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# Selective inhibition of cell-free translation by oligonucleotides targeted to a mRNA hairpin structure

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## ABSTRACT

**Using an *in vitro* selection approach we have previously isolated oligodeoxy aptamers that can bind to a DNA hairpin structure without disrupting the double-stranded stem. We report here that these oligomers can bind to the RNA version of this hairpin, mostly through pairing with a designed 6 nt anchor. The part of the aptamer selected against the DNA hairpin did not increase stability of the RNA–aptamer complex. However, it contributed to the binding site for *Escherichia coli* RNase H, leading to very efficient cleavage of the target RNA. In addition, a 2′-*O*-methyloligoribonucleotide analogue of one selected sequence selectively blocked *in vitro* translation of luciferase in wheat germ extract by binding to the hairpin region inserted upstream of the initiation codon of the reporter gene. Therefore, non-complementary oligomers can exhibit antisense properties following hybridization with the target RNA. Our study also suggests that *in vitro* selection might provide a means to extend the repertoire of sequences that can be targetted by antisense oligonucleotides to structured RNA motifs of biological importance.**

## INTRODUCTION

Numerous RNA structures play a key role in various biological processes. Bacteriophage R17 coat protein and phage T4 gene 32 protein bind to particular motifs on their own mRNA, thereby repressing translation (1,2). Aconitase regulates the lifetime of transferrin receptor mRNA and the translation efficiency of ferritin mRNA through selective binding to a stem–loop structure (3). The human immunodeficiency virus (HIV) genome contains multiple examples of functional RNA structures. The repeat region of the 5′-leader, which is reiterated at the 3′-end of the RNA, contains the *trans*-activating response element, a binding site for both viral and host proteins that is crucial for transcription, as well as a second hairpin structure with the polyadenylation signal sequence, which might be important for packaging of the viral genome and for stability of the RNA (4). An internal loop of the Rev responsive element, which binds the Rev protein, is responsible for transport of mature viral mRNA to the cytoplasm (5). The balance between translation of *gag* and *pol* genes is ensured by a frameshifting signal which involves a hairpin structure (6). Dimerization of the HIV genome is achieved through formation of a ‘kissing complex’ between the complementary loops of two hairpins (7).

High affinity ligands that can selectively bind to such RNA structures might interfere with the regulatory processes they mediate. Oligonucleotides offer several ways to design such ligands (for a review see 8). Antisense oligomers can be used if one takes advantage of peculiarities of the target structure to minimize the thermodynamic penalty of unfolding it (9). Alternatively, chemically-modified oligomers exhibiting a high affinity for RNA, such as *N*3′-phosphoramidates, 2′-*O*-methyl derivatives or those containing modified bases, are potentially useful in invading the structure (10–12). Triplex-forming oligonucleotides can be used for appropriate target sequences, i.e. double-stranded purine–pyrimidine stretches (13,14).

Recently we attempted to select hairpin binders from randomly synthesized oligodeoxynucleotide libraries (15). Indeed, we identified 26mers that were able to form complexes with a stem–loop DNA structure, thus preventing cleavage of the double-stranded stem by a restriction enzyme (16). The association of these so-called ‘aptastrucs’ (i.e. aptamers recognizing a structure) with the target hairpin was driven by formation of six Watson–Crick pairs at the bottom of the stem. Additional unidentified interactions contributed to stability of the complexes.

Within the context of antisense strategy it was of interest to determine whether similar complexes could form with the RNA version of the hairpin and could prevent translation of a message. Electrophoretic mobility shift assays (EMSAs) have allowed us to demonstrate that aptastrucs bind to the target RNA hairpin. The 6 bp in the anchoring region accounted for complex formation. RNase H footprinting of the aptastruc–RNA complexes revealed increased digestion of the target compared with the regular 6 bp heteroduplex, indicating that the 3′-part of the aptastruc provided a binding site for the enzyme, although it did not significantly contribute to oligonucleotide–RNA interactions. In addition, the target hairpin used for *in vitro* selection in our previous work was inserted in front of the luciferase gene. We demonstrated that a 2′-*O*-methyloligoribonucleotide, derived from one of the previously identified aptastrucs, was able to inhibit *in vitro* translation of a luciferase construct in a selective way.

