Site and Mechanism of Antisense Inhibition by C-5 Propyne Oligonucleotides†

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ABSTRACT: Antisense gene inhibition occurs when an oligonucleotide (ON) has sufficient binding affinity such that it hybridizes its reverse complementary target RNA and prevents translation either by causing inactivation of the RNA (possibly by RNase H) or by interfering with a cellular process such as stalling a ribosome. The mechanisms underlying these processes were explored. Cellular antisense inhibition was evaluated in a microinjection assay using ON modifications which precluded or allowed *in vitro* RNase H cleavage of ON/RNA hybrids. RNase H-independent inhibition of protein synthesis could be achieved by targeting either the 5′-untranslated region or the 5′-splice junction of SV40 large T antigen using 2′-*O*-allyl phosphodiester ONs which contained C-5 propynylpyrimidines (C-5 propyne). Inhibition at both sites was 20-fold less active than inhibition using RNase H-competent C-5 propyne 2′-deoxy phosphorothioate ONs. *In vitro* analysis of association and dissociation of the two classes of ONs with complementary RNA showed that the C-5 propyne 2′-*O*-allyl phosphodiester ON bound to RNA as well as the C-5 propyne 2′-deoxy phosphorothioate ON. *In vitro* translation assays suggested that the two classes of ONs should yield equivalent antisense effects in the absence of RNase H. Next, ON/T antigen RNA hybrids were injected into the nuclei and cytoplasm of cells. Injection of C-5 propyne 2′-*O*-allyl phosphodiester ON/RNA hybrids resulted in expression of T antigen, implying that the ONs dissociated from the RNA in cells which likely accounted for their low potency. In contrast, when C-5 propyne 2′-deoxy phosphorothioate ON/T antigen RNA complexes were injected into the nucleus, the duplexes were stable enough to completely block T antigen translation, presumably by RNA inactivation. Thus, a dramatic finding is that C-5 propyne 2′-deoxy phosphorothioate ONs, once hybridized to RNA, are completely effective at preventing mRNA translation. The implication is that further increases in complex stability coupled with effective RNase H cleavage will not result in enhanced potency. We predict that the development of more effective ONs will only come from modifications which increase the rate of ON/RNA complex formation within the nucleus.

Development of antisense oligonucleotides (ONs) has focused on modifications which improve efficacy through (i) enhanced affinity for target RNA, (ii) stability to nucleolytic cleavage, and (iii) increased permeability of ONs to cell membranes (Milligan et al., 1993; Wagner, 1994). Several reports have demonstrated advancements for ONs in each of these areas (Milligan et al., 1993; Stein & Cheng, 1993; Wagner, 1994). Despite the success in developing new antisense agents and techniques, the mechanism of antisense inhibition remains largely unexplored.

The mechanism of antisense inhibition in cells has been previously evaluated in part by using ON analogs which can either preclude or allow *in vitro* RNase H cleavage of ON/RNA complexes. RNase H can hydrolyze the RNA portion of an ON/RNA duplex, thus degrading the targeted RNA. In *in vitro* translation experiments, RNase H was required for inhibition when targeting the coding region and the 3′-end of RNA with 2′-deoxy ONs (Cazenave et al., 1986; Haeuptle et al., 1986; Minshull & Hunt, 1986; Dash et al., 1987; Walder & Walder, 1988). Cellular assays have indicated that RNase H is involved in the mechanism of antisense inhibition for 2′-deoxy ONs which contain phosphodiester or phosphorothioate linkages (Bennett et al., 1992; Monia et al., 1993; Wagner et al., 1993). Antisense ON-mediated decreases in RNA levels have been shown to be responsible for inhibition of several proteins, including intercellular adhesion molecule (ICAM) (Chiang et al., 1991; Bennett et al., 1994), procollagen (Colige et al., 1993), and protein kinase C-α (Dean et al., 1994), implicating the involvement of RNase H. Non-RNase H mechanisms of antisense inhibition occur when an ON/RNA complex sterically blocks RNA transport, processing, or translation. *In vitro* translation studies have shown that in the absence of RNase H, antisense ONs hybridized to their complementary target in the coding region of mRNA were removed by a ribosome-associated unwinding activity (Liebhaber et al., 1984; Shakin & Liebhaber, 1986; Gupta, 1987). RNase H-independent inhibition by antisense ONs has been demonstrated, but inhibition was dependent on which region of the RNA was targeted. In *in vitro* translation experiments, RNase H-independent inhibition has been demonstrated by targeting the translation initiation codon and the 5′ untranslated region of RNA (Blake et al., 1985; Maher, III, & Dolnick, 1987; Walder & Walder, 1988; Bertrand et al., 1989; Johansson et al., 1994). ON modifications which do not activate RNase H cleavage of an ON/RNA duplex, such as 2′-ribose modifications, can allow for an assessment of

