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Enzymatic amplification of translation inhibition of rabbit β-globin mRNA mediated by anti-messenger oligodeoxynucleotides covalently linked to intercalating agents

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ABSTRACT

The effects of anti-messenger oligodeoxynucleotides, covalently linked to an intercalating agent, on translation of rabbit ß-globin mRNA, were investigated both in wheat germ extract and in microinjected *Xenopus* oocytes. A specific inhibition of ß-globin synthesis was observed in both expression systems with a modified 11-mer covalently linked to an acridine derivative. In injected oocytes a more efficient block was observed with this modified oligonucleotide than with its unsubstituted homolog. This was ascribed to stacking interactions of the intercalating agent with base pairs which provide an additional stabilization of the [mRNA/DNA] hybrid. We demonstrated that in wheat germ extract, the modified and unmodified oligonucleotides behaved similarly due to the presence of a high RNaseH activity. RNaseH was also present, although to a lesser extent, in the oocyte cytoplasm. This anti-messenger DNA-induced degradation of target mRNA resulted in amplified efficiency of hybrid-arrested translation. This additional mechanism might provide anti-sense DNAs with an advantage over anti-sense RNAs.

INTRODUCTION

The role played by small RNAs as regulatory elements in gene control is receiving more and more attention. In *Escherichia coli* osmoregulation of the *OmpF* gene coding for a membrane protein is achieved by a small transcript complementary to part of the *OmpF* mRNA (1). Hybridization of these two RNAs prevents translation of the message. A similar regulation process has been reported for Tn10 transposase (2) and *repA* genes in *E. coli* (3). Overlaping transcription units on opposite DNA strands have been recently identified in drosophila (4) and mouse genomes (5,6), but natural regulation of translation by anti-sense RNAs has not been demonstrated in eukaryotic cells yet. Micro-injection of an anti-sense RNA prepared *in vitro* or cell transfection with a DNA construct specifying an anti-messenger RNA, very generally resulted in the specific inhibition of the target gene due to the formation of a double-stranded hybrid between sense and anti-sense RNAs (see reference 7 for a review). Translation is certainly a step at which these complementary RNAs do act, but the intracellular concentration of target mRNA was also reported to be affected (8) and the transit from the nucleus to the cytoplasm inhibited (9). Numerous examples demonstrating that expression of both exogenous (10-14) and endogenous genes (15-20) can be modulated by anti-sense RNAs are now available .

DNA-RNA hybrid-arrested translation was used to locate adenovirus 2 genes (21) and to

analyze mRNA mixtures (22). Translation of both prokaryotic and eukaryotic mRNAs can be efficiently inhibited by synthetic oligodeoxynucleotides in cellular extracts (23-26), in micro-injected *Xenopus* oocytes (27, 28) or in cultured cells (29, 30). Several factors can account for the differences observed in translation inhibition by various anti-messenger oligonucleotides: location of the target sequence on the mRNA, secondary structure of the complementary site,... (7). One of the crucial points in the utilization of such molecules is the chain length as it determines i) the specificity of the biological effect, ii) the hybrid stability and iii) the cellular uptake. A rough calculation shows that a sequence of seventeen nucleotides will be found once in the human genome assuming a statistical distribution of the four nucleotides. As only a small part of the DNA is transcribed, a good specificity is expected with even shorter oligonucleotides when they are targeted to messenger RNAs. A specific and significant inhibition of Rous sarcoma virus development in cultured cells was observed upon addition of a 13-mer (29). The stability of the DNA-RNA hybrid and consequently the extent of translation inhibition is directly related to the length of the hybridized region. However, the uptake by living cells will be unfavoured for long oligonucleotides as compared to short ones. As the inhibitory effect depends on the intracellular concentration of anti-messenger molecules, the optimal chain length leading to the maximal regulatory efficiency will be reached when, shortening a given anti-sense sequence, the increased cellular uptake will balance the decreased hybrid stability.

One way to reconcile these contradictory requirements is to introduce chemical modifications in the anti-messenger molecules in order to simultaneously increase their target affinity and favour their penetration into cells. A new family of oligodeoxynucleotides covalently linked to an acridine derivative was recently developed (31, 32). Intercalation of the dye in the short duplex formed upon binding of the oligonucleotide to its complementary DNA (or RNA) sequence was expected to increase its affinity for the target. As a matter of fact the presence of the dye at the end of an oligonucleotide increased the melting temperature of the complex formed with a complementary single-stranded nucleic acid either in solution (32) or immobilized on nitrocellulose filters (33), as compared to hybrids formed with unsubstituted oligonucleotides. Fluorescence and NMR studies showed that this increased affinity was actually due to stacking interactions between the acridine derivative and the nucleic acid base pairs adjacent to the dye attachment site (34; see figure 1b for a schematic drawing of the complex). Such acridine-linked oligonucleotides were shown to specifically block *in vitro* translation of a prokaryotic gene, the gene 32 of bacteriophage T4. An acridine-modified decanucleotide, complementary to a sequence adjacent to the ribosome attachment site of the gene 32 mRNA was more efficient than the homologous unmodified decanucleotide (33). We report here on the use of similar chemically modified anti-messenger oligonucleotides as translation regulators of an eukaryotic mRNA. We demonstrate that the specific inhibition of rabbit ß-globin translation observed both *in vitro* (in wheat germ extract) and *in vivo* (in micro-injected *Xenopus* oocytes) is partly due to the oligonucleotide-mediated RNaseH degradation of the target mRNA.

MATERIALS and METHODS

Oligonucleotides:

5'd[GACAGATGCACCATTCT] (17-Glo[51-67]), 5'd[TTGTGTCAAAAGCAAGT] (17-Glo[3-19]), 5'd[CACCAACTTCTTCCACA] (17-Glo[113-129]), 5'd[TTCTGTCTGTT] (11-Glo), 5'd[TTTCCTTTATTAGCCAGAAGTCAGATGCTC] (30-Glo[546-575]) complementary to rabbit ß-globin mRNA (Figure 1) and 5'd[AAGCTTGGGCTGCAGGT] not complementary to this mRNA, were synthesized on an Applied Biosystem solid phase synthesizer. Oligonucleotides 5'd[ATTCTGTCTGT] and 5'd[TGTCTGT] (complementary) and 5'd[CTTGTTTCTAC] (not complementary), linked at their 3' end to 2-methoxy,6-chloro,9-aminoacridine via a pentamethylene bridge, were synthesized according to a previously published procedure (31, 35). In the 11-mer 5'd[ATTCTGTCTGT] the phosphate group linked to the acridine derivative was substituted for a neo-pentyl phosphotriester. This generated two isomers termed Acr-11Glo[I] and Acr-11Glo[II] according to their chromatographic mobility (Thuong and Chassignol, unpublished results). The $-CH_2OH$ group of the 5'-deoxyribose of the 7-mer 5'd[TGTCTGT] (Acr-7Glo) was substituted by a methyl group. These oligonucleotides were purified in two steps by ion-exchange and reverse phase HPLC. Material from the main eluting peak was collected, end-labelled and analyzed on a 25% polyacrylamide/7M urea sequencing gel. They were assayed for rabbit globin mRNA complementarity by filter hybridization. The oligonucleotides, 5'-labelled by $\gamma[^{32}P]$ATP (Amersham; 3000Ci/mmole) were purified from residual label by chromatography on Sephadex G-50. Concentrations of acridine-modified oligonucleotide solutions were determined with a Uvikon 860 spectrophotometer using $\varepsilon^{425nm} = 8850\ M^{-1} \times cm^{-1}$ (32). For micro-injected *Xenopus* oocytes the concentrations indicated in the text and in the figures refer to internal concentrations assuming a diffusion compartment of 0.5 µl.