## MATERIALS AND METHODS

### Materials

Chemical reagents were obtained from Aldrich and [$\gamma$-$^{32}$P]ATP (37.5 MBq/mmol) from ICN. T4 polynucleotide kinase, SP6 RNA polymerase and *E.coli* RNase H were purchased from Promega. *Stu*I and *Bam*HI were from New England Biolabs.

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T7 RNA polymerase was prepared from an *E.coli* overproducing strain as described previously (17).

Oligodeoxynucleotides were obtained from Genset (Paris). 2′-*O*-Methyloligoribonucleotides were synthesized on a Millipore Expedite synthesizer. Oligonucleotides were purified by reverse phase HPLC, using an acetonitrile gradient (0–48%) in 100 mM ammonium acetate, pH 7.0, buffer and checked for purity by electrophoresis of 5′-$^{32}$P-labelled products on a 20% denaturing polyacrylamide gel.

### Plasmid construction

A *Hin*dIII–*Bam*HI DNA fragment (5′-AGCTTAGGGAGAAG-AGAGGAGCAGTTTCTCTCCTCTCTTG) containing the target hairpin (stem deoxynucleotides underlined) was inserted into plasmid pGEM-luc (Promega), thus producing pGEM-T-luc (Fig. 1). Insertion was confirmed by restriction analysis and sequencing of the recombinant plasmid.

### Electrophoretic mobility shift assays

For EMSAs 5′-$^{32}$P-end-labelled RNA target (0.1 µM) was prepared *in vitro* with T7 RNA polymerase from a template with a 17 bp minimal T7 promoter (18). The resulting transcript, ModRNA (Fig. 1), was mixed, in 50 mM Tris–acetate, pH 7.5, buffer containing 10 mM magnesium acetate (buffer A), with the desired oligonucleotide (1 µM) and loaded immediately on a 10% non-denaturing polyacrylamide gel. Alternatively, 5′-end-labeled oligonucleotides were mixed with cold ModRNA under the same conditions.

### RNase H mapping

RNase H digestion of a mixture containing 1 µM 5′-end-labelled ModRNA (prepared as described above) and 12 µM candidates in buffer A was performed on samples pre-treated for 4 min at 65°C, cooled down and incubated at 25°C for 30 min. The buffer was adjusted to 20 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM DTT. Digestion was carried out for 1 h at 25°C and samples were analysed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea.

### *In vitro* translation

For cell-free translation assays the plasmids pGEM-luc and pGEM-T-luc were linearized with *Stu*I and transcribed by SP6 RNA polymerase (Ribomax large scale RNA product system; Promega) in the presence of the cap $m^7$GpppG. mRNA (5 nM) was mixed with the oligonucleotide at the desired concentration. The mixture was pre-treated as described above for RNase H mapping and incubated in rabbit reticulocyte lysate or in wheat germ extract according to the supplier's instructions (Promega). Luciferase activity was measured in a Lumat LB9501 luminometer (Berthold) using the luciferase assay kit from Promega as per the manufacturer's instructions. Percentage inhibition was calculated relative to that of samples without added oligonucleotide.

## RESULTS

Beginning with a library of oligonucleotides randomized at 16 positions (i.e. containing ~4 billion sequences), we previously extracted, by an *in vitro* selection procedure, three 26mers, $C_1$, $C_2$ and $C_3$, that bound to a DNA hairpin (15,16). The randomized

