Photochemically and chemically activatable antisense oligonucleotides: comparison of their reactivities towards DNA and RNA targets

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ABSTRACT

Dodecaderoxyribonucleotides derivatized with 1,10-phenanthroline or psoralen were targeted to the point mutation (G->U) in codon 12 of the Ha-ras mRNA. DNA and RNA fragments, 27 nucleotides in length, and containing the complementary sequence of the 12mers, were used to compare the reactivity of the activatable dodecamers (cleavage of the target by the phenanthroline-12mer conjugates; photo-induced cross-linking of psoralen-12mer conjugates to the target). The reactivity of the RNA with the dodecamers was weaker than that of the DNA target. With psoralen-substituted oligonucleotides, it was possible to obtain complete discrimination between the mutated target (which contained a psoralen-reactive T(U) in the 12th codon) and the normal target (which contained G at the same position). When longer Ha-ras RNA fragments were used as targets (120 and 820 nucleotides), very little reactivity was observed. Part of the reactivity could be recovered by using 'helper' oligonucleotides that hybridized to adjacent sites on the substrate. A 'helper' chain length greater than 13 was required to improve the reactivity of dodecamers. However, the dodecanucleotides induced RNase H cleavage of the target RNA in the absence of 'helper' oligonucleotide. Therefore, in the absence of the RNase H enzyme, long oligonucleotides are needed to compete with the secondary structures of the mRNA. In contrast, formation of a ternary complex oligonucleotide-mRNA-RNase H led to RNAT cleavage with shorter oligonucleotides.

INTRODUCTION

Oligonucleotides can serve as potential blockers of transcription and translation through sequence-specific binding to DNA and RNA, respectively (1, 2). Antisense inhibition of gene expression using oligodeoxynucleotides has been demonstrated in a variety of in vitro models. One of the most efficient mechanisms of action of antisense oligodeoxynucleotides requires hybridization of the oligonucleotide to its complementary RNA sequence followed by cleavage of the RNA strand of RNA/DNA duplexes by ribonuclease H. As unmodified DNA oligomers lack stability in the presence of serum nucleases, investigations have been focused on designing more stable oligonucleotides such as methylphosphonates (3), phosphorothioates (4, 5), a-oligonucleotides (6), 2'-O-alkyl oligonucleotides (7, 8), etc. Although all of these analogues are resistant to exo and endonucleases and interact with mRNA, only natural phosphodiester and phosphorothioate induce RNase H cleavage. In order to overcome this problem, chemically or photochemically activatable reagents can be covalently linked to nuclease-resistant oligonucleotides. Activation of these agents should irreversibly modify the mRNA target and consequently inhibit the translation of the mRNA even in the absence of RNase H activity.

Photochemical cross-linking of psoralen-derivatized methylphosphonate oligonucleotides to rabbit β-globin messenger RNA has been shown to inhibit mRNA translation in a sequence-dependent manner (9). Chang et al (10) have used psoralen-derivatized antisense methylphosphonate oligonucleotides directed against the mRNA of either the normal human Ha-ras or the Ha-ras gene that is mutated at a single base. They have shown that the specific inhibitory activity of these oligonucleotides is increased by psoralen derivatization after light exposure (10). The antisense activity of a methylphosphonate oligonucleotide (11) or an α-oligonucleotide (12) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs can be strongly increased using psoralen-derivatized oligonucleotides and UV irradiation.

Site-specific cleavage of DNA or RNA can be achieved by linking metal chelates such as Cu-phenanthroline to complementary oligonucleotides (13–16). Using a 21 base-long oligonucleotide linked to 1,10-phenanthroline, Chen and Sigman have shown that the phosphodiester backbones of RNA and DNA have similar reactivity towards the chemical nuclease activity of 1,10-phenanthroline-copper (16). However, cleavage reactions were carried out after heat denaturation of the substrate and

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annulling of the oligonucleotide at 0°C. Such a treatment destroys potential secondary structures and consequently abolishes differences between RNA and DNA reactivity which could be due to differences in stability of secondary structures and, consequently, in accessibility to the oligonucleotide.

