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Target-specific arrest of mRNA translation by antisense 2′-O-alkyloligoribonucleotides

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ABSTRACT

We describe a novel experimental approach to investigate mRNA translation. Antisense 2′-O-allyl oligoribonucleotides (oligos) efficiently arrest translation of targeted mRNAs in rabbit reticulocyte lysate and wheat germ extract while displaying minimal non-specific effects on translation. Oligo/mRNA-hybrids positioned anywhere within the 5′ UTR or the first ~20 nucleotides of the open reading frame block cap-dependent translation initiation with high specificity. The thermodynamic stability of hybrids between 2′-O-alkyl oligos and RNA permits translational inhibition with oligos as short as 10 nucleotides. This inhibition is independent of RNase H cleavage or modifications which render the mRNA untranslatable. We show that 2′-O-alkyl oligos can also be employed to interfere with cap-independent internal initiation of translation and to arrest translation elongation. The latter is accomplished by UV-crosslinking of psoralen-tagged 2′-O-methyloligoribonucleotides to the mRNA within the open reading frame. The utility of 2′-O-alkyloligoribonucleotides to arrest translation from defined positions within an mRNA provides new approaches to investigate mRNA translation.

INTRODUCTION

Eukaryotic translation initiation is a multi-step process which requires a large number of protein factors and leads to the ordered assembly of both ribosomal subunits to form an 80S ribosome on the mRNA template [for recent reviews see (1–5)]. The 5′ m^7GpppN cap-structure of an mRNA is viewed to be first bound by eukaryotic translation initiation factor (eIF) 4F, followed by ATP-dependent unwinding of secondary structures within the 5′ untranslated region (UTR) of the mRNA. The 43S pre-initiation complex (including the 40S small ribosomal subunit as well as eIF-2 with Met-$tRNA_i$ and GTP) is thought to bind subsequently to the mRNA and 'scan' towards the AUG initiation codon (6,7). Joining of the 60S large ribosomal subunit at the initiation codon constitutes the last step of the initiation process leading to formation of the 80S ribosome. An alternative pathway of translation initiation bypasses the requirement for the cap-structure and allows the internal binding of the ribosome to a downstream region within the 5′ UTR of an mRNA. This mechanism was first discovered in picornaviral RNAs (8,9), and has recently been extended to a small group of cellular mRNAs [reviewed in (10,11)]. A complex secondary structure within the 5′ UTR, the internal ribosome entry site (IRES), is necessary and sufficient to confer internal initiation (12,13).

While antibiotics can affect translation globally, suitable techniques to arrest translation of specific mRNA templates are currently lacking, although availability of such tools could prove valuable to study the translation process itself, protein folding or the organellar import of a secretory polypeptide emerging from the ribosome. Antisense oligonucleotides, in particular phosphorothioate DNA, have been used to perturb gene expression in cultured cells and *in vitro* [reviewed in (14)]. Inhibition of gene expression frequently relies on RNase H, which cleaves targeted nuclear or cytoplasmic RNAs (15–17). In this report, we have investigated the utility of antisense 2′-O-alkyl oligoribonucleotides to study aspects of cap-dependent and cap-independent translation initiation and translation elongation *in vitro*. The chemical modification of the 2′ OH group stabilizes these 2′-O-alkyl oligoribonucleotides against degradation, and may also distinguish them sufficiently from RNA to prevent their removal by RNA helicases involved in translation.

MATERIALS AND METHODS

Materials

Restriction enzymes and RNase T1 were purchased from Boehringer Mannheim (Mannheim, Germany). Protein and nucleic acid molecular weight markers and RNase T2 were supplied by Gibco-BRL (Bethesda, MD, USA). All other chemicals were from Merck (Darmstadt, Germany). The plasmids used in this study have all been described previously [pI.19CAT: (18), pGEM4PPL (19) pGEM U1A 102/103: (20), pSKCAT and pKSRClaCAT: (21, 22)].

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Generation of RNA for translation *in vitro*

The mRNAs encoding chloramphenicol acetyltransferase (CAT), pre-prolactin (PPL) and the spliceosomal U1A protein were transcribed from linearized plasmids (I.19CAT: *Hin*dIII; PPL: *Sal*I or *Eco*RI; U1A: *Hin*dIII; SKCAT: *Bam*HI; KSRClaCAT: *Xho*I) (see also Fig. 1) in standard reactions and purified as described previously (18). The efficiency of capping was > 95%. Transcription reactions for ^{32}P-labeled mRNAs contained 0.5 mM UTP and 150 kBq/μl (4 μCi/μl) (5 μM) α[^{32}P]-UTP (Amersham Int., Amersham, UK). The concentration of ^{32}P-labeled mRNAs was determined using a Beckman LS 6000SC scintillation counter.

Oligonucleotides

2′-O-Methyl and 2′-O-allyl oligoribonucleotides as well as the DNA−oligonucleotide (Table I) were synthesized on Applied Biosystems DNA/RNA synthesizers 380A/B or 394, and purified by HPLC (23) or by the 'crush and soak' method after electrophoretic separation on denaturing polyacrylamide gels (24). Oligos were annealed to mRNAs in 20 mM Hepes−K (pH 7.6) and 100 mM KCl for 5 min at 65°C and 20 min at room temperature with a 500-fold molar excess of oligo over mRNA, unless stated otherwise. This annealing mixture was kept on ice before addition to the translation reaction. After annealing, psoralen-containing oligos (see note to Table 1) were crosslinked to the mRNA for 10 min at 4°C using a HRI-100 UV photochemical reaction chamber from ULTRA-LUM, Inc. (Carson, CA, USA).