Hybridization:

Thermal stability of oligonucleotide-mRNA complexes was determined as previously described (33). Nitrocellulose filters loaded with 1µg of rabbit globin mRNA were incubated in 2ml of a 6SSC + 10 Denhardt's solution (1 SSC = 0.15M Nacl, 0.015M sodium citrate, pH 7.2; 10 Denhardt's is 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrolidone) containing about 10^7cpm of $[^{32}P]$-labelled oligonucleotide. For thermal denaturation the filters, placed in a thermostated holder, were rinsed and eluted with 6SSC buffer while the temperature was raised at 1.2°C/mn. Fractions were collected during the thermal elution and counted. From elution profiles we determined critical temperatures Tc at which 50% of the oligonucleotide was released.

Rabbit globin mRNA translation in *Xenopus* oocytes:

X. laevis oocytes were obtained from the Laboratoire de Physiologie de la Reproduction (Université Paris 6) and maintained at 18°C in modified Barth's saline solution (36). Stage 6 oocytes were selected under stereotaxic microscope.

Usually, 80nl of rabbit globin mRNA (25µg/ml) were injected at the equator level. Taking into account the fact that the ratio of α-globin to ß-globin messenger RNA is 1.4, the concentration of ß-globin mRNA was about 0.1µM in the injected solution and 16nM inside the oocyte. Prior to injection, mRNA (B.R.L., USA) was 50% diluted either with sterile water or oligonucleotide aqueous solutions. Series of ten oocytes were injected and incubated at 18°C in 0.1ml of modified Barth's saline solution. After a 5-6 hour lag, 100µCi of [^{35}S]-methionine (1000Ci/mmole; CEA, France) diluted to 0.3mM with cold methionine were added. At the end of a 16 to 18 hours incubation, healthy oocytes (generally >80%) were collected and homogenized in 20µl (per oocyte) of 20mM Tris-HCl buffer, pH 7.6 containing 0.1M NaCl, 1% Triton X100 and 1mM PMSF (37) .

***In vitro* translation**:

0.05µg of rabbit globin mRNA in sterile water were mixed with the desired synthetic oligodeoxynucleotide and added to 30µl of the translation mixture containing wheat germ extract (Amersham, England) and amino acids (50µM each) including 15µCi of [^{35}S]-methionine. The final concentration of ß-globin mRNA was 3.9nM. The ionic conditions used were those recommended by the supplier (96mM potassium acetate, 2.4mM magnesium acetate). Translation reactions were carried out for 30 minutes at 25°C.

Analysis of protein synthesis :

[^{35}S]-labelled proteins from oocyte homogenates and cell-free translation mixtures were analyzed either by TCA precipitation after alkaline hydrolysis of amino acyl-tRNAs, or by gel electrophoresis. Labelled α- and ß-globin synthesized in the wheat germ extract were separated on 12% polyacrylamide gels containing 8mM Triton X100 and 6M urea (38). Oocyte homogenate samples were run on 13.5% SDS-polyacrylamide gels. Polyacrylamide gels were impregnated with a fluorophor (Amplify, Amersham) prior to autoradiography.

RNA analysis :

Total oocyte RNA was prepared by the phenol/chloroform method according to the procedure of Colman (37). Rabbit globin RNA from *in vitro* translation was phenol extracted, ethanol precipitated and dryed. RNA samples were fractionated either in 8% polyacylamide/ 7M urea or, following glyoxalation, on a 1.5% agarose gel. Northern blots on Compas nylon membranes (Genofit, Switzerland) were done as recommended by the supplier.

Treatment of oligonucleotide-mRNA hybrids by *E. coli* RNaseH (Genofit, Switzerland) was performed at 37°C in 20mM Tris, HCl buffer, pH 7.5, containing 10mM $MgCl_2$, 100mM KCl, and 0.1mM dithiothreitol. Oligonucleotide 5'd[CACCAACTTCTTCCACA] complementary to region [113-129] of rabbit ß-globin mRNA was used as a primer for AMV reverse transcriptase (Amersham, England). Polymerisation was performed during 1 hour at 39°C in a 100mM Tris, HCl buffer containing 10mM $MgCl_2$, 50mM KCl, 10mM dithiothreitol and 5U of RNasin (Genofit, Switzerland), in the presence of 500µM of each dNTP and 10 pmoles α[^{32}P]-dATP

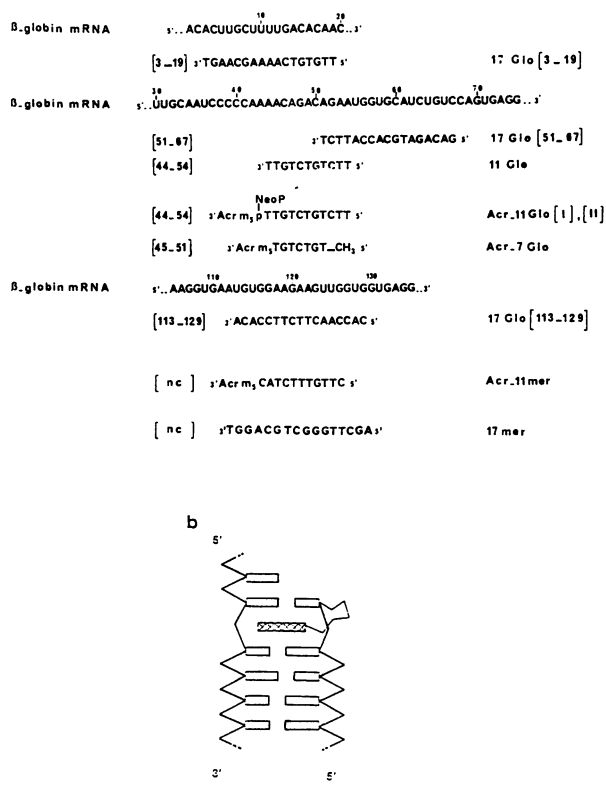

Figure 1: a) Nucleotide sequences of oligodeoxynucleotides complementary to the 5'end (top), initiation region (middle) or coding sequence (bottom) of the rabbit ß-globin messenger RNA (62). The target location on the mRNA is indicated in brackets on the left (1 referring to the transcription start). [nc] : oligonucleotides non complementary to ß-globin mRNA. The abbreviations used throughout the text are given on the right. (See "Material and Methods" for description of the chemical modifications introduced in the molecules).
b) Schematic drawing of [acridine-linked oligonucleotide/mRNA] complexes. The hatched box represents the acridine ring.

