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# Inhibition of translation initiation by antisense oligonucleotides via an RNase-H independent mechanism

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## ABSTRACT

**We have used $\alpha$-oligomers as antisense oligonucleotides complementary to three different sequences of the rabbit $\beta$-globin mRNA: a region adjacent to the cap site, a region spanning the AUG initiation codon or a sequence in the coding region. These $\alpha$-oligonucleotides were synthesized either with a free 5′ OH group or linked to an acridine derivative. The effect of these oligonucleotides on mRNA translation was investigated in cell-free extracts and in *Xenopus* oocytes. In rabbit reticulocyte lysate and in wheat germ extracts oligomers targeted to the cap site and the initiation codon reduced $\beta$-globin synthesis in a dose-dependent manner, whereas the target mRNA remained intact. The anti-cap $\alpha$-oligomer was even more efficient that its $\beta$-counterpart in rabbit reticulocyte lysate. In contrast, only the $\alpha$-oligomer, linked to the acridine derivative, complementary to the cap region displayed significant antisense properties in *Xenopus* oocytes. Therefore initiation of translation can be arrested by oligonucleotide/RNA hybrids which are not substrates for RNase-H.**

## INTRODUCTION

Antisense oligonucleotides constitute a way to specifically block the expression of a given gene. They are powerful tools for molecular genetic studies. Inhibition of the development of viruses and parasites is also documented (see (1, 2) for reviews). Despite the numerous examples available of successful inhibition of genes by antisense oligonucleotides, the precise mechanism(s) by which translation of the target mRNA is prevented is not fully understood, neither in cell-free extracts nor in intact cells.

It was initially thought that oligonucleotides could act as road-blocks to ribosomes, the RNA duplex impeding the binding of translation factors or the translocation of the ribosome on the messenger. It was first suggested by Dobberstein and co-workers that the inhibition of translation of chicken lysozyme mRNA by oligodeoxynucleotides, in wheat germ extracts, might involve RNase-H, an RNase which hydrolyses the RNA part of RNA/DNA hybrids (3). It was then reported that oligonucleotides, complementary to a region downstream of the AUG, failed to block translation in rabbit reticulocyte lysate unless the extract was doped with *E. coli* RNase-H (4). Then we demonstrated that unmodified antisense oligomers induced the degradation of the target RNA by endogenous RNase-H, both in wheat germ extracts and in *Xenopus* oocytes (5). This has now been confirmed by several reports (6−8).

The key role played by RNase-H in the inhibition of translation elongation was demonstrated by the use of antisense oligomers which do not mediate cleavage of the mRNA by RNase-H : methyl phosphonate and alpha-oligomers targeted to the coding region of the rabbit $\beta$-globin mRNA did not affect $\beta$-globin synthesis neither in wheat germ extracts nor in *Xenopus* oocytes (9).

Inhibition of the initiation step of translation by antisense oligonucleotides is far less understood. Contradictory results have been reported as regards the most effective site(s) for inhibition and the role played by RNase-H. Lawson *et al.* (10) reported that an antisense effect was observed, in reticulocyte lysate, only if the hybridization site of the oligonucleotide was adjacent to the cap of rabbit globin mRNA. Nevertheless translation inhibition by oligonucleotides complementary to other regions of the 5′ leader, including the initiation AUG codon has been described (11). From competition experiments between oligonucleotide/RNA and polyrA/polydT, Walder and Walder (12) concluded that cleavage of mRNA by RNase-H was the predominant pathway of translation inhibition by antisense oligomers even for those hybridized at the cap site. The situation was still obscured by the fact that reticulocyte lysate was considered to contain amounts of RNase-H varying from low level to no activity (4, 12). Recently, Bertrand *et al.* (13) demonstrated that an $\alpha$-oligomer, complementary to the region adjacent to the cap site, prevented the translation of rabbit $\beta$-globin mRNA. Such analogues, complementary to other RNA sequences, did not elicit RNase-H activity (9, 14).