Cell-free translations and RNA-analysis

Cell-free translations were performed as described (25) in the presence of 18.5 kBq/μl (0.5 μCi/μl) [^{35}S]methionine (Amersham Int.), 0.02 U/μl Inhibit-ACE (5Prime-3Prime Inc., Boulder, CO, USA), and 25 ng/μl *E.coli* rRNA (Sigma Chemie, Deisenhofen, Germany) (apart from in the experiment depicted in Fig. 8). Mixtures of capped *in vitro* transcribed U1A, PPL or CAT mRNAs annealed to the indicated 2′-O-alkyloligoribonucleotides were added to nuclease-treated rabbit reticulocyte lysate (RRL) (Promega, Madison, WI, USA) which was adjusted to 135 mM K^+ and 1.5 mM Mg^{2+} except in the experiment shown in Figure 7, where the concentrations were 100 mM K^+, 0.8 mM Mg^{2+}. Translation-competent extract from wheat germ (General Mills Inc., Valle Jo, CA, USA) (WGE) was prepared and used as in (18) at 120 mM K^+ and 1.9 mM Mg^{2+}. All samples were maintained on ice before incubation for 1 h at 30°C (RRL) or 25°C (WGE). After translation, an equal volume of buffer (2% SDS, 10% 2-mercaptoethanol, 100 mM Tris−Cl pH 6.8, 20% glycerol and bromophenol blue) was added and the samples were heated to 95°C for 5 min. The [^{35}S]-labeled translation products were analyzed by electrophoretic separation on 14% SDS−polyacrylamide gels and fluorography. Translation products were quantified by phosphorimaging and subsequent processing using the Image Quant-package from the manufacturer (Molecular Dynamics, Krefeld, Germany).

For RNA analysis after oligo-mediated inhibition of translation, 5 μg *E.coli* tRNA (Sigma) was added to a 14 μl aliquot of the 36 μl translation reaction and adjusted to 200 μl with H_2O. RNA was isolated by two phenol extractions and one extraction with $CHCl_3$/isoamyl alcohol (24:1) followed by precipitation with 3 volumes of ethanol. After centrifugation, the pellets were washed once with 80% ice-cold ethanol, air-dried and resuspended in 100% dye-containing formamide. Half of the sample was heated to 65°C for 5 min, chilled on ice, and electrophoresed through 1.5 % agarose gels in 0.5×TBE. Blotting onto nylon filters and subsequent probing with α[^{32}P]-dCTP labeled probes, containing the entire sequence of the transcribed mRNAs, was as described previously (26). For re-translation, RNA was extracted from 16 μl of the translation reactions as described above, except that the RNA pellets were resuspended in 100 μl of 20 mM Hepes−K pH 7.6, 1 mM EDTA and 8 M urea. The annealed 2′-O-allyl oligos were removed from the mRNA by heating to 65°C for 5 min, snap-cooling on ice for 1 min, and size separation using fine grade Sephadex-G100 (Pharmacia, Uppsala, Sweden) spin columns (24) equilibrated in the same buffer. The eluted RNA was precipitated after the addition of 1/20 volume 3 M K-acetate, pH 5.2,L ılLwith 3 volumes of ethanol. The RNA was washed and dried, resuspended in annealing buffer (see above), and re-translated in RRL (without the addition of *E.coli* rRNA) as described earlier.

RESULTS

Binding of 2′-O-allyloligoribonucleotides to any position within the 5′ UTR or just downstream of the AUG initiation codon specifically arrests cap-dependent translation

To evaluate 2′-O-allyloligoribonucleotides (oligos) as inhibitors of translation initiation, antisense oligos with complementarity to different parts of *in vitro* transcribed reporter mRNAs were synthesized. These oligos, 14−16 nts in length (Table 1), are designated with a 'C' for oligos with complementarity with the

Table I. Oligonucleotides used in this study

Oligo	Sequence
	I.19CAT
CU1	aAsGsCsUsCsGsAsAsUsUsCsGsCsCsC
CU1z	aZsGsCsUsCsGsZsZsUsUsCsGsCsCsC
C15	aGsAsUsCsCsCsCsGsGsGsUsAsCsCsGsA
C15z	aGsZsUsCsCsCsCsGsGsGsUsZsCsCsGsA
C16a	aZsUsCsCsCsCsGsGsGsUsZsCsCsG
C16b	aCsCsCsCsGsGsGsUsZsCsCsG
C16c	aCsCsGsGsGsUsZsCsCsG
C16d	aGsGsGsUsZsCsCsG
C32	aAsGsCsAsCsUsUsUsGsAsAsGsCsA
C47	aCsUsAsGsAsGsGsAsUsCsCsGsUsCsC
C95	aUsUsCsUsCsCsAsUsUsUsUsAsGsCsU
C95d	dTdTdCdTdCdCdAdTdTdTdTdAdGdCdT
C103	aGsAsUsUsUsUsUsUsUsCsUsCsCsAsU
C111	aUsAsUsCsCsAsGsUsGsAsUsUsUsUsU
C131	aZsZsCsGsGsUsGsUsZsUsZsUsCsC
C140	aGsGsAsUsZsUsZsUsCsZsZsCsGsGsU
C192	dTdC*dC*dC*mGmGmUmCmUmGmGmUmUmAmUmAmGmGmUmAdC$mA
C468	dTdAsAsCsAsCsGsCsCsAsCsAsUsCsU
C471	dTdC*dC*dC*mCmAmCmCmGmUmAmAmCmAmCmGmCmCmAdC$mA
C553	aAsCsUsGsGsUsGsAsAsAsCsUsCsAsC
C808	aAsUsCsAsCsUsUsAsUsUsCsAsGsGsC
X	aCsUsAsCsAsCsGsUsCsUsAsCsCsA
	pre-prolactin (PPL)
P15	aGsAsZsZsZsAsGsAsZsCsZsZsGsCsAsA
P61	aCsUsGsUsCsCsZsUsGsGsUsZsUsCsU
P86	aGsGsAsCsCsCsUsUsUsCsUsGsCsGsA
	U1A
CU1	aAsGsCsUsCsGsAsAsUsUsCsGsCsCsC
CU1z	aZsGsCsUsCsGsZsZsUsUsCsGsCsCsC
U47	aCsUsUsUsGsUsCsCsCsAsAsCsGsUsC
	CAT/IRES-CAT
C47	aCsUsUsUsGsUsCsCsCsAsAsCsGsUsC
C1b	aGsGsUsAsCsCsCsAsAsUsUsCsGsCsCsC
I-C1	aGsAsGsCsUsCsCsAsAsUsUsCsGsCsCsC

The sequence of the oligos is given in 5′ to 3′ direction. Abbreviations: dN: deoxyribonucleotide; aN: 2′-O-allylribonucleotide; mN: 2′-O-methylribonucleotide; Z: 2,6-diaminopurine riboside; I: inosine; dC$: 2′-deoxycytidine carrying a short spacer arm terminating in a 4′-aminomethyl-4,5′,8-trimethylpsoralen moiety (G.S.S., manuscript in preparation); dC*: biotinylated 2′-deoxycytidine (59).