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Table 1: Sequences of ONs

site[a]	region	symbol	type	sequence
19−36	5′-untranslated	5′-17	antisense	CCU CCU CAC UAC UUC UG
19−36	5′-untranslated	5′-17m2	2 base mismatch	CCU CCC CAC UAU UUC UG[b]
19−36	5′-untranslated	5′-17m4	4 base mismatch	CCU CCC CUC UAU UAC UG
19−31	5′-untranslated	5′-12	antisense	CCU CCU CAC UAC
69−86	initiation	AUG	antisense	AAC UUU AUC CAU CUU UG
69−86	initiation	AUG	4-base mismatch	AAC UCU CUC AAU UUU UG
309−326	5′-splice junction	SPL	antisense	UAC CUC AGU UGC AUC CC
309−326	5′-splice junction	SPL-m4	4-base mismatch	UAU CUA AGC UGC CUC CC
2599−2616	poly(A) signal	pA	antisense	CUA UUG CUU UAU UUG UA
2599−2616	poly(A) signal	pA	4-base mismatch	CUA UUU CUG UAU GUU UA

[a] This is an average; TAg transcription begins from multiple start sites (Pauly et al., 1992). [b] Underlined bases indicate mismatched positions.

RNase H-independent antisense inhibition in cells. For example, in confirmation of the *in vitro* translation experiments cited above, the 5′-untranslated region and the translation initiation region of RNA appear to be sites for steric block inhibition in mammalian cells (Chiang et al., 1991; Bennett et al., 1994; Dean et al., 1994) but not in the coding region (Chiang et al., 1991; Wagner et al., 1993; Bennett et al., 1994; Dean et al., 1994) or the 3′-untranslated region of RNA (Chiang et al., 1991).

Recently, potent gene-specific antisense inhibition was achieved in mammalian cells using C-5 propynylpyrimidine (C-5 propyne) 2′-deoxy phosphorothioate ONs (Wagner et al., 1993). These ONs had the required binding affinity and nuclease stability properties to yield potent antisense inhibition when microinjected into cells, and RNA inactivation was presumably achieved by an RNase H-mediated mechanism of inhibition. One of our goals was to create antisense ONs of such high affinity for RNA that they form a nearly irreversible complex, thereby inactivating the RNA via a non-RNase H, or steric block, mechanism. In an attempt to achieve this, high-affinity steric block ONs, which consisted of 2′-*O*-allyl ribose and C-5 propyne modifications, were developed (Froehler et al., 1993). In the study described in this report, we evaluated SV40 large T antigen (TAg) inhibition using 2′-deoxy and 2′-modified C-5 propyne ONs. We exploited the high-affinity, nuclease-stable 2′-*O*-allyl ONs as steric blocking agents and targeted several sites in TAg which were required for RNA processing, transport, and translation. The results showed that the steric block ONs were 20-fold less potent than modifications which had the ability to recruit RNase H. Thus, it appeared that the binding affinity of 2′-*O*-allyl ONs was not sufficient to result in potent antisense effects.