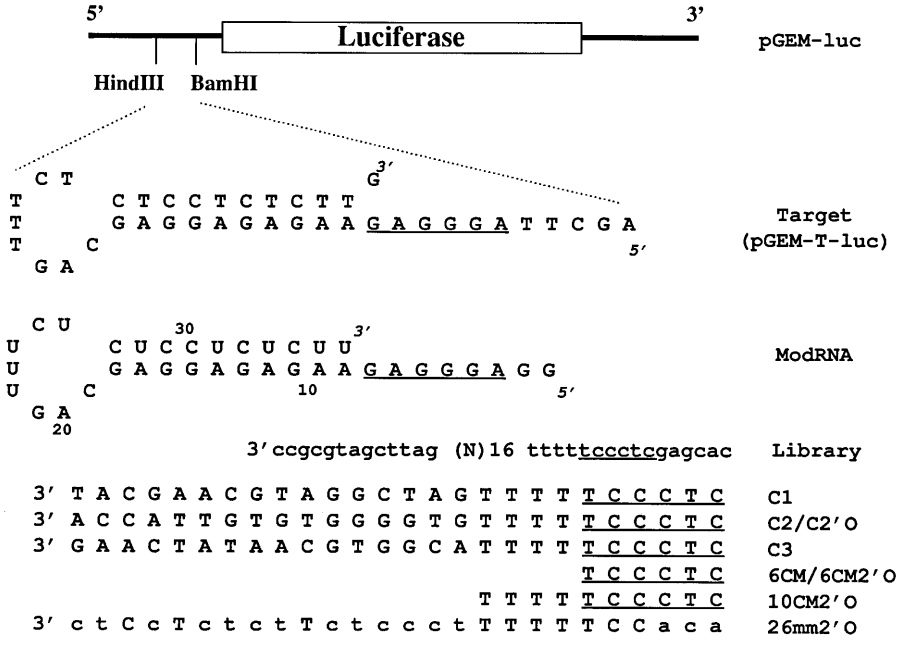



**Figure 1.** Sequences of the target and of the oligonucleotides. A hairpin structure was inserted at the *Hin*dIII and *Bam*HI sites of the pGEM plasmid, 10 nt upstream of the AUG initiation codon of the luciferase gene. The ModRNA sequence used for EMSA and RNase H mapping is also shown. A similar hairpin (with an additional 3 bp adjacent to the loop) was used in a previous work (15) to select aptastrucs from an oligonucleotide library with 16 random positions ($N_{16}$) flanked by fixed sequences, which were used for amplification during the selection procedure. In addition the 5′ fixed region contains the 6 base anchor (underlined) complementary to the single-stranded region (underlined) at the bottom of the target stem. The three selected aptastrucs $C_1$, $C_2$ and $C_3$ and the hexamer 6CM corresponding to the anchor motif are shown. The sequences 6CM, $C_2$, 10CM and 26mm were prepared as 2′-*O*-methyloligoribonucleotides. In the control oligomer 26mm the differences from $C_2$ are indicated by lower case letters.

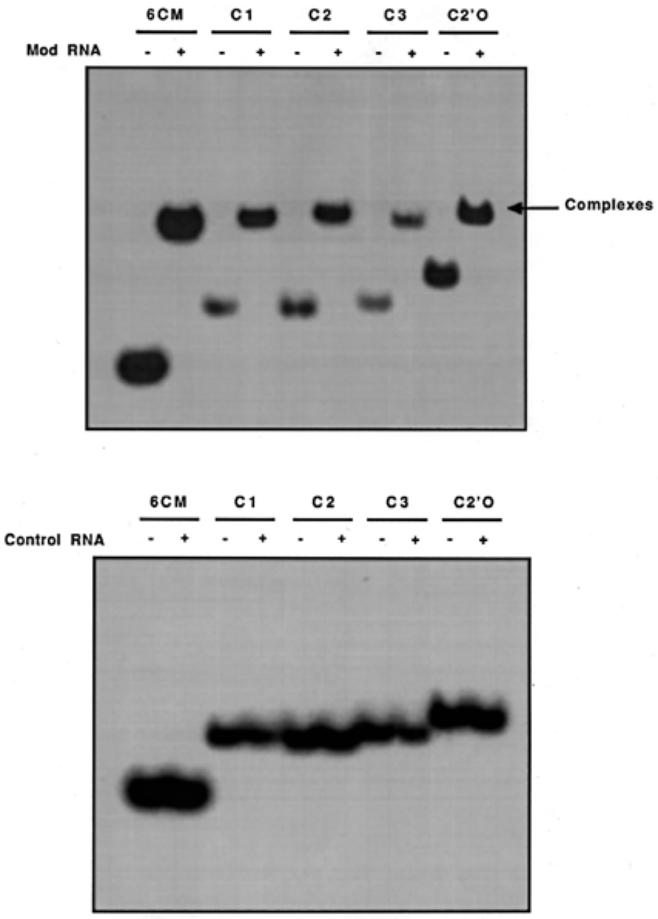



**Figure 2.** EMSA for oligonucleotide–RNA complexes. Oligonucleotides, as indicated at the top of each panel, were run either in the absence (–) or presence (+) of 1 µM ModRNA (top) or control RNA 5′-CU GUC ACG GAG CGC CUA UUA ACU GUC AGA CCU UC (bottom) as indicated (see Materials and Methods for experimental conditions).