(3000Ci/mmole, Amersham, England). [^{32}P]-labelled cDNAs were separated on a 8% urea/polyacrylamide sequencing gel.

RESULTS

Translation of rabbit ß-globin mRNA in injected *Xenopus* oocytes:

In a first set of experiments we investigated the effects on translation of oligonucleotides covalently linked at their 3' end to the acridine derivative via a pentamethylene bridge. These oligonucleotides, a 7-mer (Acr-7Glo) and an 11-mer (Acr-11Glo), are complementary to regions [45-51] and [44-54] of the rabbit ß-globin mRNA, respectively (Figure 1a). These targets are

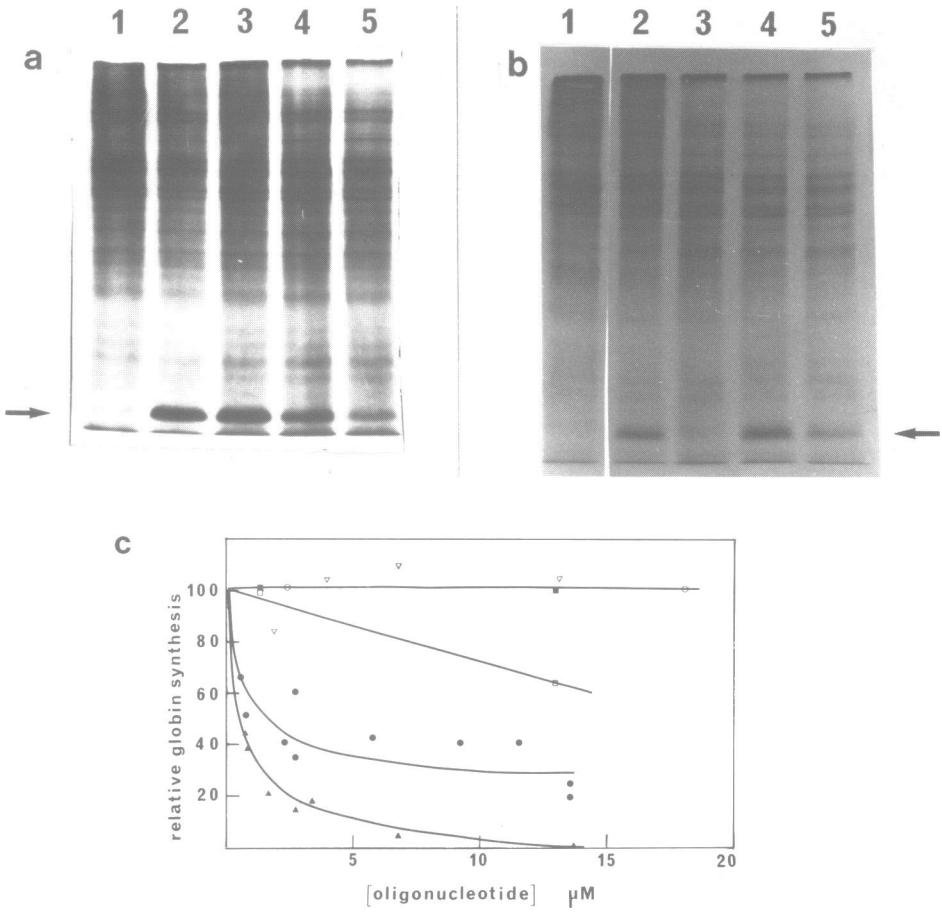

Figure 2: Effect of oligonucleotides on rabbit ß-globin synthesis in injected *Xenopus* oocytes. Autoradiographs of 12.5% SDS/polyacrylamide gels of proteins synthesized in oocytes: (a) non injected (lane 1), injected with 2ng of rabbit globin mRNA alone (lane 2) or in the presence of 13.5µM of 11Glo (lane 3), Acr-11Glo[I] (lane 4) or Acr-11Glo[II] (lane5). (b) non injected (lane 1), injected with 2ng of rabbit globin mRNA alone (lane 2). 13.5µM of Acr-11Glo[II] were co-injected (lane 3), injected 2 hours prior to (lane 4) or after (lane 5) ß-globin mRNA. The arrow indicates the position of ß-globin.
(c) Relative globin synthesis, determined from densitometer tracing of autoradiographs, in the presence of various oligonucleotides : 11-Glo (O), Acr-11mer (▽), Acr-7Glo without (■) or with pre-heating (□), Acr-11Glo[I] (●), and Acr-11Glo[II] (▲).

located immediately upstream of the initiation codon. As indicated in "Material and Methods" two diastereoisomers of Acr-11Glo were available: they resulted from the presence of a neopentyl phosphotriester at the 3' end of the oligonucleotide. Rabbit globin mRNA (a mixture of α- and ß-globin mRNAs) was injected either alone or with oligonucleotides. After a 4 to 6 hour lag the

oocytes were incubated with [^{35}S]-methionine during 15 to 18 hours. Once extracted, the proteins were analyzed by polyacrylamide/SDS gel electrophoresis. Oocytes that were microinjected with mRNA alone efficiently synthesized the globin ß-subunit (the synthesis of the α-chain requires the co-injection of haemin (39, 40)). It appeared as a low molecular weight protein, superimposed on the background of endogenous polypeptides (Figure 2a, lane 2).

Co-injection of Acr-11Glo[II] with rabbit globin mRNA resulted in the reduction of ß-globin synthesis, the extent of which increased with the anti-messenger concentration: 50% inhibition was observed at about 0.5µM (inside the oocytes) (Figure 2). Isomer [I] of Acr-11Glo was less efficient than isomer [II] although the stabilities of the two mRNA-oligonucleotide complexes were identical (Tc=36°C) as determined from thermal elutions of filter-bound hybrids (see "Material and Methods"). In contrast, the unmodified homologous oligonucleotide (11Glo) had no significant effect on ß-globin expression over the same concentration range (Figure 2a and 2c). This difference could, at least in part, result from the increased stability brought about by stacking of the acridine derivative with nucleic acid bases in the duplex, as indicated by the values of Tc : 36°C and 31°C for the modified and unmodified 11-mers, respectively. It should be noted that these Tc values determined from temperature-induced hybrid dissociation from nitrocellulose filters should not be taken as a measure of the melting temperatures (Tm) of the hybrids under the conditions used in translation experiments. However, previous experiments (33 and unpublished data) showed that the Tm values in solution follow the same order as Tc values.

