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COMPARATIVE ACTIVITY OF α- AND ß-ANOMERIC OLIGONUCLEOTIDES ON RABBIT ß GLOBIN SYNTHESIS : INHIBITORY EFFECT OF CAP TARGETED α-OLIGONUCLEOTIDES[1]

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α-anomeric oligonucleotides are resistant to nucleases and display parallel annealing to RNA complementary sequences. We compared the effect of α- and ß-oligonucleotides targeted against various mRNA regions on the rabbit ß globin *in vitro* synthesis. In order to determine the role of RNase H, experiments were performed in both rabbit reticulocyte lysate and wheat germ extract. As expected ß-oligonucleotides were found more efficient in wheat germ extract which is rich in RNase H activity and α-oligonucleotide targeted against the initiation codon or downstream had no effect because they do not induce mRNA cleavage by RNase H. However, we report, for the first time, a specific translation inhibition by α-oligonucleotides. This occurs provided they are targeted against the cap region in 5' of the mRNA.

In recent years a great interest has been devoted to oligonucleotides (oligos) as specific inhibitors of translation (1,2). Our research was focused on α-anomeric oligos which display some interesting properties: they can strongly hybridize to complementary RNA (3) with parallel annealing (4), they are not degraded by purified nucleases (5,6), they are more resistant to hydrolysis in cellular extracts than ß-oligos (7). However various attempts to find inhibition

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Abbreviations
Oligo: oligonucleotide, WGE:wheat germ extract, RRL: rabbit reticulocyte lysate, eIF:eukaryotic initiation factors, HPLC: high perfomance liquid chromatography.

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of translation by α-oligos were insuccessful (3,8). This is likely to be caused by inability of RNase H to cleave mRNA in a mRNA:α-oligo duplex (3,8). This enzyme plays indeed a key role in inhibition of translation as shown by Minshull and Hunt (9). It binds on RNA:DNA duplexes and cleaves RNA. When oligos are hybridized with either the initiation codon area or the coding region, one easily understand that mRNA can no longer give way to a fonctionnal protein. An other mechanism of translation inhibition by antimessager oligos, where the role of RNase H is likely to be less important, was described by Lawson and al (10). Oligos targeted against the 5' part of mRNA, at the cap level, interfere with the binding of translation initiation factors and therefore with ribosomal binding. Consequently translation is inhibited.

In this paper , the action of α- and ß-oligos on the synthesis of rabbit ß globin is investigated. Their effect on different targets, cap and no cap sequences was quantified. In order to assess the role of RNase H in observed inhibitions we used *in vitro* translation systems with either a high RNase H activity (wheat germ extract) or with a low RNase H activity (rabbit reticulocyte lysate). We confirm the inhibitory activity of ß-oligos targeted against the cap region even with low RNase H activity in the medium. We show for the first time that α-oligos, provided a cap targeting, are also able to inhibit translation.

Materials and Methods

- Oligonucleotides:
α- and ß-oligos, whose sequences and targets are displayed in fig. 1, were synthesized in solid phase with an automatized system (Applied Biosystems). Detritilated oligos were purified by HPLC (Waters assoc.) on Aquapore C_{18} (Applied Biosystem). Elution was performed with a 5-50% linear gradient of acetonitril in 0.1 M triethylamine acetate pH 7. Concentrations of oligos are expressed as strand concentration.

- *In vitro* translation assays:
a) Rabbit reticulocyte lysate (RRL):
0.2 µg rabbit α and ß globin mRNAs (BRL, Bethesda) were prehybridized with α- or ß-oligos at given concentrations (see legends of figures). Final volume was 5.75 µl containing 0.25 µl of 1 M CH_3COOMg and 1 µl of 32.5 M CH_3COOK. Reaction mixture temperature was brought up to 70 °C for 5 min then was allowed to cool slowly in order to get a correct hybridization. 1 µl of [^{35}S]methionine (3000 Ci/mmole, Amersham), 2.75 µl of translation cocktail (NEN), and 5 µl of lysate (NEN) were then added. Reaction mixture were then left for 1 h at 25 °C.

b) Wheat germ extract (WGE):
0.2 µg rabbit α and ß globin mRNAs were prehybridized as above. Final volume was 5.4 µl containing 1.5 µl of 1 M CH_3COOK. Reaction mixture temperature was brought up to 70 °C for 5 min and then was allowed to cool slowly. 1 µl of [^{35}S]methionine (3000 Ci/mmole, Amersham), 1 µl of amino acids solution (Amersham) and 7.5 µl of wheat germ extract (Amersham) were then added. Reaction mixture was left for 1 h at 25 °C.