CAT mRNA, a 'P' for oligos complementary to pre-prolactin mRNA, and a 'U' for antisense U1A oligos. The number identifies the 5′ most nucleotide in the target mRNA to which the oligo hybridizes. For example, C103 is an oligo which hybridizes to nucleotides 103−117 of CAT mRNA (Fig. 1). The reporter mRNAs possess relatively unstructured 5′ UTRs of comparable lengths, are efficiently capped and non-polyadenylated. 2′-O-Allyl oligos hybridized to their target mRNAs with >95% efficiency, except for oligos C32 and C47 (see below, Table I and Figure 1). The annealing, which was carried out in the presence of both the test mRNA and the control mRNA(s), was specific for the targeted transcripts, as determined by native gel electrophoresis of ^{32}P-labeled mRNAs (data not shown).

When 2′-O-allyl oligos were annealed to different parts of the 5′ UTR of I.19CAT mRNA in the presence of human pre-prolactin (PPL) and U1A mRNAs as controls, translation in reticulocyte lysate (RRL) (Fig. 2A) or wheat germ extract (WGE) (Fig. 2B) showed specific and efficient inhibition of CAT-translation (lanes 7−12). While oligos CU1, CU1z, C15 and C15z repressed translation by >80%, the lower inhibitory effect of oligos C32 and C47 (46% and 35% in RRL, lanes 11 and 12) correlates with their lower annealing efficiency (data not shown). The lack of efficient hybridization of oligos C32 and C47 may originate from the formation of intramolecular mRNA structures, which could impair oligo binding. Mock-annealing of an oligo without complementarity to any of the mRNAs ('non-specific oligo') did not affect the translation of either transcript (lanes 2−6 and 13). The partial repression of U1A translation in lanes 7 and 8 is specific, because oligos CU1 and CU1z are also complementary to the 10 first nucleotides of the U1A transcript, and a hybrid of this length suffices to partially inhibit translation in RRL (see below).

When oligo/mRNA-hybrids form around the AUG-initiator codon, CAT translation from this codon is similarly inhibited in both RRL (Fig. 3A) and WGE (Fig. 3B) (lanes 8−10). In contrast, hybridization of oligos to positions >19 nucleotides downstream of the AUG (in the ORF or in the 3′ UTR), does not affect translation of CAT or control mRNAs (lanes 11 and 13−15). Comparison of the results obtained in WGE (Fig. 3B) with those in RRL (Fig. 3A) also reveals that slightly larger translation products than the normal CAT polypeptide are more apparent in WGE with oligos annealed around the AUG codon (compare lanes 8−10 with lanes 5 and 12). These polypeptides appear to initiate at in-frame upstream non-AUG codons (data not shown). The concentrations of oligos used in the translation reactions (0.5−1.25 μM) are approximately two orders of magnitude below the threshold for non-specific inhibition of translation in either RRL or WGE (>50 μM, data not shown).

We next examined alternative target mRNAs for antisense-mediated repression of translation. 2′-O-Allyl oligos were annealed to the U1A and PPL mRNAs at the positions indicated in Figures 1 and 4, and assayed for translational inhibition in RRL. The results for the oligos hybridized to the 5′ UTR (Fig. 4A) show that the translation of U1A (lanes 9 and 13) and PPL mRNAs (lane 11) also is efficiently and specifically repressed. Oligo U47 was designed as an oligo that binds to the same position in U1A mRNA as the C47 oligo in CAT mRNA. In contrast to C47 and C32, U47 efficiently hybridizes to its target mRNA (data not shown) and inhibits its translation (lane 13), demonstrating the susceptibility of the middle portion of the 5′ UTR to oligo-mediated inhibition. The same pattern of inhibition

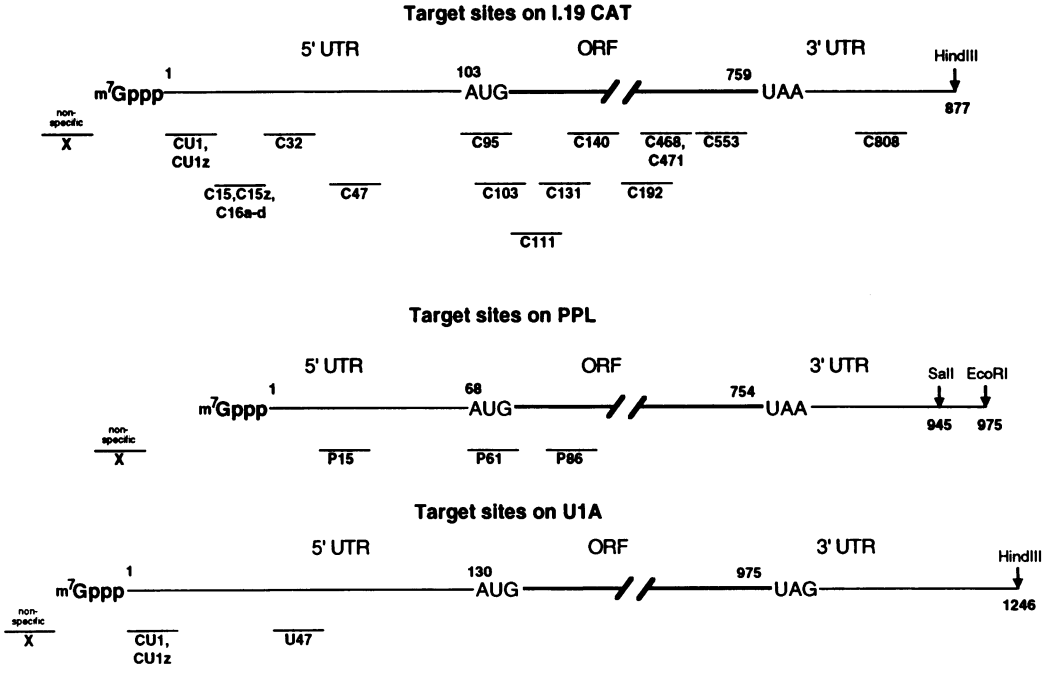

Figure 1. Diagram of the reporter mRNAs used in this study. The open reading frames encoding the human spliceosomal protein U1A, human pre-prolactin (PPL) or chloramphenicol acetyltransferase (CAT) are denoted by thick bars. All three *in vitro* transcribed capped mRNAs possess relatively unstructured 5′ UTRs of similar length to minimize differences in translatability. The target positions of the 14−16-mer 2′-O-allyloligoribonucleotides are indicated. Numbers refer to the position of the 5′ end of the antisense oligo/mRNA hybrid with reference to the distance from the m^{7}GpppG cap.