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In order to shed some light on the mechanism by which antisense oligomers prevent translation we investigated, in a systematic study, the effect of a series of antisense $\alpha$-oligomers on the translation of rabbit $\beta$-globin mRNA. $\alpha$-oligonucleotides were synthesized with either a free 5'-OH group or linked to an acridine derivative in order to increase the affinity of antisense oligomers for their target. Three target sites were selected: a region adjacent to the cap site, a sequence spanning the initiation codon and a site located in the coding region. The effect of these $\alpha$-oligonucleotides and of their unmodified equivalent on the synthesis of $\beta$-globin was investigated in cell-free extracts (wheat germ extract and rabbit reticulocyte lysate) and in microinjected *Xenopus* oocytes.

## MATERIALS AND METHODS

### Oligonucleotides

Unmodified $\beta$- and $\alpha$-oligodeoxynucleotides were synthesized on a Pharmacia automatic synthesizer. According to previous studies $\alpha$-oligomers were designed to bind in a parallel orientation with respect to the RNA strand (9, 15). Oligonucleotides linked at their 5'-end to 2-methoxy, 6-chloro, 9-aminoacridine through a pentamethylene linker were prepared as previously described (16). All oligomers were purified in one step by HPLC on a reverse phase column eluted by an acetonitrile gradient (10–50%) in a 10 mM ammonium acetate (pH 7.2) buffer. The oligomer length purity was evaluated by running [$^{32}$P]-labelled samples on 15% polyacrylamide/6M urea gels. Unmodified and $\alpha$-oligomers with a 5'OH group were 5'-end-labelled with [$^{32}$P]$\gamma$ATP and T4 polynucleotide kinase. Acridine-linked oligomers were 3'-end-labelled with [$^{32}$P]$\alpha$ddATP by nucleotidyl transferase. Although the yield of 3'-end labelling was very low the products could be detected after autoradiography. All preparations contained mainly (more than 95%) a single species.

### *In vitro* protein synthesis

Rabbit globin mRNA was purchased from BRL and used without further purification. Translation was performed either in rabbit reticulocyte lysate or in wheat germ extract according to the supplier's instructions (Promega). 0.05 $\mu$g of rabbit globin mRNA were mixed with the oligonucleotide and added to a final volume of 30 $\mu$l (wheat germ) or 25 $\mu$l (reticulocyte lysate) of the translation mixture; taking into account the presence of both $\alpha$- and $\beta$-globin mRNAs, the concentration of $\beta$-globin mRNA was 3.9 nM and 4.7 nM, respectively. The reaction was run either at 25°C for 1 h (wheat germ) or at 30°C for 45 mn (reticulocyte lysate). Each sample contained 0.925 MBq of [$^{35}$S]-methionine (37TBq/mmole) from Amersham. Reactions were generally carried out without premixing RNA with the oligomer.

[$^{35}$S]-labelled proteins were analysed either by trichloroacetic acid precipitation or by gel electrophoresis. $\alpha$- and $\beta$-globin were separated on 15% polyacrylamide gels containing 8 mM Triton X100, 7 M urea and 5% acetic acid (17). Gels were run at 10V/cm for 15 h. They were fixed in 30% methanol, 5% acetic acid and then impregnated with a fluorophor (Amersham) prior to autoradiography.

The effect of oligonucleotides on $\beta$-globin mRNA translation was determined from densitometer scanning of autoradiographs using the ratio $\beta\alpha_0/\alpha\beta_0$. The amount of $\beta$-globin synthesized in the presence ($\beta$) and in the absence ($\beta_0$) of oligonucleotide was expressed with respect to the corresponding values ($\alpha$ and $\alpha_0$) for the $\alpha$-chain in order to take into account experimental variations.

### Translation in *Xenopus* oocytes

Defolliculated oocytes, prepared by incubation with collagenase of ovaries surgically removed from *Xenopus* females, were obtained from Pr. Ozon (Laboratoire de Physiologie de la Reproduction, Paris VI University). Fully grown stage 6 oocytes were selected via stereotactic microscopy and maintained at 18°C in modified Barth's saline solution (MBS). 4 to 5 hours after injection of 80 nl of a 1/1 (v/v) mixture of globin mRNA (50 $\mu$g/ml) and oligomer, dissolved in sterile distilled water, series of ten oocytes were incubated in the wells of microtitration plates in 100 $\mu$l of MBS containing 2.6 MBq [$^{35}$S]-methionine (37TBq/mmole) for about 15 hours. After incubation the samples were homogenized in 20 $\mu$l (per oocyte) of 20 mM Tris, HCl pH 7.6, 0.1 M NaCl, 1% Triton X100 and 1 mM PMSF (18). Proteins were then analysed by SDS-polyacrylamide gel