To evaluate the underlying mechanism to the above finding, we dissected the antisense assay into individual components to assess ON/RNA stability using defined conditions at the *in vitro* and *in situ* levels. T_m analysis of ON/RNA duplexes is often used as a relative measure of binding affinity of ONs for RNA, but it is not a measure of ON/RNA hybridization and dissociation rates at physiological conditions. Thus, we evaluated the binding of ONs with RNA by gel-shift analysis using near-physiological salt conditions at 37 °C. This analysis provided information about the stability of ON/RNA complexes using cellular conditions but in the absence of proteins and translation machinery. We then introduced the translation apparatus using rabbit reticulocyte lysate and assessed the gene inhibitory properties of ON/RNA duplexes in the absence of RNase H. Finally, we compared the results above with the gene inhibitory properties of the ONs in cells by microinjecting preformed ON/RNA duplexes. By microinjecting the complexes into subcellular regions of the cell, we were able to evaluate both the site and the mechanism of antisense action. The results clearly show that while the C-5 propyne 2′-*O*-allyl ON/RNA duplexes formed *in vitro* appear to be very stable, they dissociate in cells. Also, C-5 propyne 2′-deoxy phosphorothioate ON/RNA duplexes were stable enough to completely inhibit RNA translation, presumably by allowing rapid cleavage of the RNA by RNase H in the nucleus.

MATERIALS AND METHODS

ON Synthesis, Purification, and T_m Analysis. ONs were synthesized by the H phosphonate method on an automated synthesizer (model 8750; Milligen Biosearch, Bedford, MA) using standard chemistry on controlled pore glass (CPG) support. The nucleoside analogs were prepared as described (Froehler et al., 1992, 1993; Froehler, 1993). ONs were purified by polyacrylamide gel electrophoresis (PAGE) and analyzed by PAGE and base composition analysis. ON/RNA T_m analysis was carried out essentially as described (Froehler et al., 1992) with the exception that reduced salt buffer [14 mM KCl, 1 mM $Na_2H_2PO_4$, and 1 mM $MgCl_2$ (pH 7.2)] was used in order to keep the melting temperatures on scale for comparative purposes. T_m values are ±0.5 °C.

Sequences of TAg ONs are shown in Table 1. The CAT-a ON consisted of C-5 propyne 2′-deoxy phosphorothioate modifications and consisted of the following reverse complementary sequence to the coding region of chloramphenicol acetyltransferase (CAT) mRNA: 5′-UCG UGG UAU UCA CUC CA-3′.

Microinjection and Fluorescence Microscopy. Microinjection, immunofluorescence staining, and fluorescence microscopy were carried out as described (Hanvey et al., 1992; Fisher et al., 1993; Wagner et al., 1993). Microinjection was done on a Zeiss Axiovert microscope using a pneumatic controller (PLI-188; Nikon, Garden City, NY) attached to a micromanipulator (MO-302; Narishige U.S.A., Greenvale, NY). Following injection, cells were incubated for the indicated time and then fixed with formaldehyde and immunolabeled. Analysis of the injected samples was performed using a fluorescence microscope (Diaphot; Nikon, Garden City, NY) equipped with a mercury light source, filter changer (Sutter Instruments, Novato, CA), and filters for fluorescein and Texas Red fluorochromes (Omega, Brattleboro, VT).