Here, we describe an in vitro study of the chemical and photochemical activity of oligonucleotides tethered to either 1,10-phenanthroline or psoralen and targeted to the region of the 12th codon of Ha-ras messenger mRNA. We have compared the intrinsic reactivity of RNA and DNA of different lengths with respect to targeted crosslinking by psoralen and cleavage by 1,10 phenanthroline.

MATERIALS AND METHODS

Oligonucleotides

Two orthophenanthroline-derivatized oligonucleotides (12βOP and OP12α) were synthesized (Figure 1). Covalent linkage of orthophenanthroline to the 5' end of OP12α and to the 3' end of 12βOP oligonucleotides was achieved by condensation of the oligomers carrying a thiophosphate group with a 5-iodoacetamido-1,10 phenanthroline derivative (15, 17, 18).

Introduction of a psoralen derivative at the 5' end of Pso12β1 and Pso12β2 was directly performed via its phosphoramidite derivative (18). Incorporation of psoralen at the 3'-end of the 12β3Pso was achieved by assembling the oligonucleotide on a modified support carrying the psoralen derivative (18). The Pso17β oligonucleotide was synthesized by Appligene, Strasbourg, France.

The 27-mer long oligonucleotide used as DNA substrate for the antisense Ha-ras oligonucleotides was synthesized using standard phosphoramidite chemistry (Institut Pasteur, Paris). Deprotection of the 27-mer RNA oligonucleotide (Eurogentec) was achieved using 0.01N HCl during 20h at room temperature. After Tris-acetate neutralization, the oligonucleotide was treated with n-butanol and precipitated with ethanol. 5' labelling of these oligonucleotides was performed with T4 polynucleotide kinase and [γ-32P] ATP (Amersham).

OP cleavage reactions

Cleavage reactions with 12βOP and OP12α were performed in Eppendorf tubes containing buffered solutions (100 mM NaCl, 50 mM phosphate, pH 7) of OP-derivatized oligonucleotides and 30 nM 32P-labelled RNA or DNA substrate, in the presence of 100 mM NaCl, 50 mM phosphate pH 7. Irradiation was performed in glass microtubes (OSI) with a xenon-mercury lamp (200W) in an Oriel (Stamford, CT) universal arc lamp housing system (model 66057). Light was filtered through pyrex to remove radiations below 300 nm. Then the samples were precipitated with ethanol, redissolved into formamide loading buffer, and electrophoresed. The extent of photoaddition was determined by densitometry.

Gel shift assays

Target sequences were radioactively labelled and put in the presence of unlabelled activatable oligonucleotides in 50 mM phosphate pH 7, 100 mM NaCl. After 10 min preincubation at 37°C, glycerol loading buffer (80% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue) was added, and samples were electrophoresed on a 12% native polyacrylamide gel at room temperature.

In vitro transcription

The plasmid constructs containing the entire sequence of normal and activated Ha-ras sequence were obtained as described (19) and linearized with restriction nucleases EcoR1 or PvuII. In vitro SP6 transcription was performed in 40mM Tris (pH 7.5), 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 0.5 mM ATP, CTP, UTP, 0.1 mM GTP, 0.2 mM RNA CAP analogue [m7G(5')ppp(5')G], 10 µCi [α-32P] GTP (10 mCi/mmole), 80 U RNasin (Promega), 20 U SP6 RNA polymerase (Boehringer) and 1µg of EcoR1 or PvuII linearized DNA. The reaction mixture was incubated for 1h at 40°C. After phenol-chlorophorm extraction and ethanol precipitation, RNA was redissolved in water and used for in vitro assays. The length of the transcripts was 820 nucleotides when EcoR1 was used and 120 nucleotides when PvuII was used.