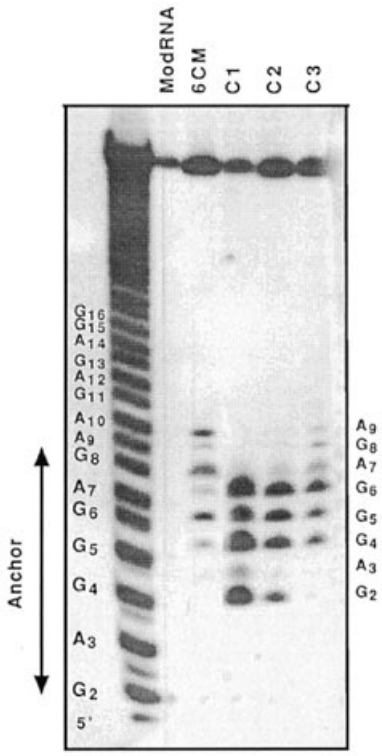



**Figure 3.** RNase H mapping of RNA–oligonucleotide complexes. $^{32}$P-Labelled ModRNA was incubated in the presence of *E.coli* RNase H with 6CM, $C_1$, $C_2$ or $C_3$. An alkaline ladder is given on the left as well as the corresponding sequence. The sequence on the right corresponds to the fragments generated by RNase H cleavage (see text).

stretch of the starting oligomers was flanked on the 5′-side by a fixed motif of 10 nt (Fig. 1). This motif contained an anchor sequence 5′-CTCCCT (termed 6CM) complementary to the single-stranded region at the bottom of the hairpin on the 5′-side. The aptastrucs identified after four rounds of selection constituted high affinity ligands that could potentially interfere with processes involving the target hairpin, such as digestion of the double-stranded stem by a restriction nuclease (16).

## Aptastruc binding to an RNA hairpin

Following our previous studies with the DNA target (15,16), we investigated the binding properties of the selected 26mers with an RNA version of the hairpin used for *in vitro* selection (Fig. 1). The stem of the RNA hairpin was actually 3 bp shorter than that of the DNA, leading to a structure of lower stability ($\Delta G$ = –49.9 kcal/mol) than the parent structure ($\Delta G$ = –56.2 kcal/mol). This modification was introduced in order to obtain a translatable transcript when the hairpin was placed upstream of the initiation codon of the luciferase gene; exceedingly stable hairpins in the 5′-leader of mRNAs are known to prevent efficient scanning by the translation initiation complex (19). The deleted base pairs, close to the apical loop, were expected not to be important for binding of the aptastruc, as previous footprinting studies performed on aptastruc–DNA hairpin complexes did not reveal any contact with the top of the stem (16). RNase mapping confirmed that a hairpin with a 13 bp stem was actually formed: the stem and the loop were clearly seen after digestion by RNase V1 and nuclease S1 respectively (not shown).

We monitored binding of oligonucleotides to the target RNA using EMSA. ModRNA was used for this study. Two G residues are present at the 5′-end, next to the anchor sequence, to allow efficient *in vitro* transcription by T7 RNA polymerase (Fig. 1). Adding any of the aptastrucs $C_1$, $C_2$ or $C_3$ resulted in reduced mobility of the $^{32}$P-end-labeled ModRNA (Fig. 2), indicating formation of aptastruc–ModRNA complexes. Moreover, the complex formed with $C_3$ had a slightly increased mobility compared with the others. As both size and charge were identical for all three complexes, this suggested formation of different structures, likely related to different kinds of interactions between the 3′-part of the aptastruc and the target RNA. This is reminiscent of our previous observations with the DNA hairpin (16). Moreover, no shift was observed when using a non-target 34mer RNA whose sequence (5′-CU GUC ACG GAG CGC CUA UUA ACU GUC AGA CCU UC) did not contain any element similar to ModRNA, indicating that complexes are specific (Fig. 3).