At concentrations lower than 15µM the translation inhibition induced by Acr-11Glos was specific *i.e.* dependent on the oligonucleotide sequence and not on the mere presence of the acridine substituent. An Acr-11mer, not complementary to the ß-globin mRNA, did not produce any effect on ß-globin synthesis (Figure 2c). Moreover, under the same conditions, the synthesis of the oocyte endogenous proteins was not affected by the injection of any acridine-linked oligonucleotide. Acr-7Glo did not block ß-globin mRNA translation even at 100µM, although its target was included in the region complementary to the active Acr-11Glos (Figure 2c). From studies in solution (32) this oligonucleotide was expected to bind to its complementary sequence at the temperature at which the oocytes were incubated (19°C) provided the target sequence was accessible. (The presence of a methyl group at the 5'end of this oligonucleotide prevented radiolabelling and therefore determination of Tc). However the binding constant could be too low to efficiently compete with mRNA secondary structures. When the [mRNA/Acr-7Glo] complex was preformed by heating the mixture 5 minutes at 80°C followed by quick chilling on ice prior to injection a weak but significant inhibition was observed (Figure 2c). This result indicated that the mRNA secondary structure involving the target sequence of Acr-7Glo was preventing complex formation in the absence of pre-heating.

It should be pointed out that injection of oligonucleotides carrying an acridine derivative at concentrations higher than 15µM resulted in a non-specific decrease of the synthesis of high molecular weight endogenous proteins (>50kd; see for instance Figure 2a, top of lane 5). The

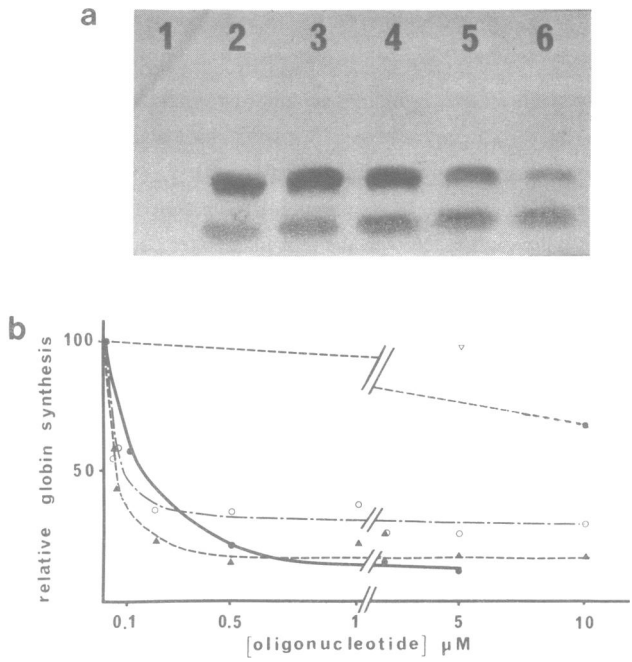

Figure 3: Effect of oligonucleotides on *in vitro* synthesis of rabbit ß-globin.
(a) Autoradiograph of 12% Triton-acetic acid-urea/polyacrylamide gels of proteins synthesized in a wheat germ extract without (lane 1) or with rabbit globin mRNAs in the absence (lane 2) or in the presence of 0.02µM (lane 3), 0.05µM (lane 4), 0.2µM (lane 5), or 0.5µM (lane 6) of Acr-11glo[II]. The upper band corresponds to ß-globin, and the lower one to α-globin.
(b) Relative globin synthesis, determined from densitometer tracing of autoradiographs, in the presence of various oligonucleotides : 11-Glo (O), Acr-11mer (▽), Acr-7Glo (■), Acr-11Glo[I] (●), and Acr-11Glo[II] (▲). Same curves were obtained when gels were cut into pieces and radioctivity was counted.

extent of inhibition was dependent on both the concentration and the length of the oligonucleotide (at the same concentration the Acr-11Glos gave a much more pronounced effect than the Acr-7Glo) but was apparently independent of its sequence. Injection of the acridine derivative alone did not produce any effect, even at a concentration as high as 50µM (results not shown). The non-specific inhibition of protein synthesis observed upon injection of high concentrations of acridine-linked oligonucleotides might be related to indirect effects either on transcription or on translation as already observed in a prokaryotic cell-free expression system (33). At high concentrations of modified oligonucleotides we observed a decrease of transcription activity in the oocytes. Injection of 25µM (internal concentration) of Acr-11mer reduced the incorporation of α[^{32}P]-CTP into acid-insoluble material by 30%. The free acridine derivative at the same concentration did not produce any effect on transcription.

We then investigated the ability of the most efficient anti-messenger oligonucleotide,

(Acr-11Glo[II]), to inhibit globin synthesis when this oligonucleotide was injected in the oocytes 2 hours either prior or after the mRNA. No effect was seen when the oligonucleotide was injected first, whereas a significant inhibition was observed when the oligonucleotide was delivered after the mRNA (Figure 2b). However, at 13.5µM, this inhibition (60%) was lower than that observed following a co-injection experiment (nearly 100% inhibition). These results are in apparent disagreement with those obtained with anti-sense RNAs (12, 41), or those reported by Kawasaki (27) with unmodified oligodeoxynucleotides. The latter author observed a higher translation inhibition upon injection of anti-messenger oligodeoxynucleotides prior to mRNAs. When injected first the oligonucleotide covalently linked to acridine could be either degraded by nucleases or trapped by intracellular structures. The acridine tethered to the 3' phosphate protects the oligonucleotide against 3' exonucleases. Therefore the modified oligonucleotide should be longer-lived than the unmodified one. Preliminary experiments indicated that the major part of oligonucleotide molecules was trapped in yolk platelets, a phenomenon probably related to the hydrophobicity of the acridine ring (C. Cazenave, unpublished results). Therefore the absence of inhibition by Acr-11Glo microinjected before the mRNA could be due to trapping in compartments not involved in mRNA translation.