was observed in WGE (data not shown). Furthermore, 2′-O-allyl oligos targeted to the AUG region of PPL mRNA inhibit translation in RRL (Fig. 4B, lanes 10 and 11) and in WGE (data not shown). The U1A mRNA is also translationally repressed by oligos targeted to the AUG-region, whereas hydrogen-bonded oligos in the ORF or the 3′ UTR do not affect translation of either of the U1A or PPL mRNAs (data not shown).

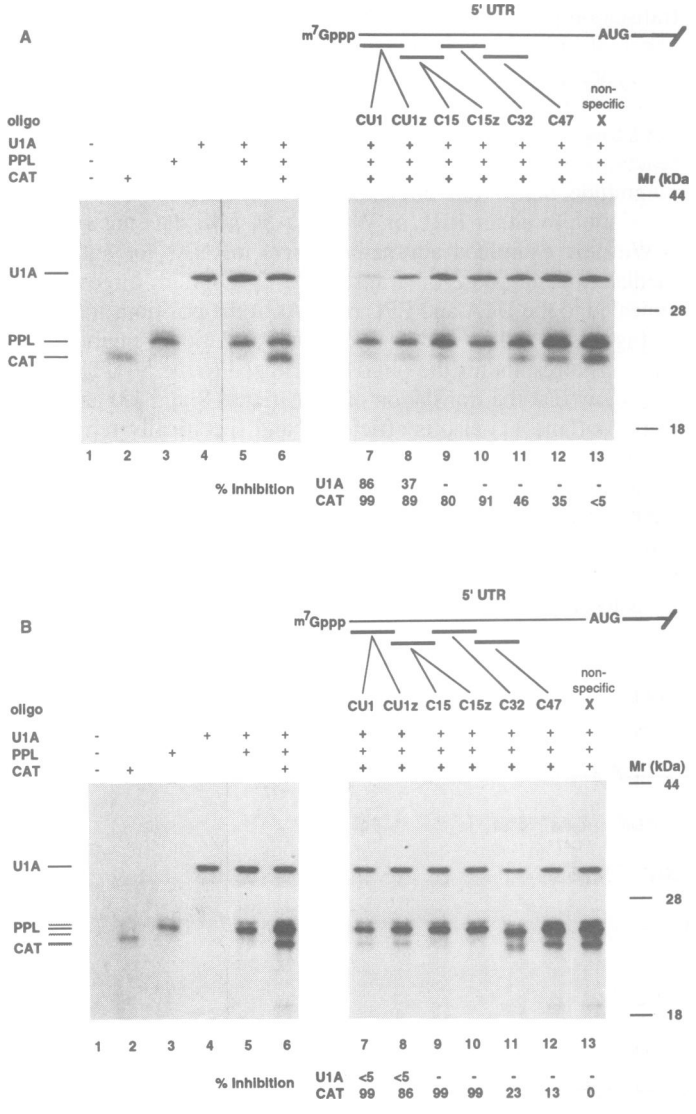

Figure 2. Inhibition of translation of I.19 CAT mRNA by 2′-O-allyl oligos annealed to the 5′ UTR. The translation products from the U1A, PPL and I.19CAT mRNAs are indicated on the left and the position of M_r markers on the right. 2′-O-Allyl oligos (Table I), either complementary to the I.19CAT-mRNA (lanes 7–12) or non-complementary (lane 13), were annealed in 500-fold molar excess to 10 fmol U1A, 20 fmol PPL or 20 fmol I.19CAT mRNAs, or mock-annealed (lanes 2–6), followed by translation in RRL (**A**) or WGE (**B**) in 12 μl reactions. Analysis of the ^{35}S-labeled products by SDS–PAGE and fluorography was as described in 'Materials and Methods'. The translational efficiency was quantified by phosphorimaging. The degree of inhibition is expressed as the percentage of translation of the targeted mRNA in comparison to the internal control mRNA (PPL). The ratio of translation of CAT and U1A to PPL in lane 6 (no oligo present) was defined as 0% inhibition, while 100% inhibition was defined as the background in lanes 3 (U1A) and 5 (CAT).

Arrest of translation by 2′-O-allyloligoribonucleotides does not affect the structural and functional integrity of the targeted mRNAs

Inhibition of translation by antisense DNA in both RRL and WGE commonly results from mRNA cleavage by RNase H (15–17). To study translation initiation and 'trap' translation intermediates for further analysis, the mRNA must remain intact.

The translationally inhibited mRNA was first examined by Northern blotting. Figure 5A demonstrates that the mRNAs remain intact, whether translationally inhibited by 2′-O-allyl oligos (lanes 6, 8 and 9) or not (lanes 2–5 and 10). In contrast, the DNA-oligo C95d (Table I) which is identical in sequence to the 2′-O-allyl oligo C95 (Table I), induces complete cleavage of the CAT mRNA (lanes 7). Thus, RNase H or other ribonucleases are not involved in the translational inhibition caused by 2′-O-allyl oligos, in spite of sufficient RNase-H activity to completely cleave the DNA oligo-targeted mRNA. In WGE, 2′-O-allyl oligo-mediated translational arrest is also independent