Antisense Assays. (1) Expression Plasmid Assay. Two expression plasmids, one which contained a mutant of SV40

TAg gene (p5080) and the other which contained the *Escherichia coli* β-galactosidase gene [β-gal; pRSVZ [American Type Culture Collection (ATCC), Rockville, MD]] were co-microinjected into the nuclei of ~100−150 African green monkey kidney cells [CV1 (ATCC)] with or without ONs (Wagner et al., 1993). For the cases of the ONs targeted to the polyadenylation signal of TAg, pCMVβgal (Clontech, Palo Alto, CA) was used instead of pRSVZ; pCMVβgal uses the CMV promoter to direct β-gal expression and does not use the TAg polyadenylation signal. The concentrations of p5080 and pRSVZ plasmids in the microinjection needle were 0.003 and 0.16 μg/μL, respectively [in 90 mM KCl, 50 mM HEPES buffer (pH 7.3)]; for pCMVβgal, the concentration in the needle was 0.025 μg/μL. The cells were incubated for 4.5 h at 37 °C, fixed, immunolabeled, and scored visually using fluorescence microscopy for TAg and β-gal expression (Hanvey et al., 1992). Each assay was repeated at least in duplicate. In some cases, results were repeated with three other cell lines, Rat2, SK-BR-3, and HUV-EC-C (ATCC), which were each cultured using the recommended media and conditions.

(2) Specific Gene Inhibition Assay. To evaluate specific inhibition of gene expression, an antisense target site in the 5′-untranslated region of TAg [termed the 5′-17 site (Table 1)] was mutated in p5080 and the wild-type sequence was placed in the +1 transcription start site of a minimal CMV promoter which directed luciferase expression. The 5′-17 site was mutated in p5080 as follows: the *Sfi*I-*Avr*II fragment of p5080 (which contained the 5′-17 target site) was replaced with the synthetic fragment 5′-CGGCCTCTGGCGGCCG-CAAGAGAAAGAGAGAGAACATCGATCGAGGC-′3. The resultant plasmid, p5080.mut2, expressed TAg in microinjected cells identically to the control p5080.

The 5′-17 site was cloned into the luciferase plasmid, pUHC13-3 (Gossen & Bujard, 1992), as follows: the 162-bp *Sac*I fragment of the pUHC13-3 CMV promoter region was replaced with the following synthetic fragment

5′-GGGGTACCCGGGTCGAGTAGGCGTGTACGGTGGGAGGCCTATATA

+1

AGCAGAGCTCGTTTAGTGAACCG CAGAAGTAGTGAGGAGGC TT

5′-17

TTTTG AGATCT GAGG CCTAGG CTCCATAGAAGACACCGGGAC

Bgl II *Avr II*

CGATCCAGCCTCCGCGGCCCCGAATTCCAGCT-3′

which places the 5′-17 site at transcription position +1. The resultant plasmid, pTAL13-3, uses a CMV minimal promoter in conjunction with seven tetracycline repressor-VP16 fusion protein binding sites to direct luciferase expression. For antisense experiments, pTAL13-3 (needle concentration, 0.034 μg/μL), pUHD15-1 [which directs the synthesis of the tetracyline repressor-VP16 fusion protein (Gossen & Bujard, 1992); 0.043 μg/μL], and p5080.mut2 (0.003 μg/μL) were co-microinjected with or without ONs into the nuclei of CV1 cells. The cells were incubated for 4.5 h and then fixed and immunolabeled for TAg and luciferase expression as described previously (Hanvey et al., 1992) with the exception that a rabbit polyclonal luciferase antibody (1:250; Cortex Biochemicals, San Leandro, CA) was used.

RNase H Analysis of ON/RNA Complexes. For RNase H analysis, a 20-nt sense RNA which contained the 5′-17 antisense ON target site of the sequence 5′-UCCA-GAAGUAGUGAGGAGGC-3′ was labeled using T4 polynucleotide kinase to a specific activity of ~6000 Ci/mmol and gel purified. The [^{32}P]RNA was bound to 5 nM ON for 1 h in RNase H buffer [50 mM Tris·Cl (pH 8.0), 10 mM $MgCl_2$, 20 mM KCl, 3 mM dithiothreitol, 20 U of RNasin (Promega Corp., Madison, WI)], and subsequently, HeLa nuclear extract (100 ng; Promega Corp.) was added (final volume of 10 μL). At 10 min, aliquots (2 μL) were removed, added to loading solution [90% formamide, 0.025% xylene cyanol, 50 mM EDTA (pH 8.0); 18 μL], heated to 95 °C for 5 min, and electrophoresed on a 20% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 8.3 M urea. Analysis was done by autoradiography. For the base cleavage ladder, [^{32}P]RNA in 1× RNase H buffer was added to the loading solution above without EDTA and heated for 5 min at 95 °C prior to loading on the gel.