Translation in wheat germ extract:

Translation of rabbit globin mRNA in a wheat germ extract gave two proteins, the α- and ß-globins, which appeared as two distinct bands on acetic acid/Triton/urea-polyacrylamide gels (Figure 3a). As observed in injected oocytes, addition of Acr-7Glo had only a small effect on the *in vitro* translation of the rabbit globin mRNA (Figure 3b). But the presence in the translation mixture of one of the two Acr-11Glo isomers complementary to the ß-globin mRNA resulted in a highly efficient and specific inhibition: 1µM decreased ß-globin synthesis by about 90%, after a 30 mn incubation at 25°C, whereas no effect was detected on α-globin synthesis (Figure 3a). At low concentration (<0.5µM) isomer [II] was slightly more efficient than isomer [I] but, under these conditions acridine-linked oligonucleotides were not more inhibitory than unmodified 11Glo, in the *in vitro* expression system, in contrast to what was observed in the oocytes (Figure 3b). The differences between the wheat germ extract and the injected oocytes indicated that the relative stability of the mRNA-oligonucleotide complexes could not account for hybrid-arrested translation in the two expression systems. This suggested the involvement of other mechanism(s) besides block of ribosome scanning of the messenger.

Evidence for RNaseH activity in the wheat germ extract and *Xenopus* oocytes:

Complexes formed between mRNAs and oligodeoxynucleotides are substrates for RNaseH. These enzymes which have been characterized in various organisms, hydrolyze the RNA moeity of RNA-DNA hybrids (42, 45). If such RNases were present in the expression systems used in our experiments they would contribute to hybrid-arrested translation, in addition to the inability of ribosomes to dissociate duplexes. It was therefore of interest to look for the presence of RNaseH both in wheat germ extract and in oocytes.

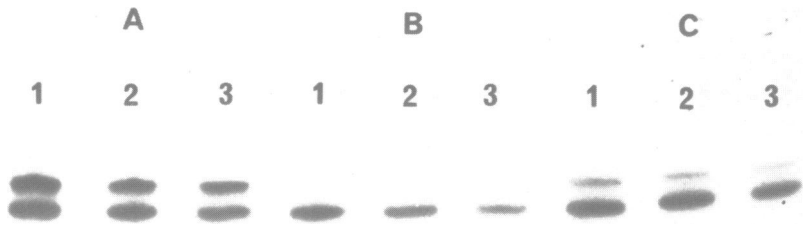

Figure 4: Effect of [oligonucleotide-mRNA] preincubation at 25°C in wheat germ extract on *in vitro* synthesis of rabbit ß-globin. In each series of experiments, translation was carried out for 30mn at 25°C after preincubation during 0mn (lanes 1), 10mn (lanes 2) or 20mn (lanes 3). In panel A, rabbit globin mRNA was preincubated in the absence of oligonucleotide and amino acids were added after the preincubation period. In panel B, mRNAs were preincubated with 0.1µM of oligonucleotide 11Glo prior to addition of the amino acids. In panel C, the RNAs were preincubated in the absence of oligonucleotide and then 11Glo (0.1µM) was added at the same time as the amino acids.

Rabbit globin mRNA was incubated in a wheat germ extract, under the conditions used for translation, in the presence of the unmodified 11-mer complementary to sequence [44-54]) of the ß-globin messenger (Figure 1). Then, after various pre-incubation times, amino acids including [^{35}S]-methionine were added and translation allowed to proceed. In the control experiment the RNA was pre-incubated in the absence of anti-messenger; then the 11-mer and the amino acids were added simultaneously. The synthesis of ß-globin was reduced in the former case as compared to the latter (Figure 4; compare panels B and C). This effect was observed only for an oligonucleotide complementary to ß-globin mRNA: it required the formation of an oligonucleotide-mRNA hybrid. (The non-specific degradation of mRNAs was taken into account by incubating and translating the rabbit globin RNA in the absence of any added oligonucleotide; Figure 4, panel A). This suggested the involvement of either a slow forming species responsible for the translation inhibition process or a hybrid-induced degradation of the mRNA. This last possibility, due to the presence of an RNaseH activity in the wheat germ extract, was demonstrated by RNA analysis. As shown on figure 5a, incubation of rabbit globin mRNAs, in the wheat germ extract, with oligonucleotides complementary to ß-globin mRNA led to the loss of the intact RNA and to the appearance of shorter fragments on a Northern blot. Using a 17-mer complementary to the very 5' end (17-Glo[3-19]) as a probe we observed an RNA shortened by about 60 nucleotides (lane 1) as compared to the intact ß-globin mRNA (lane 2) for the sample incubated in the presence of a 30-mer complementary to the sequence 546-575 of ß-globin mRNA. Unexpectedly, incubation in the presence of 17-Glo[113-129] yielded two fragments hybridizing to the probe (lane 3). Besides the primary target, computer-aided search for sequence complementarity revealed the presence of a secondary site starting at nucleotide 97. The anti-messenger used (17-Glo[113-129]) could pair with 13 bases, including 7 contiguous

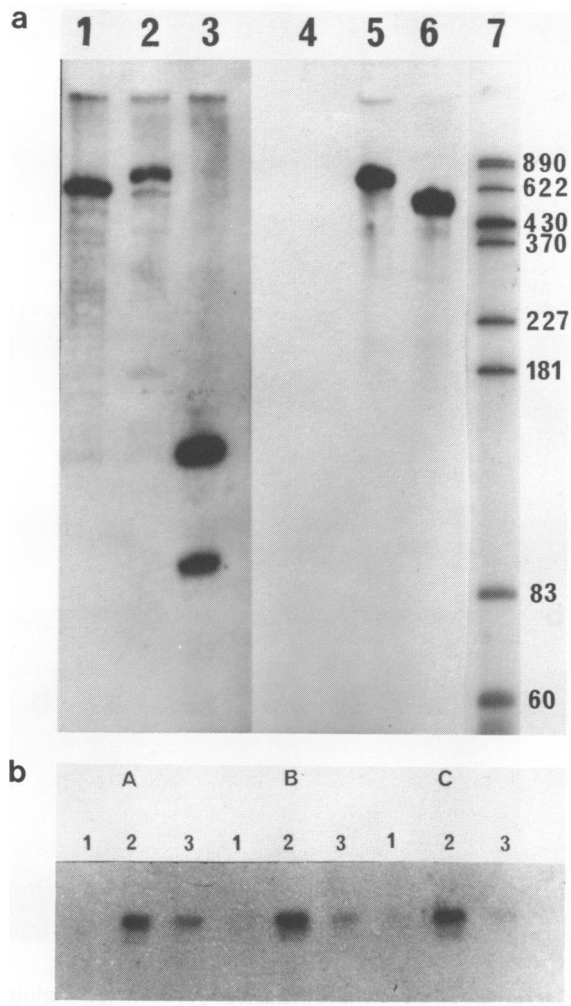

Figure 5: Northern blots of rabbit globin mRNAs incubated in wheat germ extract or injected in *Xenopus* oocytes in the presence of a complementary oligodeoxynucleotide.
(a) 0.2µg of rabbit globin mRNA were incubated (20mn at 25°C) in a wheat germ extract, in the absence of any oligonucleotide (lanes 2, 5) or in the presence of 2µM 30-Glo[546-575] (lanes 1, 4) or of 3.5µM 17-Glo[113-129] (lanes 3, 6). Phenol-extracted RNAs were electrophoresed in a 8% polyacrylamide gel containing 7M urea, and electrotransfered to a nylon membrane. The blot was probed either with 17-Glo[3-19] (lanes 1 to 3) or with 30-Glo[546-575] (lanes 4 to 6). Lane 7: DNA markers.
(b) Total RNA extracted from 10 *Xenopus* oocytes either non-injected (lanes 1), injected with 8ng of rabbit globin mRNA in the absence (lanes 2) or in the presence of 15 µM of 17-Glo[113-129] (lanes 3). RNAs were extracted after 1h30 (panel A), 4h (panel B), or 16h (panel C). Blots in (a) and (b) were probed with 5'end-labelled 17-Glo[3-19].