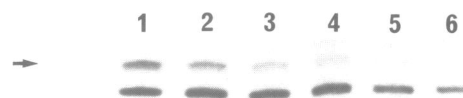


**Figure 1.** Effect of the oligonucleotide $\alpha$-Acr(aug) on the translation of rabbit globin mRNA in wheat germ extract. mRNA was incubated in the absence (lane 1) or in the presence of the oligomer at 1 $\mu$M (lane 2), 5 $\mu$M (lane 3), 10 $\mu$M (lane 4), 15 $\mu$M (lane 5) or 20 $\mu$M (lane 6). Labelled proteins were analysed by electrophoresis as indicated in Materials and Methods and the gel was autoradiographed. The arowed band corresponds to $\beta$-globin.

| Abb | Sequence | Target | $T_c$ | WGE | RRL | XOO |
|---|---|---|---|---|---|---|
| β(cap) | TTGTGTCAAAAGCAAGT | 3-19 | 47 | 0.2$^a$ | (30 %) | 0.8$^a$ |
| α(cap) | TGAACGAAAACTGTGTT | " | 47 | 1.7 | 3 | |
| α-Acr(cap) | " | " | | 0.8 | 1 | 1 |
| β(aug) | ACAGATGCACCATTCTG | 51-67 | 59 | 0.03$^a$ | (35 %) | 1.2$^a$ |
| α(aug) | TCTTACCACGTAGACA | 51-66 | | 10 | (30 %) | |
| α-Acr(aug) | " | " | | 5 | (35 %) | |
| β(sc) | CACCAACTTCTTCCACA | 113-129 | 61 | 0.02$^a$ | 2 | <0.2$^a$ |
| α(sc) | ACACCTTCTTCAACCAC | " | 49 | * | (10 %) | |
| α-Acr(sc) | " | " | | * | (10 %) | |

**Table 1.** Characteristics of antisense oligodeoxynucleotides. The abbreviations used throughout the manuscript are given in the first column. Sequences are written in the 5' to 3' direction (from the left to the right) for both $\beta$ and $\alpha$-oligonucleotides. The position of the target on the rabbit $\beta$-globin mRNA is indicated (+1 is the 1st nucleotide after the 7-met G residue). The $T_c$ value was determined as indicated in 'Materials and Methods'. The effect of antisense oligonucleotides on $\beta$-globin synthesis in wheat germ extract (WGE) rabbit reticulocyte lysate (RRL) or *Xenopus* oocytes (XOO) are indicated by the concentration ($\mu$M) at which the ratio $\beta\alpha_0/\alpha\beta_0$ was decreased by 50% (see text). When 50% reduction was not reached at the highest concentration tested, the percent of inhibition induced by 10 $\mu$M oligonucleotide is given in brackets. (*) indicates that we observed a stimulation of $\beta$-globin synthesis. a) data taken from reference (5).

electrophoresis on a 13.5% acrylamide gel. Assuming a free diffusion compartment of 0.5 μl inside the oocyte, the final intracellular concentration of β-globin mRNA was about 16 nM.

### RNA analysis

Following incubation in cell-free extracts, globin mRNA was phenol extracted and ethanol precipitated according to standard procedures (19). After dissolution in sterile water, RNA was run on a 8% polyacrylamide gel containing 7 M urea. RNA was electroblotted onto a nylon membrane and probed with [$^{32}$P] 5'-end-labelled 17-mers. Hybridization was performed at room temperature in 8×SSC (1×SSC = 0.15 M NaCl, 0.015 M sodium citrate) containing 50 mM Tris,HCl, pH 7.2, 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinyl-pyrolidone, 0.1% Na pyrophosphate, 1% SDS. Blots were washed twice for 5 mn at room temperature in 0.2×SSC.