Ribonuclease H assay

In vitro transcript (30 nM) was incubated at 37°C with oligonucleotides in the presence of 20 mM KCl, 4 mM Tris—HCl pH 7.5, 0.4 mM MgCl2, and 1U of E.coli RNase H (Pharmacia). Reaction was stopped in dry ice bath, formamide loading buffer was added, and samples were electrophoresed.

RESULTS

Three psoralen-derivatized dodecamers were targeted to the region of Ha-ras mRNA including the point mutation G → U at the 12th codon which leads to a GLY → VAL substitution in the ras p21 protein (Fig 1A, B). Upon hybridization to their complementary sequence, two of the oligonucleotides (Pso12β1 and 12β3Pso) brought psoralen close to the mutated base. Psoralen was attached to the 5' end of Pso12β1 and to the 3' end of 12β3Pso. Psoralen adducts should involve, for the most part, an uridine residue on Ha-ras mRNA. Therefore little or no reactivity was expected with wild type mRNA which contains a G instead of U at the position where psoralen is expected to react. Consequently, these psoralen-substituted oligonucleotides should achieve perfect discrimination between wild type and mutated mRNA. Pso12β2 was centered around the codon 12 point mutation and psoralen was attached to the 5' end of the dodecamer. We have shown that this oligonucleotide induced in vitro RNase H cleavage on mutated Ha-ras mRNA whereas wild type mRNA remained nearly unaffected (unpublished data).
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Figure 1. A. Activatable oligonucleotides and their Ha-ras RNA and DNA targets. 20(1), 20(2), and 20(3), are the “helper” oligonucleotides for Psol2|3, Psol2|32, and OP-oligonucleotides, respectively. The abbreviations indicate where the substituent is attached: for instance, Psol2 is a 12mer synthesized with α-anomers and substituted with psoralen at the 5’ end; for 12/3Pso, psoralen is attached to the 3’ end. B. 1,10-phenanthroline and psoralen-substituted oligonucleotides.

Therefore, Psol2|3 and 12βPso might achieve target discrimination via the specificity of psoralen addition to mRNA whereas Psol2|32 might rather discriminate via selective binding of the antisense dodecamer to mutated Ha-ras mRNA.

In order to make the oligonucleotides resistant to nucleases, several modifications of the phosphodiester backbone have been described. Most modifications (methylphosphonates, phospho-triesters, phosphorothioates, phosphorimidates, etc.) introduce a chirality at the phosphorus atom. To avoid this problem, oligonucleotides containing the α-anomers of nucleotides instead of the natural β-anomers were synthetized and shown to be very stable towards nucleases and to form double helical α-β hybrids with a parallel orientation of the two strands (20, 21).

Unfortunately, hybrids are no longer substrates for RNase H. In order to induce cleavage of the RNA substrate, a cleaving reagent—such as phenanthroline—can be attached to the oligonucleotide. The sequence of the phenanthroline conjugates of the α- and β-dodecamers whose activities are compared in the present study (12βOP and OP12α) are shown in Fig 1A, B. The G → U mutation in the target mRNA faces the 5’-end of the α-oligomer and from the 3’-end of the β-analogue. The unmodified β-dodecamer induced RNase H cleavage of the mutated target but no cleavage was observed with the wild-type target at low oligonucleotide concentration (data not shown). These two dodecamers were substituted with 1,10-phenanthroline at the 5’-end (OP12α) or at the 3’-end (12βOP).

Comparison of the activity of derivatized antisense dodecamers complementary to a structured 27mer RNA and DNA.

The sequences of the oligodeoxyribonucleotide and oligoribonucleotide targets and their complementary oligodeoxyribonucleotides are shown in Fig 1. Four types of targets were studied: two 27mers, which contained either a deoxyribonucleotide (27DNA) or a ribonucleotide (27RNA) backbone; a 120 and a 820 nucleotide-long RNA transcripts. The calculated structures of 27 and 120 nucleotide-long targets are shown in Fig 2.