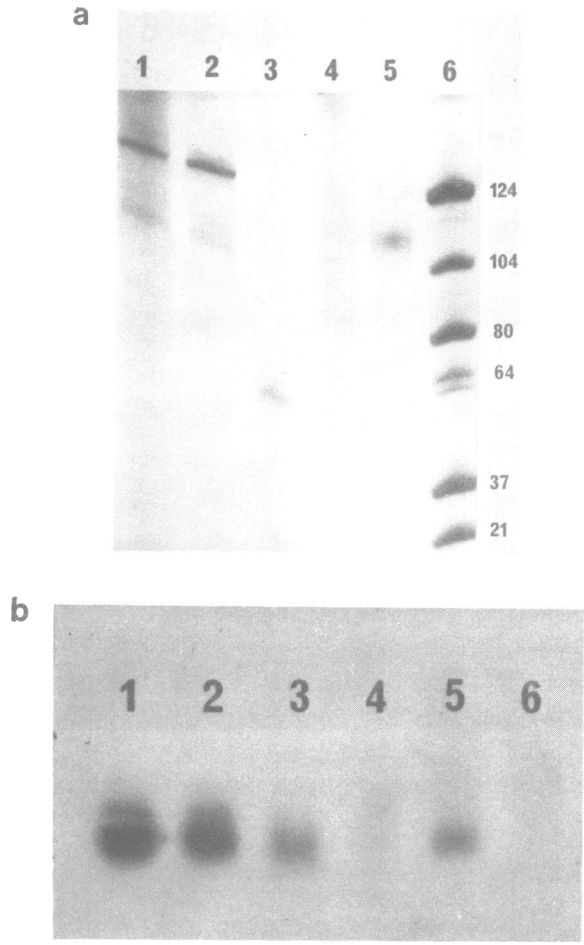

Figure 6: Effect of acridine-linked oligodeoxynucleotides on rabbit ß-globin mRNA integrity in wheat germ extract or in *Xenopus* oocytes.
a) Analysis of cDNAs primed by 17-Glo[113-129] on rabbit globin mRNA (0.2µg) (lane 1) and after 1 hour incubation with E.coli RNaseH in the absence (lane 2) or in the presence of 50µM Acr-11Glo[I] (lane 3), 17-Glo[113-129] (lane 4) or 17-Glo[3-19] (lane 5). DNA markers (lane 6). (b) Northern blots of total RNA extracted from 10 *Xenopus* oocytes injected with 8ng of rabbit globin mRNA in the absence (lanes 2) or in the presence of 10 µM of a non-complementary 17-mer (lane3), 17-Glo[113-129] (lane 4), 11Glo (lane 5) or Acr-11Glo[II] (lane 6). As a control, a [globin mRNA/17-Glo[113-129]] mixture was added to the lysis buffer of non injected oocytes (lane 1). RNAs were extracted 16h after injection. Blot was probed with 5'end-labelled 17-Glo[3-19].

positions, in this region. This hybrid could be stable enough to generate the shortest RNA piece observed in lane 3 (Fig. 5a). Formation of an anti-messenger/mRNA hybrid could block ribosomes upstream of the target, subsequently allowing RNases to attack the ribosome-free

downstream region. Alternatively mRNAs involved in DNA/RNA hybrids constitute a substrate for RNaseH. The second hypothesis seems much more likely as a 500 nucleotide-RNA fragment was detected by a probe complementary to the 3'end of ß-globin mRNA, following incubation of this mRNA in wheat germ extract in the presence of 17-Glo[113-129] (Fig. 5a, lane 6). Moreover RNA fragments observed in lanes 3 and 6 co-migrated with the digestion products of the ß-globin mRNA/17-Glo[113-129] hybrid by *E. coli* RNaseH (data not shown).

A similar analysis was performed on RNAs extracted from injected oocytes. Injection of a mixture of rabbit globin mRNAs and 17-Glo[113-129] resulted in a gradual decrease of intact ß-globin RNA with time : no more RNA was detected after a 16 hour incubation period (Figure 5b). Such a decrease was not observed when mRNA was injected in the absence of any oligonucleotide complementary to ß-globin mRNA. This result indicated the presence of an RNaseH activity in the oocyte cytoplasm. No degradation was observed when 17-Glo[113-129] and mRNA were added to oocytes in the lysis buffer immediately prior to RNA purification (Figure 6b, lane 1): this ruled out RNaseH cleavage during the time course of RNA extraction. We failed to detect in oocytes the RNA pieces seen in the wheat germ extract. This was probably due to a fast degradation of the RNA fragments in oocytes. It should be pointed out that in the wheat germ extract the amount of shorter RNA fragments varied from one experiment to the other probably reflecting differences in RNase content from batch to batch. This anti-messenger-induced degradation of the target mRNA will therefore increase the efficiency of hybrid-arrested translation.

In the case of an acridine-linked anti-messenger, it was crucial to check whether intercalation of the dye in the duplex prevented RNA degradation. Rabbit globin mRNA and Acr-11Glo[II], were incubated with *E. coli* RNaseH. Extension of 17-Glo[113-129] used as a primer for reverse transcription of RNA products, showed that RNaseH treatment of the [Acr-11Glo[II]/globin mRNA] hybrid led to the synthesis of a short cDNA, about 70 nucleotides long. The ß-globin mRNA was completely converted to the cleaved species with this modified oligonucleotide as well as with unsubstituted 17-mers (Figure 6a). The shortening by about 50 nucleotides with respect to the cDNA synthesized from intact ß-globin mRNA was expected if the duplex was attacked by the nuclease. Therefore the presence of the intercalated dye did not prevent the *E. coli* RNaseH attack. Neither did it block RNaseH activities in wheat germ extract and injected oocytes. Sixteen hours after injection into *Xenopus* oocytes Acr-11Glo[II] led to a complete degradation of the ß-globin mRNA whereas the unmodified 11Glo induced only a partial cleavage (Fig. 6b). Injection of a non-complementary 17-mer resulted in a partial loss of the ß-globin mRNA. After such a long incubation time this was not totally unexpected as it was reported that the formation of only four base pairs constituted a substrate for the *E. coli* and calf thymus RNaseH (45). It should be pointed out that neither this non complementary 17-mer nor 11Glo inhibited ß-globin synthesis. This suggested that partial degradation occurred rather late with respect to translation. In the wheat germ extract addition of Acr-11Glo[II] resulted in a faster degradation of ß-globin