### RNase-H assay

Assays were carried out according to the method described by Büsen and Hausen (20). About 100 pmol of [$^{3}$H]-labelled RNA/DNA hybrids (poly(rA).poly(dT) or the transcription product of nicked calf-thymus DNA by *E. coli* RNA polymerase) were incubated for 10 mn at 37°C, with 1 μl of cell-free extracts, in 500 μl of RH buffer containing 30 mM Tris, HCl pH 7.8, 30 mM ammonium sulfate, 0.01% β-mercaptoethanol and 10 mM $MgCl_2$. Then, 500 μl of 8% trichloroacetic acid and 0.6 mg yeast tRNA were added to each sample to precipitate acid-insoluble material. After centrifugation the supernatant was removed and counted. One RNase-H unit is the amount of enzyme which renders 100 pmol of RNA nucleotide acid-soluble under optimal conditions in 10 minutes at 37°C. RNase-H activity was also measured in RH buffer at 30 and 25°C on the one hand and under the conditions used for in vitro translation on the other hand ; in this latter case, 100 pmoles of hybrid were added to 30 μl of cell free extracts and incubated for 10 mn at 25 or 30°C for wheat germ and reticulocyte extracts, respectively.

### Hybridization studies

1 μg of rabbit globin mRNA was bound to a nitrocellulose filter by heating at 80°C for 2 hours. The filter was incubated in 2 ml of 8×SSC/10×Denhardt's solution (10×Denhardt's is 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinyl-pyrolidone) containing about $10^7$ cpm of [$^{32}$P]-labelled oligomer. The filters were then placed in a thermostated holder, and were eluted with 8×SSC as the temperature was increased at a rate of 1.2°C/min. Thermal elution profiles were constructed, and the Tc determined to be that temperature at which 50% of the total counts had been eluted (21, 22).

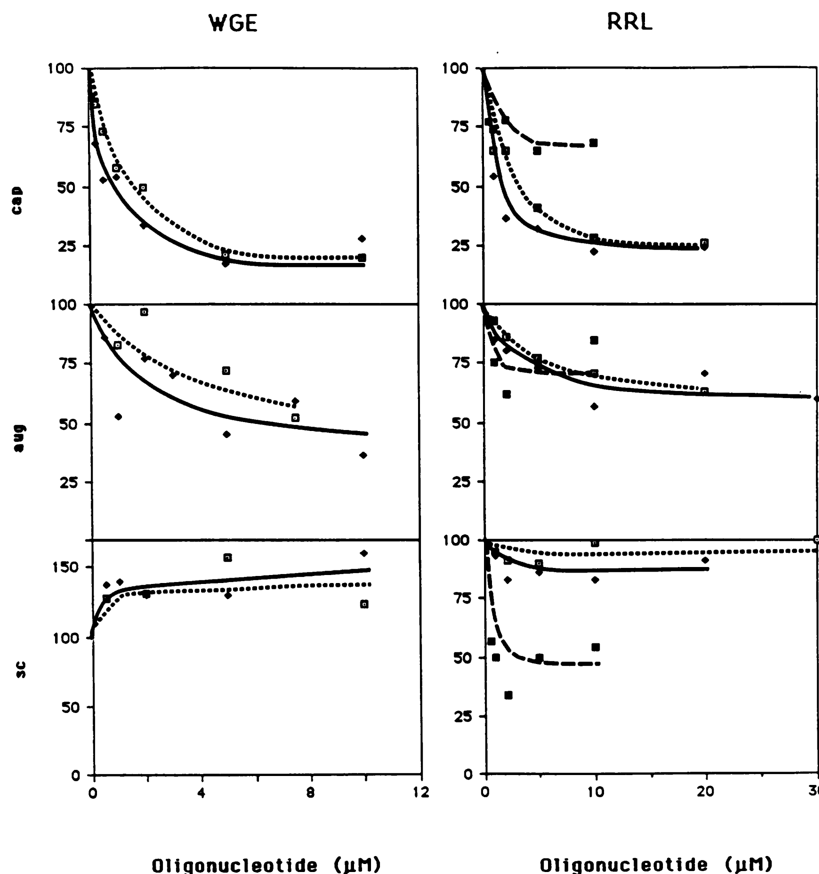


**Figure 2.** Inhibition of rabbit β-globin synthesis in wheat germ extract (left) or rabbit reticulocyte lysate (right). Unmodified oligomers (n), or α-oligonucleotides without (r) or with an acridine derivative at their 5'-end (◆), targeted to the cap site (top), the initiation region (middle) or the coding sequence (bottom) were added to cell-free extracts as indicated in Material and Methods. [$^{35}$S]-labelled proteins were analysed by electrophoresis and the gel was autoradiographed. The percentage of inhibition was calculated as indicated in the text.