## RNase H mapping of ModRNA–aptastruc complexes

That the ModRNA–aptastruc complexes adopt different overall structure was confirmed by RNase H mapping: incubation of 5′-$^{32}$P-end-labelled RNA–aptastruc complexes in the presence of

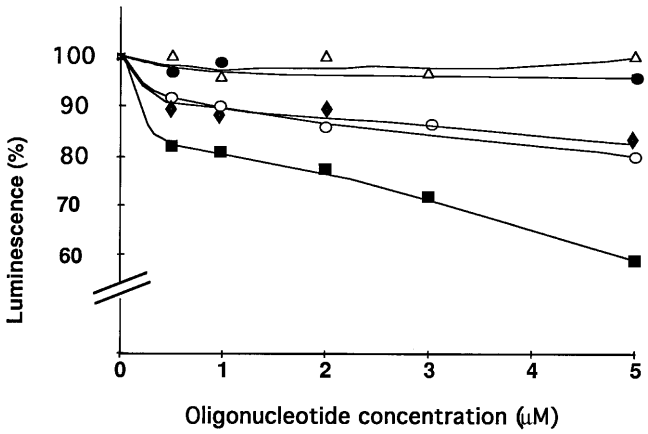
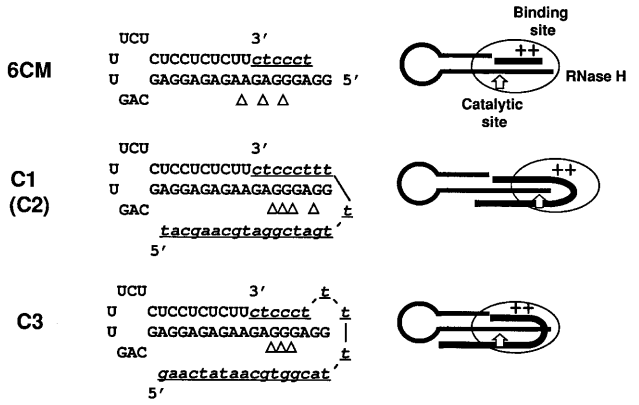



**Figure 4.** Inhibition of translation of the luciferase reporter gene in wheatgerm extract by 2′-*O*-methyloligoribonucleotides. The pGEM-T-luc transcript was translated in the presence of C2′O (■) 6CM2′O (○) 10CM2′O (◆) or 26mm2′O (●). As a control the pGEM-luc RNA was translated in the presence of C2′O (Δ).




**Scheme 1.** The major cleavage sites induced by RNase H on complexes formed by ModRNA with 6CM, $C_1$, $C_2$ or $C_3$ are indicated (arrow heads) on the left. The sequences of the oligonucleotides are indicated by italicized underlined lower case letters. The right part of the scheme shows location of the binding region (indicated by ++) and of the catalytic site (arrow) of the enzyme.

*E.coli* RNase H, which cleaves the RNA strand of RNA–DNA duplexes, produced different patterns; both the band position and the extent of digestion varied for $C_1$ (or $C_2$) and $C_3$ complexes (Fig. 3). The cleavage profile obtained with the aptastrucs was also different from that generated by the ModRNA–6CM complex.

The antisense hexamer 6CM yielded three major fragments. Assignment of cleavage sites was made with respect to the alkaline ladder and RNase T1 digestion of ModRNA (not shown). The RNase H fragments exhibit a slower mobility than the alkaline breakdown products, as the former have a 3′-OH and the latter a 2′,3′-cyclic phosphate. In addition, examination of the cleavage pattern indicates that the distance between two successive bands varies depending on the sequence (Fig. 3). Assuming a shorter distance for 5′-GpA than for 5′-ApG or 5′-GpG steps allows unambiguous assignment of the bands: the fragments induced by 6CM corresponded to cleavage at G(5), A(7) and A(9), while the binding site extends from A(3) to G(8).