DNA target. Phenanthroline-substituted oligonucleotides were used at a concentration of 5 μM in the presence of 2.5 μM CuSO4 in order to form the phenanthroline–copper chelate, and 2 mM MPA was used as a reducing agent. Under these conditions, the phenanthroline-copper complex generates oxidative species that are responsible for the nucleolytic activity (13, 16). The efficiency of cleavage was evaluated after incubation for 1 hour at 37°C. Both derivatized oligonucleotides cleaved the 27DNA substrate with an efficiency of 30% (data not shown). The cleavage sites were located in the G-rich region of the target near the 5’-end (12βOP) and the 5’-end (OP12α) of the oligonucleotides hybridized specifically to their target. At 5 μM concentration, the OP-oligonucleotide conjugates did not discriminate mutant (27DNA VAL) from wild type (27DNA GLY) target. At lower concentrations, where discrimination due
to the mismatch should be observed, the reactivity of the OP-oligonucleotide conjugates was too low to make meaningful comparisons.

Assays with psoralen-oligonucleotide conjugates were performed using the same substrate and oligonucleotide concentrations as those used with the OP- derivatized oligonucleotides. Irradiations were performed for either 10 or 30 min at 37°C. Following 365 nm irradiation, psoralen can be covalently linked to thymines in DNA. Psoralen-derivatized oligonucleotides induced 37 to 80% of photoadducts after 10 min irradiation at 37°C. Psol2/32 was the most efficient (80% adducts). The high efficiency of this derivatized oligonucleotide could be explained by the fact that either of two thymines, separated by one base, could react with the psoralen moiety of the oligonucleotide (Figure 1). Psol2/31 and 12/33Pso were designed in order to achieve complete discrimination between wild type and mutated target, using the properties of 5' (Psol2/31) and 3' (12/33Pso) psoralen to react with the thymine base present on the mutated target. According to the proposed structure shown schematically in Fig 2, 12^3Pso was targeted more to the single-stranded region contained in 27DNA than Pso 12/31. The slight difference in reactivity (56% for 12β3Pso versus 37% for Pso12β1) could be explained by the availability of the target sequence to form the hybrid but it might also be due to the different linkers used to attach psoralen to the 3'-end and 5'-end of the oligonucleotides (Figure 1B).

We then compared the reactivity of Pso12β1 and Pso12β2 with the mutated DNA target (27DNA VAL) and the wild type target (27DNA GLY). As expected, Pso12β1 reacted with the thymine present in the mutated target and did not react with the wild type target which contains G instead of T (Figure 3A) (Table 1). After irradiation for 30 min at 37°C, we obtained 62% of photoproducts on mutated 27DNA VAL and less than 1% on wild type 27DNA GLY. In contrast, the psoralen moiety of Pso12β2 could form adducts with thymines present in both the wild type and the mutated targets. The yield of photoproducts was nearly identical with the two targets (~92% after 30 min irradiation) at 5 μM concentration of the oligonucleotide (Figure 3A).

**Table 1.** Fraction (as %) of the target DNA cross-linked to psoralen derivatized oligonucleotides (5 μM) in the presence of mutated (27DNA VAL) or unmutated (27DNA GLY) target DNA (30 nM) after 30 minutes of irradiation at 37°C