## RESULTS

### Alpha-oligomers specifically inhibit rabbit β-globin synthesis in cell-free extracts

Addition of rabbit globin mRNA to cell-free extracts (rabbit reticulocyte lysate and wheat germ extract) leads to the production of two major polypeptides: the α- and β-chains of hemoglobin (figure 1). We investigated the effect of a series of antisense oligonucleotides, complementary to the β-globin message, on the synthesis of the encoded protein. Three different regions of the mRNA were targeted: the cap site (cap), the region of the AUG initiation codon (aug) and the coding sequence (sc). For each site both unmodified β- and nuclease-resistant α-oligomers were synthesized, the latter ones being prepared either with a free 5'-hydroxyl group or with an acridine (Acr) derivative covalently linked at their 5'-end (see Table 1 for the location of the target, the sequence and the abbreviation of the oligonucleotides).

As shown on figure 1, addition of the oligonucleotide α-Acr (aug) to wheat germ extract resulted in the specific inhibition of β-globin synthesis at concentrations below 10 μM. At higher concentration a slight decrease of α-globin was observed as well which might be due to partial homology between the initiation regions of α- and β-globin mRNA. Translation inhibition was induced by α- and α-Acr oligomers, complementary to the cap or to the AUG regions, in both cell-free extracts (figure 2). Oligomers complementary to the cap site were better inhibitors than those complementary to the AUG region. In the wheat germ system 50% inhibition was reached at 0.8 μM α-Acr(cap) and 5 μM α-Acr(aug). In the rabbit reticulocyte lysate half-reduction was observed in the presence of 1 μM α-Acr(cap) whereas α-Acr(aug) induced only a 35% decrease at 10 μM. Oligomers linked to the acridine derivative were slightly more efficient than the parent compounds (Figure 2 and Table 1). This should be ascribed to the additional energy of interaction brought by the intercalation of the dye into the oligo/mRNA duplex (5, 21, 23). In contrast, neither the oligonucleotide α-(sc) nor its acridine-linked analogue α-Acr(sc) produced any decrease of β-globin mRNA translation at concentrations up to 30 μM. In wheat germ extracts, these oligomers rather stimulated the protein synthesis.

### Inhibition of translation by α-oligomers is not due to RNA cleavage by RNase-H

It was previously reported that α-oligomers, complementary either to the AUG region or to the coding sequence did not prevent translation (9, 13, 14). This was ascribed to the failure of these α-oligomer/RNA hybrids to be recognized as substrates by RNases-H. With the above results in mind it was of interest to determine whether the inhibition of translation induced by α(aug) oligomers resulted from the cleavage of the complementary region of the rabbit β-globin mRNA. Northern blots of globin RNA, incubated in wheat germ extracts in the presence of α-Acr(aug), did not show any RNA cleavage product even at 10 μM oligomer, *i.e.* at a concentration which reduced β-globin synthesis by about 75% (figure 3). Similar results were obtained with α-Acr(aug) in reticulocyte lysate (not shown). Therefore RNase-H is not involved in the translation inhibition of β-globin mRNA in cell-free extracts by these modified oligonucleotides.

### Only the acridine-linked α-oligomer targeted to the cap site prevents translation in frog oocytes

Micro-injection of rabbit globin mRNA in *Xenopus* oocytes allows the synthesis of β-globin which can be detected after gel electrophoresis in a low background of endogenous proteins (figure 4). The six α-oligomers used for *in vitro* studies were co-injected with globin mRNAs at a concentration of 7 μM. As in cell-free extracts, no effect was seen in the presence of either α(sc) or of α-Acr(sc). But, in contrast to the *in vitro* situation, the α-oligonucleotides targeted to the cap and to the AUG regions were not efficient either. At this concentration, only α-Acr(cap) reduced significantly the β-globin synthesis whereas a slight effect was observed in the presence of α-Acr(aug) (figure 4). Fifty per cent reduction was achieved at about 1 μM α-Acr(cap) (Table 1).