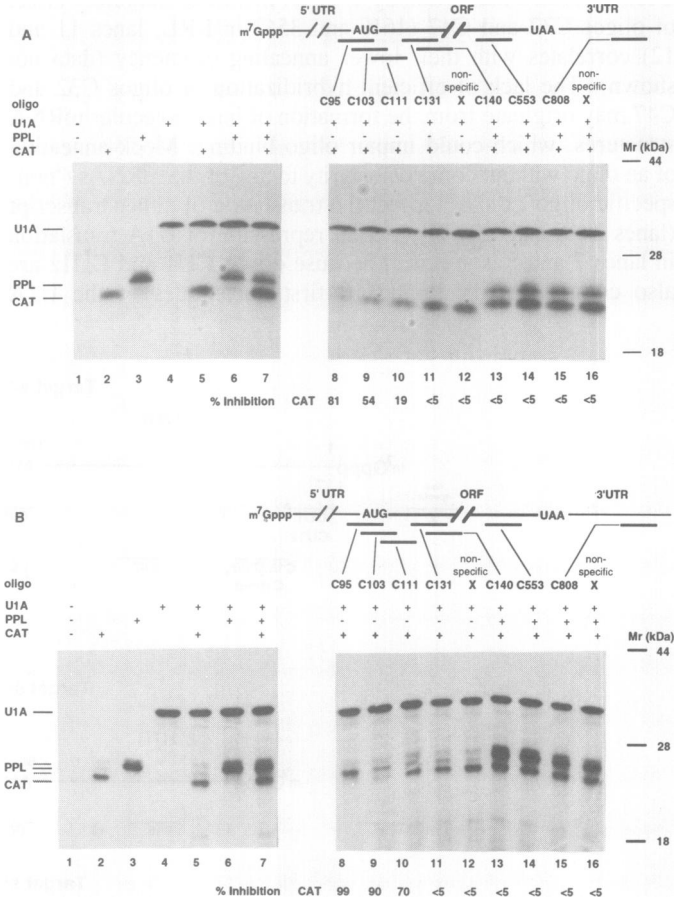

Figure 3. Effect of 2′-O-allyl oligos annealed over the AUG initiation codon, within the open reading frame (ORF) or to the 3′ UTR on inhibition of I.19CAT mRNA translation. The translation products from the U1A, PPL and CAT mRNAs are indicated on the left and the position of M_r markers on the right. Thick bars symbolize 2′-O-allyl oligos (Table I), either complementary to the CAT-mRNA (lanes 8–11 and 13–15) or non-complementary (lanes 12 and 16) were annealed and translated in RRL (**A**) or WGE (**B**). The AUG initiation codon is positioned at 103–105. Translational inhibition is expressed using U1A as an internal control as described in Figure 2.

of mRNA cleavage (data not shown). We next assessed the functional integrity of the inhibited mRNAs. After the inhibition assay, the oligo was removed from the mRNA by heat denaturation and passage through a size-exclusion column in a denaturing buffer. Re-translation of the mRNAs demonstrates complete recovery of translational efficiency of CAT (Fig. 5B, lanes 3), PPL (Fig. 5B, lanes 6) and U1A (Fig. 5B, lanes 9) mRNAs. The same results were obtained when the oligo/mRNA-hybrids were assayed in WGE (data not shown). Antisense 2′-O-allyl oligos thus appear to arrest translation by physical blockage independent of cleavage or modification of the targeted mRNAs.

Length requirements for antisense 2′-O-allyloligoribonucleotides

The 2′-O-allyl modification confers enhanced thermodynamic stability to oligo/mRNA hybrids when compared to other commonly used types of antisense oligos (27). The substitution of adenine by 2,6-diaminopurine further increases the T_m of oligos hybridized to U-rich stretches of target RNAs (28). We thus assessed the minimal length of an antisense 2′-O-allyl oligo required for efficient translational inhibition. A non-complementary oligo or serially shortened complementary oligos were annealed to a site 15 nucleotides downstream from the cap-structure of I.19CAT mRNA (Fig. 6, lanes 3–8) in the presence of U1A mRNA as a control. Translation in reticulocyte lysate was almost completely inhibited (96–99%) with oligos of 16 or 14 nucleotides in length (Fig. 6, lanes 3–5). Oligo/mRNA hybrids of 12 or 10 base pairs still allowed efficient albeit reduced repression (80%) of CAT mRNA translation (Fig. 6, lanes 6–7), and an oligo of only 8 nucleotides still inhibited CAT expression by 46% (lane 8).

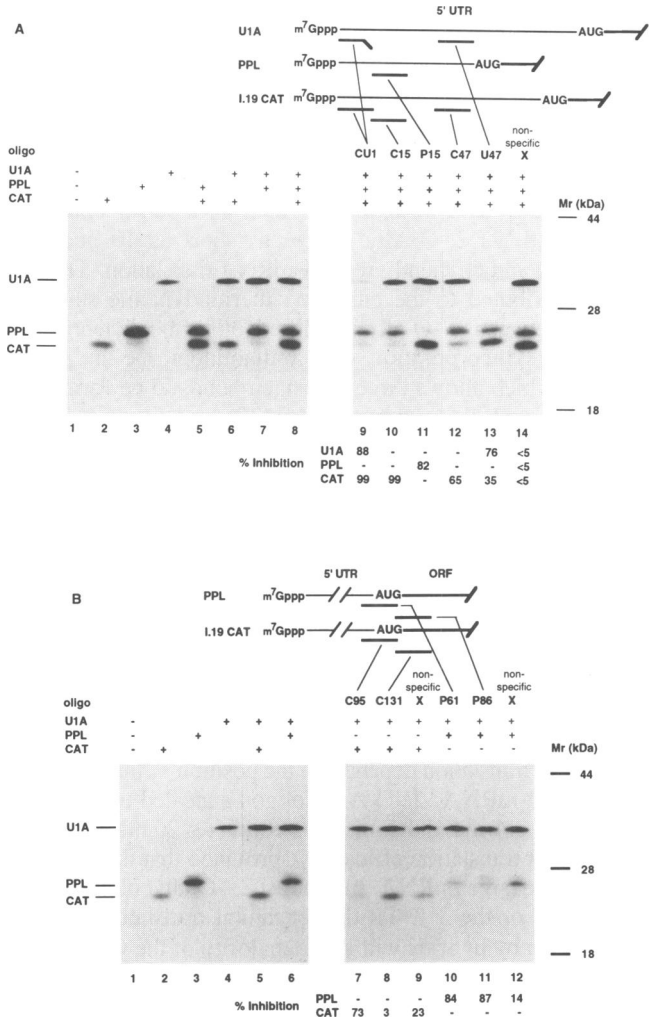

Figure 4. Inhibition of PPL and U1A mRNA translation by 2′-O-allyl oligos annealed to different positions in the 5′ UTR (**A**) or over the AUG initiation codon (**B**). The translation products from the U1A, PPL and CAT mRNAs are indicated on the left and the position of M_r markers on the right. (A): 2′-O-allyl oligos (Table I), either complementary to U1A mRNA, PPL-mRNA, CAT-mRNA or non-complementary were annealed and translated in RRL. The extent of translational inhibition is expressed as in Figure 2. The 35% inhibition of CAT translation by oligo U47 was not reproducible in several repeat experiments.