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<th>27DNA GLY</th>
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<tr>
<td>Pso12β1</td>
<td>&lt;1%</td>
<td>62%</td>
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<td>Pso12β2</td>
<td>92%</td>
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**RNA target.** In order to directly compare the reactivity of the OP and psoralen-derivatized oligomers with RNA and DNA, we used a 27RNA substrate with the same sequence as 27DNA (Figure 1). The psoralen-derivatized oligonucleotides reacted with the 27RNA target with a lower efficiency as compared to 27DNA (18% for Pso12β1 and 35% for Pso12β2) (Figure 3B). The two phenanthroline-substituted oligonucleotides, α12OP and 12βOP, did not induce any detectable cleavage of the 27RNA target. The
higher stability of the RNA secondary structure as compared with dodecamers to the RNA substrate is more likely to reflect the DNA-DNA hybrids. Therefore the weaker binding of the target. RNA DNA hybrids are expected to be more stable than (175nM) was required to achieve 50% association with the RNA oligonucleotide while more than 10-fold higher concentration of the oligonucleotides. As shown in Fig 5, 50% association of Psol2/32 to DNA was achieved with 15nM oligonucleotides saturated RNA and DNA targets at 5 \text{ nM} \text{ target (27RNA or 27DNA). Target alone (Lane 1), with OP12a (Lane 2), with 12/3OP (Lane 3), with Psol2/32 (Lane 4), with Psol2/32 (Lane 5). It should be noted that the retardation of 27DNA induced upon binding of the \alpha-oïoligonucleotide OP12a was less than that induced by \beta-oïoligonucleotide.

Figure 4. Gel shift experiment in the presence of 5 \mu M oligonucleotide and 30 nM target (27RNA or 27DNA). Target alone (Lane 1), with OP12a (Lane 2), with 12/3OP (Lane 3), with Psol2/32 (Lane 4), with Psol2/32 (Lane 5). It should be noted that the retardation of 27DNA induced upon binding of the \alpha-oïoligonucleotide OP12a was less than that induced by \beta-oïoligonucleotide.

Figure 5. Psol2/32 binding to 27mer RNA and DNA targets as determined from gel shift experiments. The fraction of 27RNA (\circled{O}) and 27DNA (\circled{A}) shifted is plotted versus Psol2/32 concentration. Target concentration was 10nM.

The difference in reactivity towards DNA and RNA targets could reflect the difference in binding affinity of the oligonucleotides which might be a consequence of a more stable secondary structure in RNA than in DNA; alternatively it could reflect an intrinsic difference of reactivity with ribonucleic acid as compared to deoxyribonucleic acid sequences.

In order to compare RNA and DNA oligonucleotide binding, gel electrophoresis under non-denaturing conditions was carried out. These binding studies were performed using the same buffer and the same concentrations of template and modified oligonucleotides as those used for the 'activity' assays. Using these conditions, we could observe that the psoralen-modified oligonucleotides saturated RNA and DNA targets at 5 \mu M concentration of the oligonucleotides. As shown in Fig 5, 50% association of Psol2/32 to DNA was achieved with 15nM oligonucleotide while more than 10-fold higher concentration (175nM) was required to achieve 50% association with the RNA target. RNA-DNA hybrids are expected to be more stable than DNA-DNA hybrids. Therefore the weaker binding of the dodecamers to the RNA substrate is more likely to reflect the higher stability of the RNA secondary structure as compared with the DNA substrate. However, at the concentrations used for 'activity' assays, the targets (RNA or DNA) were saturated with the psoralen-derivatized oligonucleotides, suggesting that the difference in stability between RNA and DNA structures could not be the only explanation for the weaker reactivity of the psoralen-oligonucleotide conjugates with RNA as compared with DNA. The structure of the hybrids, especially at the psoralen binding site, and the difference in reactivity of U as compared to T clearly contribute to the difference in reactivity. With the phosphorhline-derivatized oligonucleotides, although the DNA target was saturated under the conditions used for the 'activity' assays, the RNA target was not completely saturated (Figure 4, lane 2, 3). Therefore, the difference in stability of the RNA and DNA structures contribute to the lack of reactivity of phosphorhline-derivatized oligonucleotides towards the RNA target.

