17PO- α . Therefore, although the α -oligonucleotide was bound to its target, the hybrid was not recognized as a substrate by *E.coli* RNase-H. In contrast the phosphorothioate analogue was able to induce the cleavage of the complementary RNA as previously observed with homooligomers (12).

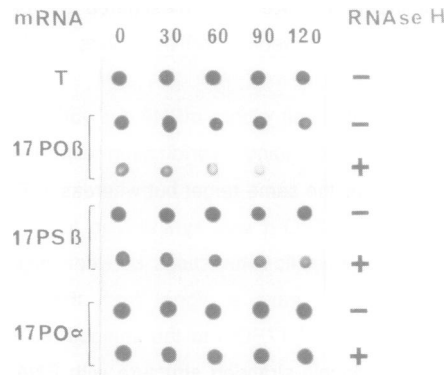


Figure 8 : Oligonucleotide-induced cleavage of rabbit β -globin mRNA by RNase-H. Dot-blot of globin mRNA incubated in the absence (T) or in the presence of 17-mers listed on the left side, in the absence (-) or in the presence of *E.coli* RNase-H (+), during the time indicated (in minutes) at the top of the autoradiograph. The blot was probed with ^{32}P -5'-end-labelled 17PO- β .

DISCUSSION

As part of our studies on the use of oligodeoxynucleotides as specific inhibitors of gene expression, we have chosen to examine modified oligomers with respect to their ability to inhibit protein translation. We have focused on two modifications, phosphorothioate DNA and α -DNA which render oligonucleotides resistant to nucleases, and have compared these with the normal analogues.

The expected inhibition of globin synthesis in the reticulocyte lysate system and in the wheat germ extract was observed with normal antisense oligomers in agreement with previous reports (8, 28, 42, 43). By contrast, the situation with modified oligonucleotides was more complicated. In wheat germ extract no effect on β -globin synthesis was detected in the presence of α -oligomers (either a parallel 17-mer or an antiparallel 16-mer), targeted to the coding region of the β -globin mRNA, even at high concentrations ($>30 \mu\text{M}$). The same results were obtained in *Xenopus* oocytes: none of the two α -oligodeoxynucleotides inhibited translation of microinjected rabbit globin mRNA. This anti- β -globin sequence was synthesized in both orientations because, although it has been known for some time that α -DNA forms parallel-stranded structure with β -DNA (19-23), it was more recently reported that α -dT $_{\beta}$, linked to a phenanthroline-copper complex, binds to polyrA in the antiparallel orientation (25). Our studies with filter-immobilized globin mRNA showed that only the parallel 17PO- α hybridized to rabbit globin mRNA, in fair agreement with a recent work using two other mRNA species (27). Therefore it seems possible that the orientation of the two strands in an α -DNA/ β -RNA hybrid depends on the base sequence of the α -oligodeoxynucleotide or that

α -oligothymidylates represent a unique case where the two strands are antiparallel rather than parallel.

The absence of inhibitory effect of the parallel 17PO- α is clearly due to the lack of activity of RNase-H on the hybrid formed with β -globin mRNA. Preliminary investigations performed with a methylphosphonate derivative of the 17-mer complementary to the region 113-129 of the β -globin mRNA support this conclusion. This phosphonate oligomer (a gift from Dr. Zon) did not prevent β -globin synthesis neither in the wheat germ cell-free system nor in micro-injected *Xenopus* oocytes at any concentration (up to 50 μ M). This methylphosphonate 17-mer failed to induce the degradation of the target mRNA by the *E. coli* RNase-H.

Such an RNase-H activity was previously shown to be present both in wheat germ extracts and in microinjected oocytes (8). Both the normal 17PO and its phosphorothioate analogue 17PS induced the RNaseH activity and were strongly inhibitory in both systems. But hybrids formed by rabbit β -globin with 17PS were not more susceptible to RNase-H than the ones formed with the PO analogue in contrast to what was observed with oligo(dT)'s (12). This could arise either from differences in hybrid structures or from enzyme specificity. Since RNase-H appears to be an important component in translation inhibition by complementary oligodeoxynucleotides it might be of interest to target A-rich sequences by phosphorothioate oligomers in order to take full advantage of mRNA degradation.

The experiments with phosphorothioate oligomers in microinjected *Xenopus* oocytes confirm what was surmised from cell-free experiments. The nuclease resistance properties of the S-oligonucleotides make them very active even if microinjected long before the message (Table 1). As a consequence of both DNase resistance and RNase-H activation, nearly complete inhibition could be achieved at concentrations in the low nanomolar range, i.e. at a stoichiometry of about one oligonucleotide per four mRNA molecules indicating a catalytic effect for this oligonucleotide. Results obtained from competition experiments between 17PO- α on the one hand and 17PO- β or 17PS on the other hand are also relevant : inhibition of β -globin translation by either of the latter two oligomers can be reduced by co-injection of 17PO- α . But the concentration of the 17PO- α competitor required to get a 50% reduction of the inhibitory effect in the presence of 17PO- β was lower than that in the presence of 17PS (data not shown). This could be ascribed to the intracellular degradation of 17PO- β by DNases which results in a decreasing concentration of active antimessenger during the time course of the translation experiment. These conclusions with respect to inhibition of translation are summarized in Table 2.