- Quantification of synthesized proteins:
Proteins were denatured by addition of an equal volume of the following solution: 1.5 mM CH_3COOH, 7 M urea, 1.2 mM 2-mercaptoethanol, 0.8 mM

pyronin Y, and dropped on a 12% polyacrylamide acid-urea-triton gel (10). Electrophoresis, fluorography and autoradiography were then performed. Synthetised protein bands in the gel were localized by autoradiography and isolated. The rate of protein synthesis was determined by scintillation counting with the use of a Beckman LS 1800 apparatus.

Results

I) Action of α- and ß-anomeric oligos targeted against various regions of mRNA.

Lysates were programmed with mRNA coding for rabbit α and ß globin. α- and ß-anomeric oligos were targeted against regions indicated in Fig 1.

a) In the RRL translation system (Fig 2A), with a low RNase H activity, no inhibition is observed when the target of α- and ß-oligos is at the level of the initiation codon (oligo 1) or downstream (oligos 2 and 30). However α- and ß-oligos targeted against the cap structure of ß globin mRNA display a specific inhibition of ß globin synthesis. The α-oligo is more efficient than the ß-oligo. α globin synthesis (which is used as a control) shows an unexpected stimulation by the various oligos. This effect appears to be linearly dependant on the length of oligos for a given strand concentration of 10 μM (Fig 3).

b) In the WGE translation system (Fig 2B), with a RNase H activity, ß-oligos targeted against the AUG codon (oligo 1) and downstream (oligos 2 and 30) inhibit ß globin synthesis. α-oligos display no activity under the same experimental conditions. ß-Oligo 1 is the only one which does not inhibit, even partially, α globin synthesis. As in Fig 2A, α- and ß-oligos targeted against the

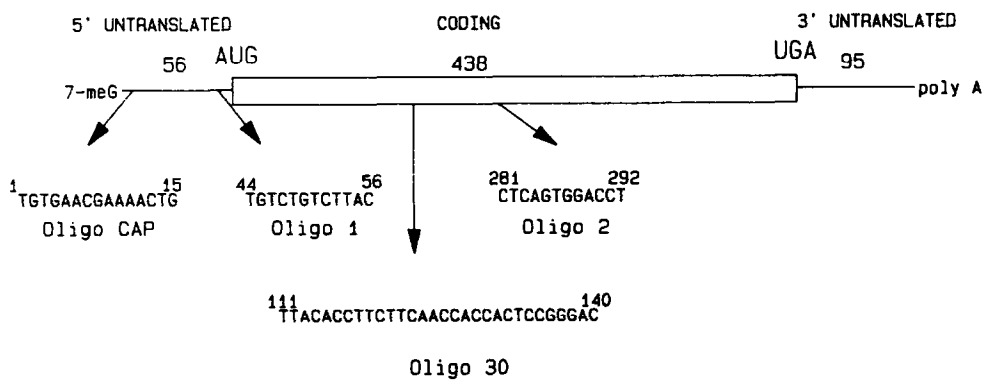

Figure 1
Positions and sequences of α- and ß-oligos complementary to rabbit ß globin mRNA.
In all cases α-oligos are in parallel orientation and ß-oligos are in antiparallel one.