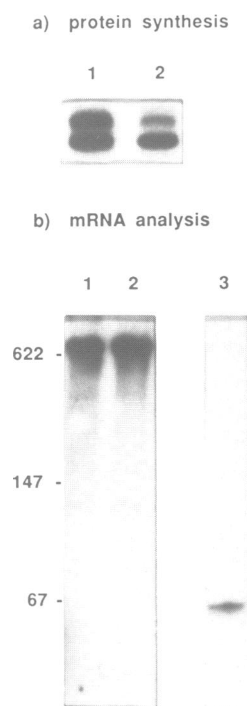


**Figure 3.** Effect of α-oligonucleotide on *in vitro* protein synthesis and mRNA integrity. Protein (a) and RNA (b) were analysed after in vitro translation of 150 ng of rabbit globin mRNA in the absence (lanes 1) or in the presence (lanes 2) of 10 μM of α-Acr(aug). In lane 3 of panel b) globin mRNA was incubated with *E. coli* RNase-H in the presence of 1 μM β-aug. The Northern blot (b) was probed with 5' end-labelled β-cap. Positions of size markers are indicated on the left of the figure.

### A methylphosphonate oligomer targeted to the cap does not block translation

Like α-oligomers, methylphosphonate derivatives do not elicit RNase-H activity. Consequently, a 17-mer targeted to the coding region of rabbit globin mRNA did not prevent translation (9). We investigated the effect of the methylphosphonate 17-mer complementary to the region 3–19 of the rabbit β-globin mRNA. To our surprise this oligonucleotide did not reduce β-globin synthesis either in cell-free extracts or in micro-injected *Xenopus* oocytes at concentrations up to 50 μM (not shown).

### RNase-H activity in rabbit reticulocyte lysate

The effects of unmodified oligonucleotides, β(cap), β(aug) and β(sc) on the synthesis of β-globin in wheat germ extract have been reported previously (5). In this cell-free translation system the antisense properties of these oligomers are mainly driven by the RNase-H sensitivity of the hybrids they form with the target RNA. In rabbit reticulocyte lysate the contribution of RNase-H

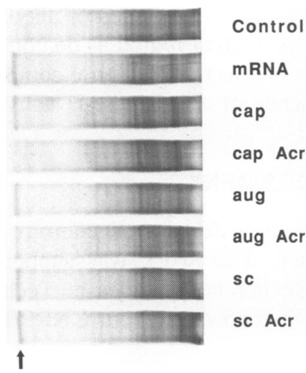


**Figure 4.** Effect of α-oligonucleotides on translation of rabbit β-globin mRNA in *Xenopus* oocytes. [$^{35}$S]-labelled proteins from oocytes not injected (control) or injected with the mRNA in the absence (mRNA) or in the presence of 7 μM of α(cap), α-Acr(cap), α(aug), α-Acr(aug), α(sc) or α-Acr(sc) were analysed by electrophoresis on a SDS/polyacrylamide gel. The β-globin band is indicated by an arrow.

to antisense effect is still a matter of debate (4, 12). We therefore compared the effects of α- and β-oligomers in this cell free system. All three unmodified oligonucleotides decreased the synthesis of β-globin; the oligomer β(sc) was more efficient than the two other ones (figure 2). Northern blotting analysis of the β-globin mRNA after translation in reticulocyte lysate indicated a partial cleavage at the expected sites, indicating the presence of RNase-H activity in the lysate (not shown). Indeed, the standard assay described in Material and Methods, revealed that RNase-H amounted to 5.8 units/mg of proteins in our batch of lysate (under the conditions used for *in vitro* translation experiments). The corresponding value for wheat germ extracts was 15.3 units/mg. It is worth mentionning that, in both cell-free systems, the RNase-H activity measured under the conditions used for in vitro translation experiments was reduced to a few percent of that determined in RH buffer at the same temperature (25 and 30°C for the wheat germ and the reticulocyte lysate, respectively).