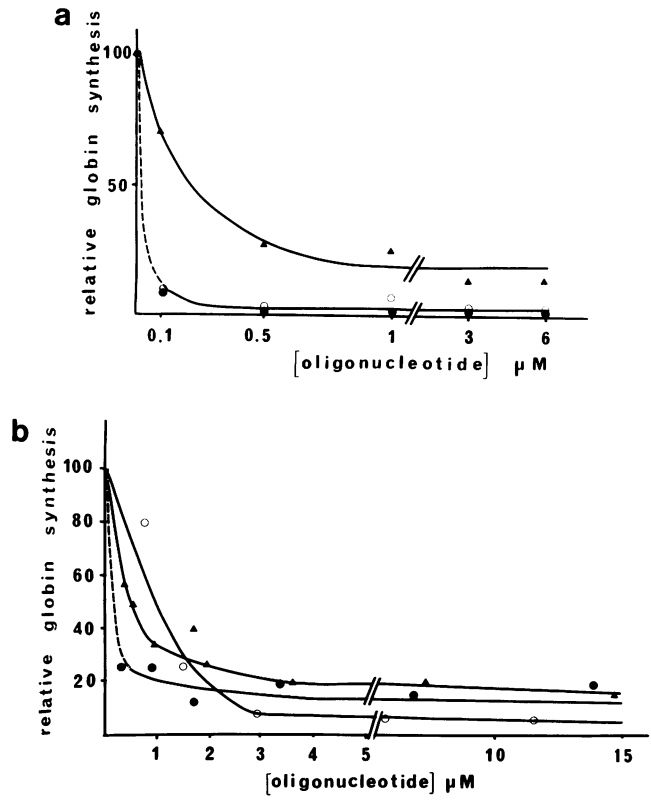

Figure 7: Effect of oligonucleotides on rabbit ß-globin synthesis in wheat germ extract (a) and in microinjected oocytes (b). Relative globin synthesis in the presence of various oligonucleotides was determined from densitometer tracing of autoradiographs: 17-Glo[3-19](▲), 17-Glo[51-67](O), 17-Glo[113-129](●).

mRNA than that due to the homologous unmodified 11Glo. The mRNA cleavage induced by 5µM of the two 11-mers (either modified or unmodified) was not as important as that due to the presence of 3µM of 17-Glo[113-129]. This might reflect the relative stabilities of [mRNA/oligonucleotide] complexes. The cleavage product was detected only in the case of the 17-Glo[113-129]; this could be due to a less efficient binding to the membrane of the small RNA piece resulting from RNaseH attack of the other hybrids. Therefore RNaseH activity contributed to hybrid-arrested translation both in wheat germ extract and in injected oocytes.

Translation inhibition by unmodified 17-mers complementary to various regions of the rabbit ß-globin mRNA :

It was previously reported that in eukaryotic and prokaryotic cells anti-sense RNAs targeted upstream of the initiation codon were more inhibitory than those complementary to the coding

sequence (8, 12, 14). Similarly, it was reported that in a rabbit reticulocyte lysate, DNA fragments complementary to sequences downstream of the AUG failed to arrest translation (46). However, if the RNaseH contribution to translation inhibition by oligodeoxynucleotides is important, we expect an effect even with anti-messengers targeted against the coding sequence. The translation inhibitory effect of three unmodified 17-mers (Figure 1) complementary to the very 5'end (17-Glo[3-19]), the region of translation initiation (17-Glo[51-67]) or the coding sequence (17-Glo[113-129]) of the rabbit ß-globin mRNA was investigated both *in vitro* (in a wheat germ extract) and in injected oocytes.

Addition to a wheat germ extract or micro-injection in *Xenopus* oocytes of any one of these three unmodified 17-mers, reduced the synthesis of [^{35}S]-methionine-labelled proteins directed by rabbit globin mRNA (Figure 7). Electrophoretic analysis of the translation products showed that inhibition was restricted to the ß-globin chain: at concentrations above 20µM the ß-globin band could hardly be detected on an overexposed autoradiograph whereas the synthesis of α-globin (in the wheat germ extract) and of endogenous proteins (in oocytes) was not affected (data not shown). The inhibition was specific for the target mRNA and likely resulted from the formation of a [ß-globin mRNA/oligonucleotide] hybrid. In a wheat germ extract 17-Glo[3-19] was by far less inhibitory than the two other 17-mers. From densitometer tracing of the autoradiographs, the ß-globin band was 30, 91 and 93% inhibited in the presence of 0.1µM of oligonucleotides complementary to the cap, AUG and coding regions, respectively. Independently of the location of the target on the mRNA and of the inhibition mechanisms (see "Discussion") these results can be in part accounted for by the G-C content of the three oligonucleotide-mRNA hybrids and the relative stabilities of the secondary structures involving the target sequences. The 17-Glo[3-19] forms only 6 G-C base pairs with the target mRNA instead of 8 for the two other 17-mers. On the other hand a computer calculation of the stability of local hairpins in the target region showed that they decreased in the order [113-129]<[3-19]<[51-67] (Auron *et al.*, 1982). The efficiency of the three 17-mers to block ß-globin synthesis in oocytes decreased in this same order (Figure 7). The most efficient was 17-Glo[113-129] as observed in the wheat germ extract. However there seemed to be a difference in the relative efficiencies of 17-Glo[3-19] and 17-Glo[51-67] in the oocytes as compared to wheat germ extract.

DISCUSSION

Our objective was to produce short synthetic oligodeoxynucleotides of high enough affinity to block translation by specific hybridization with a target mRNA. We chose to link the oligonucleotide to an intercalating agent. Previous physico-chemical studies demonstrated that stacking of the dye with base pairs of the mRNA/anti-messenger duplex should provide the modified molecule with an additional, sequence independent, interaction (31, 32). Such oligonucleotides linked to an acridine derivative efficiently and specifically repressed translation of a prokaryotic gene in an *in vitro* expression system (33). It was also previously reported that

unmodified oligonucleotides were able to block translation of ß-globin mRNA both in a wheat germ extract (24, 28) and in microinjected oocytes (27, 28). The results presented in this paper clearly demonstrate that a specific and significant inhibition was reached with an acridine-linked oligonucleotide containing only 11 nucleotide units. However, in a wheat germ extract, in contrast to what was previously observed in an *E. coli* extract (33), the addition of the acridine-linked oligonucleotide did not result in a marked increase of the inhibition of translation, compared to the homologous unmodified one. This was not related to a peculiarity of the anti-messenger sequence as a large difference between the inhibitions produced by the conventional (11Glo) and the acridine-modified (Acr-11Glo) anti-messengers was observed in *Xenopus* oocytes injected with rabbit globin mRNA.