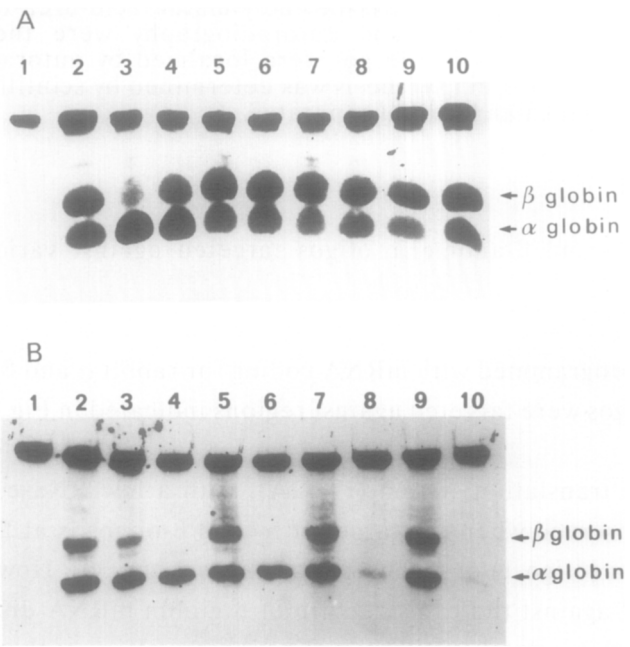

Figure 2
Translation arrest assay mediated by α- or ß-oligos.
Panel A: Rabbit reticulocite lysate programed with rabbit α and ß globin mRNAs.
Panel B: Wheat germ extract programed with rabbit α and ß globin mRNAs.
Samples were analysed on Acid-urea-triton polyacrilamide gel electrophoresis and autoradiographied as indicated in materials and methods. Prehybridation step is omited.
No mRNA (lane 1): no oligo (lane 2); oligos added: 10 μM α-oligo cap (lane 3): 10 μM ß-oligo cap (lane 4): 10 μM α-oligo 1 (lane 5): 10 μM ß-oligo 1 (lane 6): 10 μM α-oligo 2 (lane 7): 10 μM ß-oligo 2 (lane 8): 10 μM α-oligo 30 (lane 9): 10 μM ß-oligo 30 (lane 10).

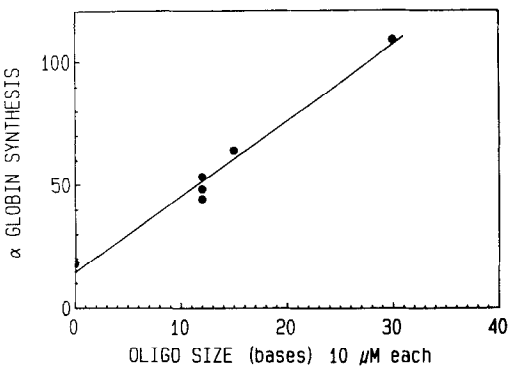

Figure 3
Stimulation of α globin mRNA translation by α-oligos in RRL.
RRL were programed with α and ß globin mRNA in presence of 10 μM α-oligos (12 to 30 mers). Samples were analysed on acid-urea-triton gel as indicated in materials and methods. α globin synthesis was calculated by scanning the autoradiography.