Effect of 2′-O-allyloligoribonucleotides on internal initiation of translation

An mRNA translated by a cap-dependent mechanism is sensitive to antisense inhibition by oligo/mRNA-hybrids formed close to the cap-structure (Figs 2 and 4A) or over the AUG initiation codon (Figs 3 and 4B). An mRNA translated by an internal initiation mechanism would, however, be expected to be unaffected by an oligo hybridized to the cap-proximal region. Two sets of antisense 2′-O-allyl oligos, 16 and 15 nucleotides in length, were annealed to CAT mRNAs, one similar to I.19CAT and the other harboring the internal ribosome entry site (IRES) from foot-and-mouth disease virus (IRES-CAT) (Fig. 7A), which allows internal initiation of translation in reticulocyte lysate (21,22). Both CAT mRNAs are identical for the eleven 5′ most nucleotides and have the same G–C content for the following five nucleotides. Therefore, the cap-proximal oligos C1b and I-C1 (Table I and Fig. 7A) form hybrids of virtually identical stability with their respective target mRNAs. The oligos were designed to form hybrids with the IRES-CAT mRNA outside of the IRES sequence, and should therefore not interfere with the function of this element *per se*.

The 2′-O-allyl oligo over the AUG-initiation codon efficiently blocks translation of both CAT mRNAs (Fig. 7B, lanes 4 and 8). The cap-proximal oligos interfere with cap-dependent translation, but not internal initiation (Fig. 3B, compare lanes 3 and 7). The lack of inhibition of IRES-CAT translation by the cap-proximal oligo (lane 7) cannot be attributed to inefficient hybridization, as assessed by analysis of ^{32}P-labeled mRNAs on native gels after annealing with complementary or non-complementary 2′-O-allyl oligos (data not shown).

2′-O-methyloligoribonucleotides cross-linked to the ORF inhibit translation

As shown in Figure 3, 2′-O-alkyl oligos annealed to the middle region of the ORF fail to arrest translation. We finally assessed the effect of cross-linking oligos to two different regions within the ORF of the I.19CAT mRNA. Antisense 2′-O-allyl and 2′-O-methyl (2′-O-Me) oligos were annealed, and the 2′-O-methyl oligos UV-crosslinked via their psoralen tags. Psoralen-tagged oligos were made and purified in a similar fashion to the previously described psoralen-modified oligodeoxyribonucleotides (29). Subsequently, the oligo/mRNA hybrids were co-translated with U1A mRNA as a control in reticulocyte lysate. UV-irradiation of the CAT mRNA without oligo only marginally affected its translation (Fig. 8, lanes 3 and 4). Annealing of the

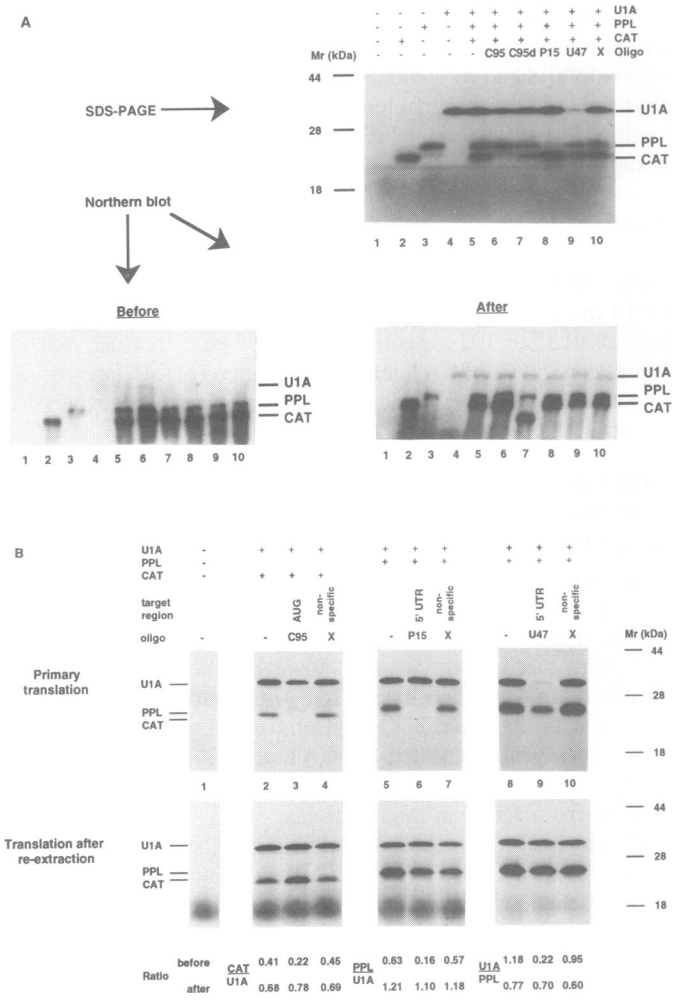

Figure 5. (**A**) Translational inhibition conferred by 2′-O-allyl oligoribonucleotides is independent of mRNA cleavage. 60 fmol CAT mRNA, 45 fmol PPL mRNA and/or 30 fmol U1A mRNA were annealed to the indicated 2′-O-allyl oligos (lanes 6, 8–10) or DNA-oligo (lane 7) and translated in reticulocyte lysate in 36 μl reactions. Translation products are indicated on the right and the position of M_r markers on the left. The integrity of the mRNAs before and after translation was assessed by Northern analysis as described in 'Materials and Methods'. The positions for the CAT, PPL and U1A mRNAs are indicated on the right. (**B**) Translational inhibition induced by 2′-O-allyl oligo-ribonucleotides is reversible. Upper panel: 80 fmol CAT mRNA and 40 fmol U1A mRNA (lanes 2–4), 80 fmol PPL mRNA and 40 fmol U1A mRNA (lanes 5–7) or 40 fmol U1A mRNA and 80 fmol PPL mRNA (lanes 8–10) were annealed to the indicated oligos, followed by translation in RRL in 24 μl reactions. Lower panel: RNA was re-extracted and re-translated as described in 'Materials and Methods'. Inhibition of translation is expressed as the ratio between the indicated translation products at the bottom of the figure.