## DISCUSSION

mRNA translation can be inhibited by antisense oligonucleotides via two different ways: induced cleavage of the RNA by RNase-H and hybrid-arrested binding (or scanning) of translation complexes. The relative contribution of these two processes to protein synthesis inhibition is different for the initiation and the elongation steps of translation and will likely vary from one biological system to the other. It is now well established that in wheat germ extract and in *Xenopus* oocytes the complex RNA/oligonucleotide is attacked by RNase-H (5−7). Indeed, the truncated mRNA can no longer support translation. Moreover, the cleavage product is further processed by other RNases. This is the only way by which antisense oligonucleotides that do not modify the mRNA can block polypeptide chain elongation. Consequently, modified oligonucleotides that do not mediate RNase-H activity do not inhibit protein synthesis when targeted to the coding region. Methylphosphonate and α-oligomers fall into this category (9).

As demonstrated in this paper, antisense oligonucleotides that bind to the 5′-leader region can prevent translation via a different mechanism. We observed a selective inhibition of β-globin synthesis, both in wheat germ extract and in rabbit reticulocyte lysate by oligonucleotides α-cap and α-aug. Northern blot analysis clearly demonstrated that the target mRNA remained intact after *in vitro* translation. Therefore, α-oligomers targeted to the 5′ leader region prevent translation through competition with initiation complexes. The result that we obtained with α-cap agrees quite well with a previous report by Bertrand *et al.* (13). However, they (and others (14)) failed to inhibit protein synthesis using an α-oligomer spanning the AUG initiation codon of the rabbit β-globin or of the p26 mRNAs. We are presently unable to explain this discrepancy between the results obtained with different mRNA and oligonucleotide sequences.

α-oligonucleotides are much more resistant to nucleases than unmodified β-oligomers (24, 25). Indeed no degradation of α(cap), α(aug) or α(sc) was detected following incubation in cell-free extracts during the time course of in vitro translation experiments (Boiziau, unpublished results). It was previously reported than the half life of an α-16mer was longer than 8 hours in frog oocytes, compared to 10 minutes for the unmodified homologue (26). Therefore the differences observed in antisense efficiency cannot be ascribed to the degradation of α-oligonucleotides.

It was previously reported that oligomethylphosphonate, 9 to 12 nucleotides long, complementary to the cap or to the AUG region induced a decrease of rabbit β globin synthesis *in vitro* (27). This inhibition, observed at high oligonucleotide concentration (25−100 μM), was essentially non-specific. Since that report various effects on translation have been described with these analogues (28−31). For instance, a series of methyl phosphonate oligomers, targeted to the 5′ leader region of the mRNA did not reduce CAT gene expression even at 400 μM (32). We confirm here that such derivatives targeted to the rabbit β-globin mRNA are very poor translation inhibitors in cell-free extracts and in *Xenopus* oocytes. In contrast to α-oligonucleotides α-cap and α-(Acr)cap, the methylphosphonate 17-mer was not able to compete with the formation of the initiation complex. But, recently the expression of the MDR gene was turned off in human erythroleukemia cells using a 15-mer methylphosphonate complementary to the 5′-leader of the mRNA (29). However, in this case the antisense oligonucleotide might play a role outside of translation. Indeed, such oligomers can block maturation of RNA (33).

An acridine residue was linked at the 5′-end of oligomers in order to increase the affinity of the antisense molecule for its target. It has been demonstrated that intercalation of the dye into the RNA/oligomer duplex provided the oligonucleotide with a higher binding constant compared to homologous unmodified oligos (21, 34). This subsequently led to an increased antisense efficiency both in prokaryotic and eukaryotic *in vitro* expression systems (5, 21, 35). Physico-chemical studies have also demonstrated that acridine-linked α-oligonucleotides exhibited a higher affinity for a DNA target than regular α-oligomers (25). From the *in vitro* studies described here it can be seen that acridine oligomers α-(Acr)cap and α-(Acr)aug are slightly more efficient at inhibiting translation than the parent oligonucleotides α-cap and α-aug, in cell-free extracts. This is likely due to an increased stability of the duplexes, although we were not able to measure the stability of α-Acr/RNA duplexes using thermal elution of filter-bound complexes (the acridine derivative linked at the 5′-end of the oligonucleotides, prevented 5′-end labelling and 3′-end labelling had a very low yield). In *Xenopus* oocytes the role of acridine was crucial: α-(Acr)cap was much more efficient