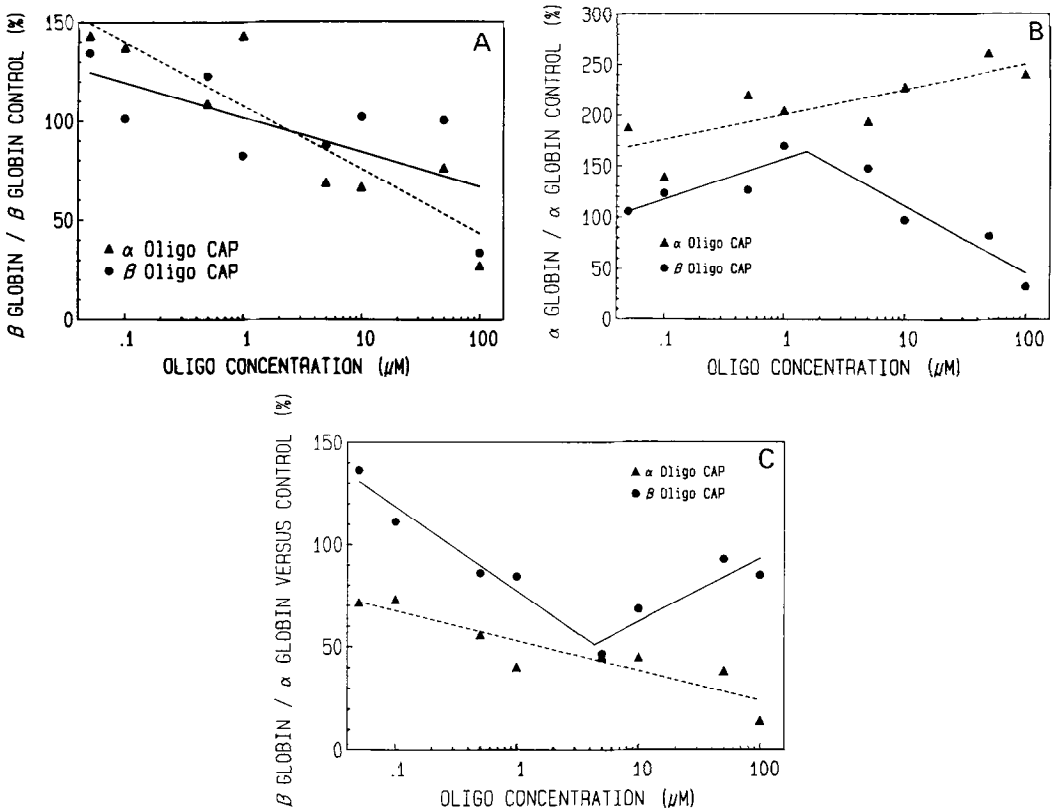

Figure 4
Translation inhibition of Rabbit globin mRNAs by α- or ß- cap targeted oligos in RRL.
RRL were programed with α and ß globin mRNAs with increasing amount of α- or ß-oligos cap. Samples were analysed on acid-urea-triton gel. Bands were excised from the gel, and radioactivity was measured to determine the level of synthesis of each subunit.
Panel A: Linear regression of ß globin synthesis versus control in presence of α- or ß-oligos cap.
Panel B: Linear regression of α globin synthesis versus control in presence of α- or ß-oligo cap.
Panel C: Linear regression of ß globin on α globin ratio versus control in presence of α- or ß-oligos cap. 50% inhibition is 2 μM for the α-oligo and 4 μM for the ß.

cap structure inhibit specificaly ß globin synthesis. However the ß-anomer is more efficient than the α-anomer in the WGE assay.

II) Inhibition of ß globin translation by cap targeted oligos as a function of oligonucleotide concentration.

The oligo amount which specifically inhibits by 50% the translation of ß globin was determined. In RRL, α- and ß-anomeric oligos stimulate in an unspecific way α and ß globin synthesis (Fig 3). It seems that this stimulation is identical for α and ß globin.

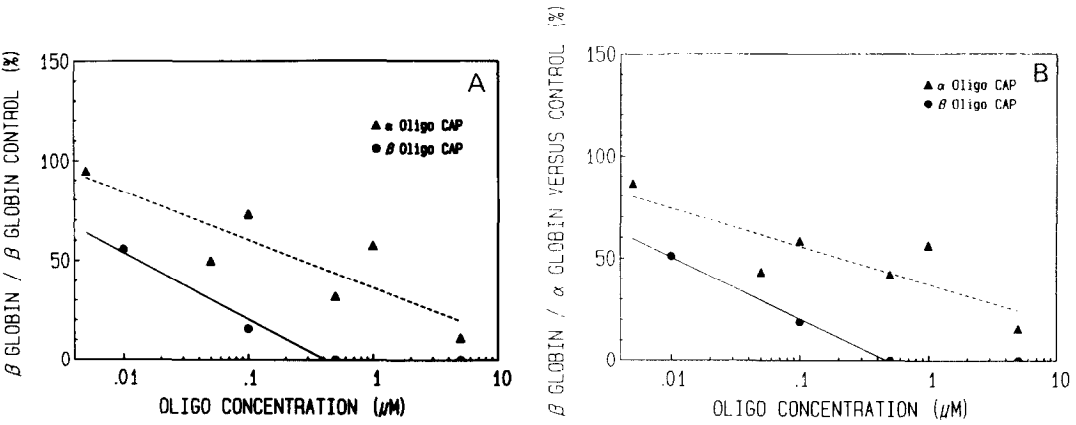

Figure 5
Translation inhibition of rabbit globin mRNAs by α- or ß-oligo cap in WGE. WGE were programed with α and globin mRNAs with increasing amount of α- or ß-oligo cap. Samples were analysed on acid-urea-triton gel electrophoresis. Bands were excised from the gel, and radioactivity was mesured to determine the level of synthesis of each subunit.
Panel A: Linear regression of ß globin synthesis versus control in presence of α- or ß-oligo cap.
Panel B: Linear regression of ß globin on α globin ratio versus control in presence of α- or ß-oligo cap. 50% inhibition is 0.01 μM for the ß-oligo and 0.2 μM for the α.

On Fig 4A and 4B, ß and α globins are quantified taking into account the controls without oligos. The slope of the regression line in Fig 4A, indicates dose dependent activity of cap targeted α- and ß-oligos, however because of unspecific stimulation values are higher than 100% for low oligo concentrations. It appeared that the ß globin on α globin ratio allows a normalisation of ß globin specific inhibition taking into account an unspecific stimulation of protein synthesis. The unspecific action on α globin, shown in Fig 4B, is therefore taken into account in Fig 4C where one can observe that α- and ß- cap targeted oligos display the following 50% inhibitory activities: 2 μM for the α-oligo and 4 μM for the ß-oligo. Because of an unspecific action on α globin synthesis inhibition triggered by the ß-oligo is lowered for concentration higher than 5 μM.