All three aptastrucs induced cleavage of ModRNA in the anchor binding region (Fig. 3), indicating that the 3′-end of the oligomers prevented neither binding of the enzyme to the complex nor recognition of the anchor region as RNA–DNA heteroduplex. Generation of different cleavage patterns from the aptastruc–RNA mixtures (Fig. 3 and Scheme 1) suggests that availability of the anchor duplex and location of the 3′-part of the oligomer were not the same in complexes formed with $C_3$, on the one hand, and with either $C_1$ or $C_2$, on the other, leading to a different location of the enzyme on the substrate. $C_3$ induced cleavage in the same region as the antisense hexamer 6CM, although the relative sensitivity of the sites to RNase H was different. The similar pattern obtained with $C_1$ and $C_2$ was shifted 5′ (relative to the RNA orientation) by 2 nt, i.e. closer to the 5′-end of the hybrid compared with 6CM. Moreover, the extent of digestion was much greater with $C_1$ and $C_2$ than with 6CM and $C_3$, although the binding constants were of the same order of magnitude (see below). Therefore, the local structure in the vicinity of the heteroduplex modulates RNase H activity in some unknown way.

## Inhibition of *in vitro* translation by aptastruc–mRNA complexes

We investigated the effect of the three aptastrucs on *in vitro* translation. For this purpose we transcribed the pGEM-T-luc construct, in which the target hairpin was inserted 10 nt upstream of the AUG initiation codon of the luciferase gene (Fig. 1). The message was translated with an efficiency similar to that of mRNA without the hairpin. No effect on luciferase activity level was observed following addition of any of the oligomers $C_1$, $C_2$ or $C_3$ to rabbit reticulocyte lysate (RRL) programmed with the T-luc transcript (not shown). In contrast, when *in vitro* translation was carried out in wheatgerm extract (WGE) luciferase synthesis was reduced to nearly zero in the presence of 5 μM oligonucleotide $C_1$, $C_2$ or $C_3$. However, as a similar result was obtained when the WGE was programmed with a transcript derived from the parent pGEM-luc, i.e. from the plasmid without the hairpin, this inhibition was non-specific (not shown). This inhibition was likely related to RNase H-mediated cleavage of the luciferase message at non-target sites (see Discussion).

In order to confirm this RNase H contribution to translation and to investigate the potential interest of chemically-modified aptamers we studied the effect of 2′-*O*-methyl analogues. These nuclease-resistant oligomers display a strong affinity for complementary RNA sequences (11). Although they do not elicit RNase H activity, they were reported to block *in vitro* reverse transcription (20) and translation when targeted upstream of the AUG initiation codon (11,21). We arbitrarily synthesized the 2′-*O*-methyl derivative of $C_2$ (C2′O). This oligomer was able to bind to ModRNA, as shown by EMSA (Fig. 2). Moreover, C2′O displayed a specific inhibitory effect on translation in WGE: 40% inhibition of luciferase activity from pGEM-T-luc was achieved at 5 μM oligomer, whereas a <5% decrease in luciferase synthesis from the parent transcript pGEM-luc devoid of the target hairpin was observed (Fig. 4). The amplitude of inhibition induced by C2′O was significantly higher than that produced by 6CM2′O, the 2′-*O*-methyl analogue of 6CM. The decanucleotide 10CM2′O, composed of the conserved part of the aptamers, i.e. the anchor region and the consecutive T residues, behaved similarly to 6CM2′O. It is unlikely that a difference in degradation of C2′O and 6CM2′O (or 10CM2′O) is responsible for the greater inhibition of translation with C2′O, as we never observed cleavage of oligonucleotides, even unmodified ones, under these