Oligonucleotides tethered to OP or psoralen need help to react with Ha-ras mRNA

We tested the reactivity of the phosphorhline- and the psoralen-derivatized dodecamers using in vitro transcribed RNAs. The reactivity of the psoralen-oligonucleotide conjugates Psol2/32 and Psol2/32 was investigated using a 120 nucleotide-long Ha-ras RNA transcript (transcribed from a PvuII-linearized plasmid). The length of the target allowed us to separate the photoadducts from unreacted RNA by PAGE. The OP-oligonucleotide reactivity was tested on a 820 nt-long transcript in the presence of 12/3OP, Psol2/32, and Psol2/32. Cleavage was performed in the presence of 5 \mu M oligonucleotide, 30 nM target and 1 U E.coli RNase H during 3 min at 37°C. The arrow indicates the long cleavage product (~700 nucleotides). Lane 1 : Ha-ras transcript alone. Lane 2 : Ha-ras transcript in the presence of 12/3OP and RNase H. Lane 3 : Ha-ras transcript in the presence of Psol2/32 and RNase H. Lane 4 : Ha-ras transcript in the presence of Psol2/32 and RNase H. B. 12/3OP-induced cleavage of 820 nt-long Ha-ras in vitro transcript in the presence of a 'helper' oligonucleotide (20mer(3)). Lane 1 : Transcript alone. Lane 2 : Transcript in the presence of 5 \mu M of 12/3OP and 5 \mu M of 'helper' 20mer(3) oligonucleotide (Figure 1). The arrow indicates the long (~700 nucleotides) cleavage product. Gel migration in this case was carried out for a shorter period as compared to the migration in parts A and C. This explains the shorter distance between the 820 and 700 nt-long fragments. C. OP12a induced cleavage of 820 nt-long Ha-ras in vitro transcript in the presence of 'helper' 20mer (3) oligonucleotide. The arrow indicates the cleavage product (~700 nucleotides). Same conditions as in part B. No reaction was observed in the absence of 'helper' oligonucleotide with both 12/3OP and OP12a.
derivatized dodecamers induced specific RNase H cleavage of their respective RNA targets (Figure 6A), showing that at least a fraction of the oligonucleotides hybridized to the RNA in the presence of RNase H. Therefore the lack of reactivity was not due to the inability of the oligonucleotides to bind the RNA. However, the lifetime of the hybrid could be sufficient to induce RNase H cleavage but not long enough for a chemical or photochemical reaction to take place. In order to increase the interaction of derivatized dodecamers with the target mRNA, we added a ‘helper’ oligonucleotides (20 nucleotides in length) which bind continguously to the dodecamers (Figure 1). Under these conditions, 12βOP and OP12α induced specific cleavage (Figure 6B, C) and psoralen-derivatized oligonucleotides could be cross-linked to the RNA target upon irradiation, albeit with a low efficacy (2% and 10% cross-linking were obtained with Pso12β1 and Pso12β2, respectively). These results suggest that chemical and photochemical modifications of Ha-ras mRNA in the targeted region cannot be achieved with short antisense oligonucleotides. We have tested the activity of a 17 nucleotide-long psoralen-derivatized oligonucleotide (Figure 1) without any success suggesting that longer oligonucleotides are needed in the case of Ha-ras mRNA. Efficient cleavage could be achieved on β-globin mRNA (589 nucleotides) with a phenanthroline-derivatized β-17mer (data not shown). This result suggests that secondary structures of the targeted sequence which are different for Ha-ras as compared with β-globin mRNA limit the efficacy of the activated oligonucleotides (22).

Specific cleavage and cross-linking were induced in the presence of 20 mer ‘helper’ oligonucleotides which bind to adjacent sites. Shorter ‘helper’ oligonucleotides were less efficient at improving the reactivity of dodecamers. The minimum ‘helper’ length which was required to observe an activity was 13 with psoralen-oligonucleotides (data not shown). It is likely that cooperative binding of the ‘helper’ oligonucleotides increases the stability of the activatable dodecamers, allowing them to react with the RNA target. In contrast, dodecamers alone induced RNase H cleavage under the same conditions suggesting that the hybrid stability was sufficient to induce the endonucleolytic activity of RNase H.