The unspecific stimulation of protein synthesis is much lower in WGE. We can therefore observe in Fig 5A that the cap targeted ß-oligo is more efficient than the α-oligo on ß globin synthesis. In order to compare the oligos activities in WGE with those in RRL a normalised representation is shown in Fig 5B. The 50% inhibitory dose is 0.01 μM for the ß-anomer and 0.2 μM for the α-anomer in WGE.

Discussion

We first compared the action of α- and ß-oligos on the rabbit ß globin synthesis. Two *in vitro* translation assays were used: RRL with a low RNase H

activity and WGE with a high one. Oligos were targeted on various regions of the ß globin mRNA (Fig 1).

With a low RNase H activity α- and ß-oligos targeted against the AUG initiation codon or downstream do not inhibit ß globin translation. Even a 30-mers oligonucleotide, with an expected greater stability of the oligo:mRNA duplex, does not display an inhibitory action. This likely results from an efficient enzymatic activity linked to the ribosomal complex which is able to dissociate the mRNA hybrids during protein synthesis.

However α and ß-oligos targeted against the cap area in 5' of the ß globin mRNA display a specific inhibition of ß globin synthesis. This result is quite stimulating. One can postulate that at the cap level protein synthesis can be inhibited specificaly by interfeence of oligos with initiation factors. This could take place even without mRNA cleavage, because of the low RNase H activity (9,12). We observed in RRL an unspecific stimulation of protein synthesis by α- and ß-oligos if they are prehybridized to mRNA prior translation. This stimulation is proportional to the oligos length. Three hypothesis could therefore account for this result: (i) inhibition of RNase activity by oligos acting as polyanionic compounds, (ii) increase of the life of mRNAs due to protection against RNases by unspecific hybridisation of oligobases sequences , (iii) unspecific hybridisation could destabilize the superstructure of mRNAs and therefore facilitate the action of ribosomes. Experiments performed in the presence of RNasin, which is a ribonuclease inhibitor, did not show a stimulation of protein synthesis (result not shown). Futhermore stimulation is more important when prehybridation of mRNA with oligos occurs (Fig 3). In that case mRNA is denatured before hybridization which allows probably unspecific hybridizations. These results favor the third hypothesis.

In WGE all tested ß-oligos displayed an inhibitory activity on ß globin synthesis. Among α-oligos, only the cap targeted one is able to inhibit translation. These results are in agrement with previous observations (3,8). ß-Oligos are efficient only in the presence of RNase H (WGE). In both RRL and WGE, stable hybrids between mRNA and α-oligos do not trigger the RNase H induced cleavage of mRNA (3,8) and therefore do not inhibit translation. The inhibitory action of α-oligos targeted against the cap region on 5' of mRNA shows that it is possible to perturb ribosomal activity by a different mechanism. As proposed by Lawson and al (10) oligos hybridized in 5' of mRNA can prevent the binding of initiation factors such as eIF-4A or eIF-4B on the cap sequence and therefore inhibit initiation of translation.

We compared the efficiency of cap targeted α- and ß-oligos in WGE and in RRL (*in vitro* translation systems). Because of the unspecific stimulation of protein synthesis in RRL this comparison was made possible by normalizing the inhibition values through the α globin synthesis considered as a control. Cap

targeted oligos are 10 to 100 times more efficient in WGE than in RRL. This suggests that even in the cap region and without mRNA induced clivage, RNase H could play a role in the observed inhibition. However cleavage stil increases the activity and therefore a higher efficiency for a 50% inhibition of ß-oligos (10 nM) is observed when compared to α-oligos (200 nM) in WGE. The opposite is true in RRL: 2 μM for α-oligos against 4 μM for ß-oligos. This slight difference could be caused by a better stability of mRNA:α-oligos duplexes when compared to mRNA:ß-oligos duplexes (3).

We show in this paper that cap targeted α-oligos inhibit rabbit ß globin translation with specificity even at low RNase H activity. This constitutes the first report of antimessager activity of the non natural α-oligos. This could constituted an improvement for the specificity of oligos as antimessenger agents. It is known that RNase H sensitive oligos have non specific targets in a whole genome (8,13). For instance a 20-mers oligo designed for 20 bases specific sequence will have numerous recognitions with 12 bases triggering possible cleavage by RNase H and therefore unspecific proteins inhibition (result not shown). α-Oligos which are only efficient in the cap region through a RNase H partially independent action mecanism will not be subject to the same causes of unspecific action because unspecific hybridisation, exept in cap areas, will not create undesired translation inhibition.

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