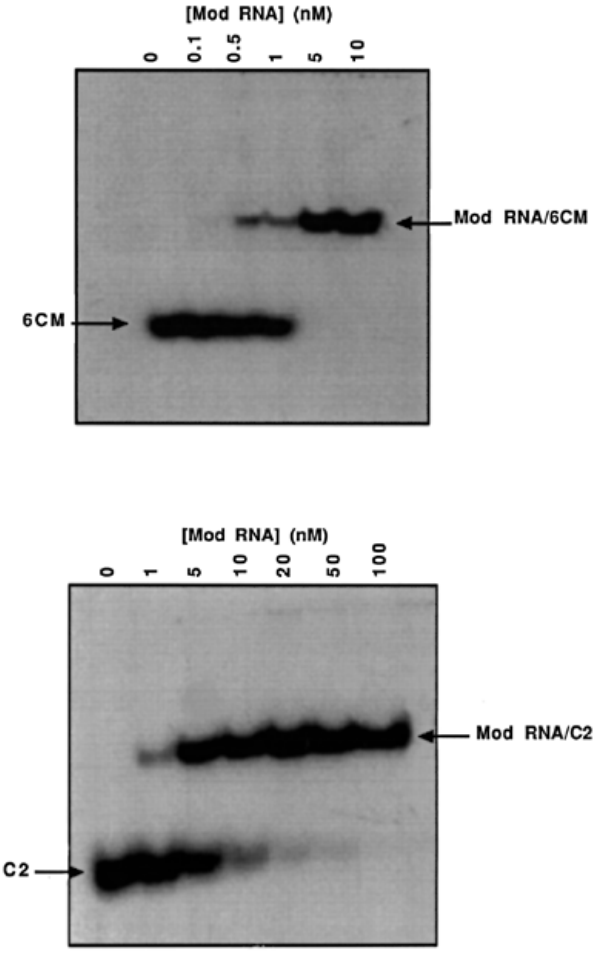



**Figure 5.** EMSA for oligonucleotide–RNA complexes. 5′-$^{32}$P-End-labeled oligomers 6CM (top) and $C_2$ (bottom) were titrated with increasing amounts of ModRNA, as indicated at the top of the lanes, under the conditions given in Materials and Methods. The arrows refer to the free oligomers and to the complexes.

conditions. Last, the selectivity of the effect was demonstrated using 26mm2′O, a mismatched 26mer: no inhibition of translation was observed in this case (Fig. 4).

### Quantitative evaluation of aptastruc–mRNA complexes

In order to determine whether the increased efficiency of C2′O compared with 6CM2′O was due to additional stability of the RNA–oligonucleotide complex due to the 3′-part of the aptastruc sequence we evaluated the affinity of these oligomers and of the unmodified oligonucleotides for their target by EMSA. We titrated 5′-$^{32}$P-end-labelled oligonucleotides with ModRNA. As shown in Figure 5, the oligomer 6CM binds ~3- to 5-fold better to the RNA hairpin than $C_2$. A similar result was obtained for the 2′-*O*-modified 6mer and 26mer (not shown). Last, the oligomers $C_1$, $C_2$ and $C_3$ displayed a similar affinity for the RNA, with $K_d$ values of ~5 nM. Therefore, the 3′-end of the 26mers did not contribute to stability of the complexes and this did not acount for the improved translation inhibitory properties of C2′O.

## DISCUSSION

We had previously demonstrated that oligodeoxynucleotides able to bind to a DNA hairpin can be identified through *in vitro* selection (15,16). We report here that such anti-DNA aptastrucs can prevent *in vitro* translation following binding to an mRNA in which the hairpin used for selection was inserted upstream of the initiation codon.

The association between the anti-DNA aptastrucs and the RNA target was due to formation of 6 bp involving the designed anchor motif (Fig. 1). The 16 nt at the 3′-end of the selected oligomers, corresponding to the randomized sequence in the library, did not contribute to stability of the aptastruc–RNA complexes. This contrasts with the results obtained previously with the DNA target, for which a 100-fold difference was observed between the binding constants of aptastrucs and of hexamer 6CM (16). These results with the RNA target were not unexpected: post-selection modification of either the target or of the aptamer generally weakened the properties for which the aptamer was selected (22,23).

Aptastrucs $C_1$, $C_2$ and $C_3$ did not block protein synthesis in RRL but were able to non-specifically prevent *in vitro* translation in WGE. With respect to antisense experiments, the major difference between RRL and WGE resides in the contribution of RNase H to the effects induced by oligonucleotides. Antisense oligonucleotides act through an RNase H-independent mechanism in RRL (24,25); in contrast, oligonucleotide-induced degradation of RNA by RNase H accounts for most of the observed effects in WGE (26,27). In this latter case RNase H has also been reported to be responsible for non-specific inhibition, due to cleavage of imperfect hybrids resulting from binding of oligonucleotides to non-targetted RNA (28,29). This explains fairly well the results obtained with the three aptastrucs. On the one hand, the complex that these oligomers formed with the target cannot impede the scanning translation machinery in RRL. On the other hand, in WGE inhibition of luciferase synthesis might originate in RNase H-mediated degradation of oligonucleotide–mRNA duplexes. A search for complementarity between oligonucleotides and the luciferase mRNA revealed the potential formation of mismatched hybrids ~300 nt downstream of the initiator AUG. The best match (with $C_1$) had eight contiguous base pairs which might serve as a substrate for RNase H (30).