**DISCUSSION**

Several nuclease-resistant oligonucleotides are refractory to RNase H action, a cellular enzyme that cleaves the RNA moiety in an oligodeoxyribonucleotide-mRNA hybrid at the oligonucleotide binding site. Cleaving reagents or photocative groups have been attached to these oligonucleotides in order to induce irreversible damage to complementary sequences and therefore develop nuclease-resistant, efficient inhibitors of mRNA translation. In the present study, we used dodecamers targeted to the point mutation region (GλU) in codon 12 of Ha-ras mRNA and derivatized them with phenanthroline or psoralen in order to compare their chemical and photochemical reactivity towards RNA and DNA substrates. In most cases, oligodeoxyribonucleotides with natural phosphodiester linkages were used in order to compare targeted reactions and RNase H cleavage induced by the same derivatized oligonucleotides.

27mer DNA and RNA targets containing a stem and loop structure were chosen to address the question of chemical and photochemical reactivity of dodecamers. The activatable oligonucleotides were designed to alter specifically the Ha-ras mRNA which contains a GλU point mutation at the 12th position of the 12th codon. Discrimination between the wild type and mutated Ha-ras mRNA was achieved using unmodified dodecamers centered around the 12th codon which specifically induced RNase H cleavage of the mutant target at low concentrations (data to be published). All the activatable oligonucleotides that we have tested reacted with the 27mer DNA substrate. We observed that the reactivity of the psoralen-derivatized oligonucleotides was dependent on the target sequence. Pso12β2 was the most efficient in generating photoadducts, probably because its psoralen moiety could form adducts with two thymines present in the target. We also compared the activities of two psoralen-oligonucleotides (Pso12β1 and Pso12β2) on mutated (27DNA VAL) and wild type (27DNA GLY) targets (Table 1). Pso12β1, which was designed to specifically form adducts with the thymine of mutated codon 12 completely discriminated between these two targets. Under the same concentration conditions, no discrimination was observed with Pso12β2, OP12α or 12βOP, which were centered around the mutation.

Psoralen-derivatized oligonucleotides were about 3 times less reactive with the 27RNA substrate than with the 27DNA substrate, while no reactivity was observed with the phenanthroline-derivatized oligonucleotides on the 27RNA target. In order to determine if the RNA target was saturated by derivatized oligonucleotides under our experimental conditions, we performed gel shift assays using the same reaction conditions as those used in the ‘reactivity’ assays. Although higher concentrations of psoralen-derivatized oligonucleotides were required to saturate the RNA target as compared to the DNA target (Figure 5), RNA and DNA were saturated under the experimental conditions used for ‘reactivity’ assays (5 μM of oligonucleotide). Therefore these results suggest that the low reactivity of psoralen-derivatized oligonucleotides towards Ha-ras RNA is due not only to a more stable RNA structure, but also to differences in psoralen photoreactivity with thymine in DNA and uracil in RNA together with different structures of the hybrids at the site of psoralen intercalation. RNA has been shown to react to a lesser extent than DNA with different psoralens (23). Recently Kean and Miller compared the reactivity of 29mer RNA and DNA fragments expected to form hairpin structures (consisting of a 7-base pair stem and a 15-base loop) and four different complementary psoralen-derivatized oligomethylphosphonates. They have shown that although significant levels of cross-linking were achieved with all four oligomers with the DNA hairpin target, very little cross-linking was obtained with the RNA hairpin (24). In contrast, two of the oligomers cross-linked to the same extent to linear RNA and DNA targets (24). However, it is not sufficient to target the oligonucleotide to a single-stranded region within an RNA in order to increase its binding and therefore its ‘reactivity’. For example Lima et al have shown that a decarboxyribonucleotide targeted to the 5′-side of a 16-nucleotide loop of a 47-mer hairpin target had an association constant of approximately 3 × 1010 M⁻¹ whereas a decarboxyribonucleotide targeted to the 3′-side of the loop had an association constant of less than 1 × 10⁸ M⁻¹. In contrast, Kean and Miller have shown that psoralen-derivatized octamers formed more stable complexes when targeted to the 3′-side than to the 5′-side of a 15-nucleotide loop in a 29mer hairpin target. Teare and Wollenzien have described efficient photochemical addition of oligonucleotides linked to a psoralen moiety by making
DNA/RNA heteroduplexes containing unpaired RNA nucleotides in order to facilitate intercalation of psoralen (25). Only weak photoaddition reactions were observed with a target containing no extra-base, i.e., fully complementary to the psoralen-substituted oligonucleotides.