2′-O-methyl oligo with psoralen (lanes 5 and 9), or a 2′-O-allyl oligo without psoralen (lane 7) does not inhibit translation. However, cross-linking of the psoralen-containing oligos to the mRNA (>95% as determined by denaturing gel-electrophoresis of the labeled mRNAs before and after UV-irradiation, data not shown) efficiently inhibits translation (lanes 6 and 10). Thus, elongation seems to be unaffected by solely hydrogen-bonded oligos within the ORF, whereas ribosomal translocation along the mRNA appears to be arrested by the covalently attached oligo. We attempted unsuccessfully to identify shorter translation products resulting from arrest of elongation. It is possible that ribosomal arrest within the ORF limits translation to one round per transcript, which would not allow sufficient ^{35}S-methionine incorporation for detection by fluorography. Moreover, the ribosome may release the peptide bound to the last tRNA, and the released peptide could be unstable in reticulocyte lysate (30,31) and therefore be undetectable.

DISCUSSION

Cell-free translation experiments have been used to evaluate different types of oligonucleotides for their properties as antisense reagents (14). In this report, we describe the properties of 2′-O-alkyl oligoribonucleotides as novel antisense probes to study mRNA translation *in vitro*. Our experiments establish the following features of 2′-O-alkyl oligoribonucleotides: 1) Non-specific effects of 2′-O-alkyl oligos on mRNA translation are minimal. The lack of non-specific effects in part results from diminished binding of proteins to the oligos, which is conferred by the 2′-O-alkyl modifications (23). This contrasts with some other nuclease-resistant oligos, e.g. those containing sulfur modifications (32). Furthermore, activation of protein kinase R (PKR) by the oligo/mRNA hybrids leading to inhibition of translation by phosphorylation of the α-subunit of eIF-2 (33) can be excluded, as translation of internal control mRNAs is unperturbed. 2) 2′-O-Allyl oligos as short as 10 nucleotides specifically and efficiently repress mRNA translation. This result can be attributed to the enhanced thermodynamic stability of hybrids between 2′-O-allyl-modified oligoribonucleotides and RNA (27). Incorporation of 2,6-diaminopurine in place of adenine, which allows three hydrogen bonds to be formed with uracil in the mRNA target sequence (28), provides an additional possibility to raise the T_m of the oligo/mRNA hybrid (compare oligo CU1 to CU1z and C15 to C15z in Fig. 2) and allows U-rich stretches to be targeted. 3) Translationally arrested mRNAs remain structurally and functionally intact. In contrast to DNA and several other types of oligos, inhibition by antisense 2′-O-allyl oligos is independent of cleavage by RNase H or other nucleases (Fig. 5A). The functional integrity of the mRNA is not affected during translational arrest [as assessed by re-translation of the mRNA following oligo removal (Fig. 5B)], and oligo/mRNA hybrids thus do not appear to be substrates for dsRNA-helicase/deaminases (34,35).

Arrest of translation depends on the position of the oligo within the targeted mRNA. 2′-O-Allyl oligos annealed to any part of the 5′ UTR or around the initiation codon (≤20 nts downstream of it) inhibit translation efficiently, provided that they hybridize well with the target mRNA. In contrast, 2′-O-allyl oligos annealed to the ORF or the 3′ UTR do not inhibit translation. The lack of inhibition by hybrids within the majority of the ORF is likely due to removal of the oligo by the elongating ribosome (see below), whereas an oligo bound downstream of the termination signal may remain bound. Position dependence has also been observed for other types of antisense probes, such as α-DNA (36), DNA oligos or cDNA (37–45). The capacity of 2′-O-allyl oligos hybridized to the 5′ UTR to arrest translation is in good agreement with the effects seen by intramolecular mRNA helices, and may have implications for the mechanism(s) by which they interfere with the translation initiation pathway. When located close to the cap structure, intramolecular hairpins affect early steps in the initiation pathway and may preclude the stable association of the 43S pre-initiation complex with the mRNA

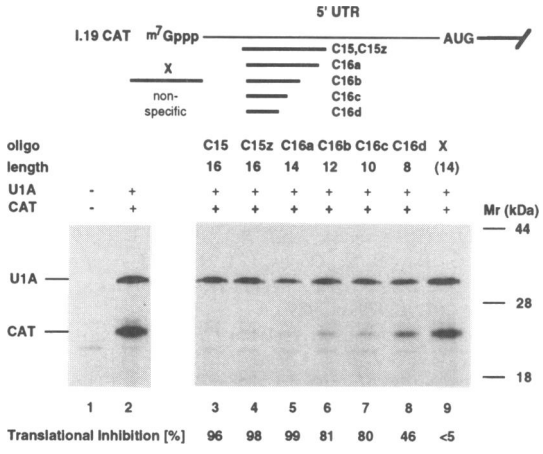

Figure 6. Length requirement for translational arrest by 2′-O-allyl oligoribonucleotides. 2′-O-Allyl oligos, either complementary to the CAT-mRNA (lanes 3–8) or non-complementary (lane 9) were annealed in 400-fold molar excess to a mixture of 15 fmol U1A and 30 fmol I.19CAT mRNAs or mock-annealed (lane 2), followed by translation in reticulocyte lysate in 12 μl reactions. The translation products are indicated on the left and the position of M_r markers on the right. The extent of translational inhibition was calculated and expressed as the percentage of inhibition in comparison to lane 2.