The 2′-*O*-methyl analogue of aptamer $C_2$ was a selective inhibitor of translation, more efficient than hexamer 6CM(2′O), although the two oligomers have similar affinities for ModRNA. Similar binding properties were observed with a long RNA fragment containing the hairpin, obtained by *in vitro* transcription of plasmid pGEM-luc linearized with *Bam*HI, ruling out interactions with the upstream mRNA sequence. This was surprising, as generally inhibition efficiency of antisense sequences is correlated with oligonucleotide–RNA hybrid stability (31). This suggests that even if the 3′-part of the oligomer does not stabilize the complex it might adopt some conformation that interferes with ribosome scanning (although one cannot exclude the possibility that the structure adopted in the WGE is different from that under the conditions used for binding studies). This is reminiscent of the situation observed with so-called clamped antisense sequences which are efficient inhibitors of polypeptide elongation, whereas conventional antisense sequences are not (32).

RNase H mapping of ModRNA–6CM complexes indicates that RNase H cuts at an internucleoside linkage outside the RNA–DNA hybrid, in a region corresponding to the junction between the

regular double-stranded RNA stem and the heteroduplex (Fig. 3). The enzyme may have sensed a local distortion resulting from juxtaposition of an A-form double helix (the hairpin stem) and the target RNA–antisense DNA helix, for which recent studies indicate a heteronomous structure: whereas the RNA strand adopts an A-type structure, the DNA strand is neither A-form nor B-form (33). Several examples of abnormal cleavage by RNase H have been described, in particular digestion of RNA bound to chemically modified oligonucleotides such as 2′-*O*-methyl derivatives (21). Similarly, the presence of mismatched pairs was recognized by human RNase HII, even when the corresponding site in the perfect duplex was not cleaved (34).

The distribution of cleavage sites on the ModRNA–6CM hybrid is restricted to the 3′-part (with respect to the RNA strand), indicating that the catalytic site is located downstream of the binding site (Scheme 1). A similar result was obtained with human RNase HII: no cleavage occured within the four terminal nucleotides of the hybrid on the 5′-side (35). The binding of *E.coli* RNase HI to the substrate is ensured by a basic residue-containing domain, the so-called basic protrusion (36). The similar cleavage patterns observed with *E.coli* RNase HI and human RNase HII suggests a similar organisation of the two enzymes: a binding domain positioning the catalytic center 4 nt away, in the 3′-direction, along the RNA strand (Scheme 1). This seems to be a hallmark of class II eukaryotic RNases H (Pileur and Cazenave, unpublished results). As cleavage sites close to the 5′-end of the RNA were observed with the 26mers, in particular $C_1$ and $C_2$, and because a short nucleic acid stretch upstream of the cleavage site is required for RNase H binding, this indicates that the aptastruc sequence adjacent to the anchor duplex provides the binding site. In Scheme 1 the 'loop regions' downstream of the anchor site were tentatively written differently for $C_1$ ($C_2$) and $C_3$ to fit with the different cleavage patterns, although similar complexes could be drawn for the three aptamers. We indicate two possible GT pairs at the very 5′-end of the RNA bound to $C_1$ ($C_2$), but we have no proof for that. Whatever the actual interactions between the oligomers and the RNA, the conformation of this region likely varies from one complex to the other, leading to different interactions with the enzyme and, subsequently, to the different cleavage patterns observed with $C_1$ or $C_2$, on the one hand, and $C_3$, on the other.

In conclusion, our results suggest that efficient antisense oligonucleotides can be identified through an *in vitro* selection procedure. They might also explain some non-specific antisense effects of oligonucleotides. Numerous examples of non-antisense effects resulting from interactions with proteins, either in a sequence-dependent or sequence-independent way, have been reported (37). Oligonucleotides can also perturb a pre-existing structure, thus generating unexpected effects (38). Our results demonstrate that oligonucleotide-mediated inhibition of translation can be due to direct interaction with mRNA, even in the absence of detected complementarity at the level of the primary sequences, through an RNase H-independent mechanism. We also demonstrated that RNase H activity can be significantly modulated by local structures in the vicinity of RNA–DNA heteroduplexes. This might be relevant for efficiency and specificity of antisense oligonucleotides

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