However there are still unanswered questions. For example 17PS binds more weakly to globin RNA than 17PO (Table 1) and it is less active at inducing cleavage of mRNA by *E. coli* RNase-H (Figure 8). In wheat germ extracts 17PO and 17PS are equally active at inhibiting β -globin synthesis. In microinjected oocytes 17PS is about ten times more active than in the

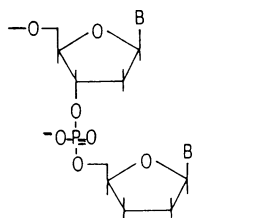
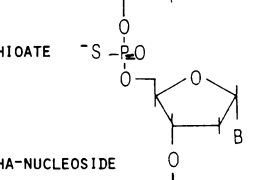

	RESISTANCE TO DNASES	ACTIVATION OF RNASE H	INHIBITION OF TRANSLATION
 <p>PHOSPHODIESTER</p>	NO	YES	YES
 <p>PHOSPHOROTHIOATE</p>	YES	YES	YES
 <p>ALPHA-NUCLEOSIDE</p>	YES	NO	NO

Table 2 : Properties of unmodified, phosphorothioate and alpha-antisense oligodeoxynucleotides.

cell-free system whereas 17PO has quite similar activity. Therefore there might be other factors than nuclease resistance, hybrid stability and RNase-H susceptibility which are playing a role in the efficacy of oligodeoxynucleotides at inhibiting protein synthesis. One such factor could be different compartmentalization of the two oligomers inside oocytes. The kinetics of RNase-H cleavage could also be different in the two systems.

At least at low concentrations ($<1\mu\text{M}$ for 17PS) phosphorothioate analogues fulfill the criteria of anti-sense specificity: 17PS inhibits the synthesis of its target only, i.e., β -globin. But in this paper, we have demonstrated that these derivatives may be non-sequence specific inhibitors of protein synthesis if the concentration is not optimized. We have shown that this effect is highly concentration and length dependent: in wheat germ extract a 5-mer PS was not toxic at $100\ \mu\text{M}$ but a 15-mer containing two blocks of 5 contiguous phosphorothioates behaved like a 10-mer PS. In contrast, in the reticulocyte lysate, a 15-mer phosphodiester capped with phosphorothioate units at both ends exhibited specific behavior close to that of the all-phosphodiester analogue. The results presented in this paper can be discussed with respect to the effects of S-oligomers in other systems. For instance studies on HIV replication and protein expression have revealed two distinct mechanisms of inhibition by phosphorothioate oligodeoxynucleotides: one sequence (antisense) specific, the other non-sequence specific (15, 44, Matsukura et al., unpublished results). Kinetic studies of cellular uptake of fluorescent oligomers have identified an 80kD protein as a possible cell surface receptor for oligonucleotides and related substances (45, 46). In addition, Zhang et al. (unpublished results), have shown that the 80kD protein binds phosphorothioate with greater avidity than normal DNA. A similar observation was noted for HIV reverse transcriptase, which also binds phosphorothioate DNA with higher affinity than its oxygen congener (50). In subsequent

experiments, it has been shown that although both phosphorothioate oligomers and their normal counterparts bind to ribosomes, the former is not displaceable (Stein and Neckers, unpublished results). It is thus of interest to note that when 17PS was injected into *Xenopus* oocytes in high enough concentration (16 μ M), total protein synthesis was abolished and the oocytes exhibited altered pigmentation and then underwent extensive cytolysis. These observations may account for the non-specific cellular cytotoxicity observed when cells are exposed to concentrations of phosphorothioate DNA above 25-50 μ M for extended times. However: it is worth noting that in *Xenopus* oocytes specific translation inhibition by a 17-mer was achieved in the nanomolar range i.e. at concentrations three orders of magnitude lower than that at which toxic effects took place.

From the standpoint of antisense strategy, phosphorothioate DNA appears to be one promising member of the class of modified oligonucleotides. These compounds will very likely be alternative tools to unmodified derivatives in the field of developmental biology and in drug research. These compounds could allow one to specifically ablate the expression of a gene in cells at very low concentrations of antisense molecules, without detrimental effect or interference with the intracellular nucleotide pool, in contrast to recent reports in which unmodified phosphodiester oligonucleotides have been used (47-49).

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Abbreviations:

ODN, oligodeoxynucleotide; PO, phosphodiester oligomer; PS, phosphorothioate oligomer; α , alpha oligomer.

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