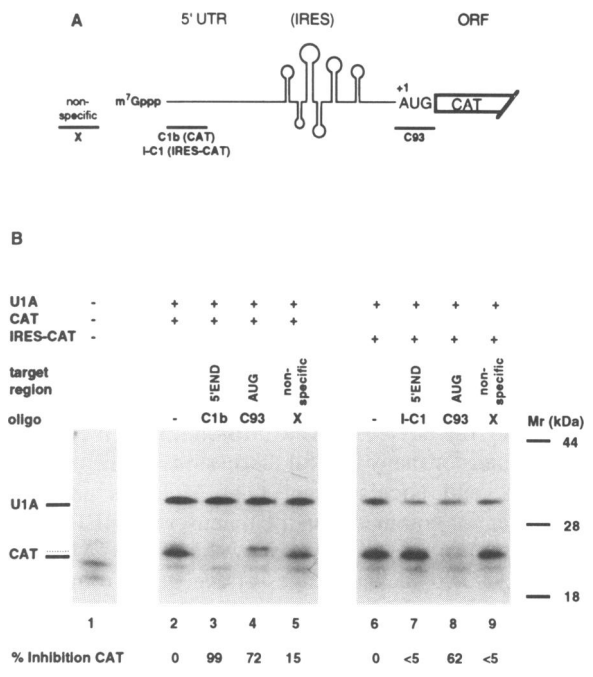

Figure 7. Effect of antisense 2′-O-allyloligoribonucleotides on internal initiation of translation. The indicated 2′-O-allyl oligoribonucleotides (A and Table I) were annealed to a mixture of 14 fmol U1A mRNA and 14 fmol of either SKCAT (CAT) mRNA (A, B: lanes 2–5) or KSRClaCAT (IRES-CAT) mRNA (A, B: lanes 6–9) in 400 mM KCl, 20 mM Hepes (pH 7.6), and translated in RRL. The degree of inhibition of translation is expressed as described in Figure 2.

(46,47). We suspect that cap-proximal antisense 2′-O-allyl oligos could act in a similar way. Highly stable intramolecular structures further downstream within the 5′ UTR have been suggested to impede 'scanning' of the 43S complex (47). 2′-O-Alkyl oligo/mRNA hybrids in similar positions are probably not affected by eIF-4A or other helicases involved in the initiation phase of translation and may thus also prohibit scanning. The downstream boundary for antisense-mediated inhibition (~20 nts downstream from the initiation codon) may be defined by the leading edge of a 43S pre-initiation complex positioned over the AUG and the ~25 nucleotides that an 80S ribosome at the AUG extends into the ORF, as determined by 'toe-print' (48,49) and 'finger-print' analyses (50). Thus, 2′-O-allyl oligos probably arrest mRNA translation by different mechanisms depending on the position of the oligo within the mRNA.

The inability of hydrogen bonded oligos within the open reading frame to arrest translation is most likely due to removal of the oligo by the elongating ribosome (but not by the pre-initiation complex) (51–53). Reports on the use of antisense oligos to arrest translation elongation have shown that hybrids within the ORF require RNase H for efficient inhibition (15–17,36,54,55), with one possible exception (56). We show that translation can also be inhibited by UV-crosslinking of psoralen-tagged 2′-O-methyl oligos to the ORF (Fig. 8). To our knowledge, this is the first demonstration of highly efficient arrest of translation by oligos bound within the open reading frame without RNase H activation. Covalent attachment of oligos containing either methylphosphonate DNA or α-DNA via psoralen or alkylating reagents has previously been shown to inhibit translation by 40–60% (57,58). We consider it most probable that the elongation phase of translation is arrested, although C-terminally shortened translation products have not been detected.

Finally, antisense 2′-O-allyl oligos could prove useful as tools to study internal initiation of translation as they can be used to discriminate between this and the cap-dependent mode of initiation

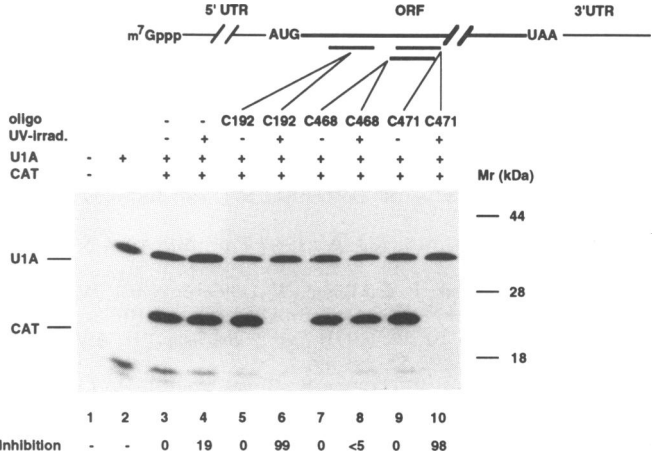

Figure 8. Covalent cross-linking of psoralen tagged 2′-O-alkyloligoribonucleotides to the ORF inhibits CAT mRNA translation. 2′-O-Allyl oligos (lanes 7 and 8) or 2′-O-methyl oligos bearing psoralen at their 3′ ends (Table I) (lanes 5–6 and 9–10) were annealed to 30 fmol ^{32}P-labeled I.19CAT (CAT) mRNA, irradiated with UV-light (lanes 4, 6, 8 and 10) and translated together with 20 fmol U1A in reticulocyte lysate. The extent of cross-linking was greater than 95% for the 2′-O-methyl oligos as determined by denaturing gel-electrophoresis of the annealing mixtures before and after UV-irradiation (not shown). After translation, the ^{32}P-labeled mRNA was degraded by addition of 100 U RNases T1 and 1 U RNase T2 and incubation for 30 min at 30°C. The slight variation in translatability, whether the mRNA was UV-irradiated in the presence (lane 8) or absence (lane 4) of a non-cross-linkable oligo, is most likely due to experimental variation rather than a protective effect on the mRNA by the presence of the oligo, as a smaller difference was observed when the experiment was repeated.

of translation. Antisense 2′-O-allyl oligos thus provide a novel and versatile molecular tool for further dissection of the translation initiation pathway. They may permit analysis of long-standing issues including the definition of the binding regions of ribosomal complexes to mRNAs in the cap-dependent and internal initiation modes.

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