In the case of 1,10 phenanthroline-derivatized oligonucleotides, our results suggest that the stability of the RNA hairpin structure is the major parameter which influences the cleavage reaction since the OP-oligonucleotide failed to compete efficiently with intramolecular RNA/RNA interactions as shown in gel shift experiments. Chen and Sigman have shown that, after denaturation and annealing, a 21-base long OP-oligonucleotide induced efficient cleavage of an 81 base-long RNA and a 187 base-long DNA fragment containing the same target sequence (16). Recently Chen et al. have shown that the RNA target could be efficiently cleaved by an heptamer linked to 1,10 phenanthroline without denaturation (26). This short oligonucleotide was directed to a single-stranded loop of the lac operon RNA fragment.

Having found with 27RNA or 27DNA targets that reaction efficiency was dependent on RNA secondary structure, we addressed the question of reactivity of these compounds towards longer RNAs. For this purpose, we used 120 and 820 nucleotide-long in vitro Ha-ras transcripts. Neither OP-derivatized oligonucleotides, nor Pso-derivatized oligonucleotides reacted with these substrates. Maher and Dolnick have shown that the efficiency of translation arrest could be increased if two oligonucleotides were targeted to tandem binding sites (27). Contiguously bound oligonucleotides have also been shown to increase the stability of Oligo/RNA duplexes and to enhance translation inhibition by ellipticine-derivatized oligonucleotides (28). Reynolds et al. obtained efficient photoadducts with AMT-derivatized 19mer methylphosphonate oligonucleotides targeted to a 440 base-long bcr/abl RNA target, provided they used two additional tandem oligonucleotides to induce the reaction (29). In our case, addition of a 'helper' oligonucleotide, which binds to the substrate at a site adjacent to the active dodecamers, was required to enhance the chemical and photochemical activity of dodecamers. Since helper chain length greater than 13 improved dodecamers reactivity, this suggests that long-enough oligonucleotides are needed to 'melt' the secondary structure of Ha-ras RNA and consequently give rise to target modification after oligonucleotide binding. We failed to induce modifications of Ha-ras mRNA using a 17mer oligonucleotide linked to psoralen. These results show that there is a minimum oligonucleotide length requirement to unfold Ha-ras mRNA secondary structure. A β-globin 589 base-long mRNA was cleaved by a phenanthroline-derivatized 17mer with a good efficiency. The difference in RNA structure around the target site may explain the difference in reactivity of Ha-ras and β-globin mRNA (22, 30).

Using the same experimental conditions, we have shown that all derivatized β-oligoodeoxyxynucleotides induced RNase H cleavage of Ha-ras mRNA, even though they were not able to cross-link (Pso) or cleave (OP) their target in the absence of RNase H. Ribonuclease H, which binds and cleaves the RNA moiety of RNA/oligodeoxynucleotide hybrids, is expected to shift the equilibrium towards hybrid formation and to make the reaction irreversible. Altogether, these results suggest that mRNA secondary and tertiary structures are a limiting step in the design of efficient antisense oligonucleotides in the absence of RNase H activity. In addition, the difference in chemical or photochemical reactivity of RNA versus DNA substrates, together with the local structures of the hybrids at the reactive site, must be taken into account when designing activatable oligonucleotides.

REFERENCES