Hybrid-arrested translation can occur through various processes. It was first assumed that binding of a complementary sequence to a mRNA prevented its translation. However it was more recently reported that several other steps were also affected by anti-sense polynucleotides. Transcription of the *crp* gene in *E. coli* is inhibited by a short divergent RNA which binds to the nascent mRNA chain upstream of an A+U rich region, thus producing a structure similar to a ρ-independent terminator (48). In cultured L cells export of the thymidine kinase mRNA from the nucleus to the cytoplasm was prevented by anti-sense RNAs (9). Oligonucleoside methylphosphonate complementary to the splice junction of herpes virus pre-mRNAs had an anti-viral effect probably related to splicing inhibition (49). In our case ß-globin synthesis can be affected only at the level of translation as oligonucleotides were injected into the oocyte cytoplasm together with the target mRNA. Depending on the location of the target with respect to the initiation codon they might interfere either with initiation or with elongation steps. Anti-sense RNAs were reported to be poor inhibitors of protein synthesis if targeted downstream of the initiation codon (7). Liebhaber and co-workers (46, 50) claimed that they failed to block translation in rabbit reticulocytes lysates by using DNA complementary to mRNA coding sequences. This was ascribed to the presence of an unwinding activity in these extracts associated with elongating 80S ribosomes. From our results it is clear that complementary oligodeoxynucleotide-induced degradation of the mRNA can play a major role in hybrid-arrested translation in cells or extracts containing RNaseH activities.

The presence of a high RNaseH activity in wheat germ extracts undoubtedly contributed to the inhibition of protein synthesis. At concentrations leading to complete translation inhibition, a pre-incubation as short as 10mn of a [11Glo/globin mRNA] mixture was sufficient to lead to an almost total degradation of the RNA. Therefore under the conditions used for translation (30 mn) this likely constituted the major mechanism of inhibition. The presence of the acridine derivative did not give any advantage to the modified anti-messenger due to the high RNaseH activity of this extract. Differences previously observed between wheat germ extracts and reticulocyte lysates for oligodeoxynucleotide-arrested translation might arise from the low RNaseH activity in the latter cell-free system (24). This agreed fairly well with a recent report (51). RNaseH activity explains

preliminary observations about the effects of anti-messenger oligodeoxynucleotides either *in vitro* (52) or in injected oocytes (27).

The differences observed between the wheat germ extract and microinjected oocytes could arise from several contributions: i) the incubation temperatures were different (19°C in oocytes and 25°C in wheat germ extract) which affect both the stability of the mRNA-oligonucleotide hybrid and the kinetics of RNaseH-induced degradation of the mRNA; ii) in the wheat germ extract RNaseH-induced hydrolysis of the mRNA was fast as compared to the time required for translation in contrast to oocytes where the RNaseH activity was much slower (compare figures 4B and 5b). Although the oocyte cytoplasm clearly contained an RNaseH activity, its relative contribution to complementary oligonucleotide-arrested translation was probably not as large as in wheat germ extract as the acridine-linked 11-mer was a more potent inhibitor of ß-globin synthesis than the unmodified one. In the case of an oligonucleotide directed downstream of the AUG, such as 17-Glo[113-129] degradation of the target mRNA by an RNaseH certainly plays the prominent role in hybrid-arrested translation. In wheat germ extract, a 17-mer complementary to the very 5'end of the ß-globin mRNA was by far less efficient than 17-mers targeted to other parts of the mRNA. Besides nucleotide composition and secondary structure differences this could be related to the formation of the initiation complex involved in translation and/or to competition with cap-binding proteins. Blake *et al.* (24) reported a stimulation of ß-globin synthesis in the presence of a 9-mer complementary to the region [4-12] of the ß-globin mRNA both in wheat germ extract and in reticulocyte lysate. It should also be noted that identical inhibitions of trypanosome mRNA translation by oligonucleotides complementary to the "cap" region, were obtained in wheat germ extract and in rabbit reticulocyte lysate (25) suggesting that in this case RNaseH contribution might not be as important as for oligonucleotides complementary to other RNA regions. Alltogether these results indicate that oligonucleotides directed against the "cap" could interfere with other mechanisms and could be useful tools to investigate translation initiation processes.

The above results suggest that an increased efficiency can be expected from anti-messenger oligodeoxynucleotides compared to homologous anti-sense RNAs in cells containing RNaseH activity. Consequently, introduction of chemical modifications in the oligodeoxynucleotides must be restricted to sites which do not alter recognition of the DNA/RNA hybrid or prevent RNA cleavage by RNases. Alternatively an increased efficiency of anti-sense molecules could be obtained if the oligonucleotides were covalently linked to a substituent which irreversibly damage the target mRNA. The RNA modification would be restricted to the target sequence as a result of specific hybrid formation. It has been previously reported that EDTA-Fe(II) chelates could induce a targeted cleavage of single-stranded DNA sequences when tethered to an oligonucleotide (53-55). A more efficient cleavage is obtained when the oligonucleotide is further substituted by an acridine derivative to stabilize the complex with the target sequence (56). A specific cleavage of phage T4 32 mRNA was obtained upon incubation of part of this RNA with a complementary oligodeoxynucleotide linked to the acridine derivative at its 3'end and to an EDTA-Fe^{2+} complex

at its 5' end (Toulmé, unpublished results). Other metal complexes such as Cu(I)-phenantroline (57) and Fe(II)-porphyrin (58) can be used to target reactions to specific sequences. Another improvement will also result from the synthesis of oligodeoxynucleotides resistant to DNase attack. Degradation of anti-messengers obviously weaken their inhibitory efficiency. We observed a partial degradation of acridine-oligonucleotides in wheat germ extracts, the extent of which varied from batch to batch. Substituting phosphate groups for phosphonates results in nuclease-resistant oligonucleotides. Such oligonucleotides were successfully used to regulate mRNA translation both in cell-free systems (59, 60) and in intact cells (49, 61). This chemical modification generates a family of diastereoisomers of variable affinity for the target sequence. Studies on cultured cells and future developments of modified oligonucleotides as therapeutic agents also require a more efficient transport through the cell membranes. Design of synthetic oligonucleotides that fulfil the above requirements constitutes an exciting challenge to DNA chemists.

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