than $\alpha$-cap. From preliminary experiments it seems that this might result from a kinetic effect. No inhibition was observed in the presence of $\alpha$-cap when protein labelling was performed for 15 hours following addition of [$^{35}$S]-methionine. However, at shorter incubation times (less than 7 h) a significant inhibition of $\beta$-globin synthesis was observed (C. Cazenave, unpublished result). The different behaviour exhibited by $\alpha$-(Acr)cap and $\alpha$-cap might reflect the longer lifetime of $\alpha$-(Acr)cap/RNA complexes compared to $\alpha$-cap/RNA ones. This suggested that the oligonucleotide/RNA hybrid can no longer form in the oocyte once dissociated. It should be recalled that an 11-mer complementary to the AUG region was inefficient in oocytes unless it was linked to an acridine residue (5).

Defining the most effective target site for antisense oligonucleotides is complicated by the role that RNase-H plays in the inhibition process. In the case of reticulocyte lysate the situation is far from clear due to contradictory results. In this system specific inhibition has been achieved by oligomers complementary to the cap region, and to a lesser extent, to the region of the initiation codon (11). In several studies cDNA or oligonucleotides that did not cover part of the 5′-leader region were ineffective antisense molecules (10, 36). In contrast, in wheat germ extracts, inhibition has been observed whatever the target site on the mRNA as far as the hybrids elicit RNase-H activity (5). The difference between the two systems has been ascribed to their RNase-H content : high in wheat germ, low (or reduced to zero) in reticulocytes. Our study confirms the presence of RNase-H activity in commercial lysates as previously pointed out (12). In our hands, the 17-mer complementary to the coding region of the rabbit globin mRNA was the most effective antisense of the three unmodified oligonucleotides tested both in cell-free extracts and in micro-injected oocytes. Whether this was due to this particular sequence remains to be determined. Contradictory results obtained by different laboratories might be due to variation of RNase-H from batch to batch (although we found the same level in two different batches from Promega). In all studies but one, RNase-H activity was not quantitated which precludes any conclusion. We would strongly recommend checking for its presence when antisense experiments are carried out. For oligonucleotides that do not mediate RNase-H activity (and probably in media which do not contain RNase-H) the cap site is the most effective target.

At least one more question remains to be answered: why is $\alpha$(cap) more effective than $\beta$(cap)? The unmodified oligonucleotide can block translation by two different mechanisms (induced cleavage and physical block) whereas the $\alpha$-oligonucleotide can only act as a stopper. The Tc values which characterize the stability of the oligonucleotide/RNA duplex are identical (47°C) for the two oligomers (Table 1). Making the reasonable assumption that $\Delta H$ is the same for $\alpha$- and $\beta$-17-mers, these molecules should have the same binding constant under translation conditions. Moreover, no significant degradation of $\beta$-cap was detected during the time course of *in vitro* translation experiments. Therefore, besides RNase-H another component should be involved which would favour $\alpha$-oligonucleotides. Lawson *et al.* (10) have demonstrated the existence of a melting activity associated with initiation factors. We might speculate that this activity is less efficient on $\alpha$- than on $\beta$-oligonucleotides.

In this study we have shown that $\alpha$-oligonucleotides can inhibit translation when targeted to the 5′ leader region. An $\alpha$-oligomer complementary to the cap site was more efficient than the $\beta$-homologous sequence. The long lifetime of these compounds due to their nuclease resistance could make them very interesting compounds with intact cells. Moreover, linking these oligomers to reactive groups such as psoralen or alkylating reagents could allow targeting the coding region as demonstrated recently (37, 38). This results in effective antisense compounds that could act even in the absence of any RNase-H activity.

## ACKNOWLEDGEMENTS

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