Kinetics of Hybridization and Dissociation of ON/RNA Complexes in Vitro. A gel-shift assay was used to analyze rates of hybridization and dissociation of ONs targeted to the 5′-untranslated region of TAg RNA (5′-17; Table 1). A 20-nt sense RNA which contained the 5′-17 antisense ON target site, 5′-UCCAGAAGUAGUGAGGAGGC-3′, was 5′-endlabeled using T4 polynucleotide kinase to a specific activity of ~6000 Ci/mmol and gel purified. Rates of hybridization were set up as follows: [^{32}P]RNA (5000 cpm/reaction) and ON were each heated to 95 °C for 2 min and subsequently mixed in buffered salts [40 mM MOPS buffer (pH 7.2), 140 mM KCl] in a total reaction volume of 80 μL; rates of hybridization were determined at 37 °C for four different concentrations of ON (200, 400, 800, and 1600 pM) over a period of seven time points (0, 1, 2, 4, 8, 16, 32, and 64 min). Aliquots (8 μL) were removed from the reactions and the reactions were quenched by adding sense ON (unlabeled) to 10 μM. Samples were electrophoresed on a 15% native PAGE (19:1 acrylamide:bisacrylamide, 0.4 mm thick, 750 V) at 4 °C. Gels were quantitated using a β^- particle detector (Ambis 4000; Ambis, San Diego, CA). Under these conditions, the [^{32}P]RNA/DNA duplex runs as a single retarded band on the gel relative to the faster migrating single-stranded [^{32}P]RNA.

A similar assay was performed for off-rate analysis. [^{32}P]-RNA (5000 cpm/reaction) was prebound to ON (1600 pM) for 2 h using the same conditions as described above. Sense ON was then added to the reaction at a final concentration of 10 μM, and aliquots of the reaction were taken over seven time points (0, 0.75, 1.5, 3, 6, 12, and 24 h) and placed on ice. Electrophoresis and gel analysis were performed as described above. In control experiments, heating of the reaction mixture (including the sense ON) to 95 °C for 5 min followed by a 2 h incubation at 37 °C showed complete complex dissociation, indicating that the sense ON functioned properly to completely bind any dissociated antisense ON.

Preparation of TAg and CAT RNAs. To generate unspliced TAg RNA for microinjection, a 2695-bp *Avr*II/*Bam*HI fragment of pSV40 (ATCC), containing TAg, was subcloned into *Bam*HI/*Eco*RI-cleaved pGEM3Z (Promega Corp., Madison, WI) together with a linker insert (containing *Eco*RI and *Avr*II ends) which replaced the 5′-leader region of TAg (sequence of the insert was 5′-AATTCCAGAAG-TAGTGAGGAGGCTTTTTTGGAGGC-3′) such that the 5′-17 antisense ON target site was 10 nt downstream from the T7 transcription start site (creating p3ZTAg). The 2700-nt,

unspliced, ^{m7}GpppG-capped TAg RNA was synthesized using the *Bam*HI-cleaved construct and T7 RNA polymerase (mMessage machine kit, as per instructions; Ambion, Austin, TX). The RNA was purified by sequential ethanol precipitations. Similarly, the 1632-bp *Hind*III/*Bam*HI fragment of CAT was cloned into pGEM3Z and cut with *Bam*HI, and the 1645-nt RNA was synthesized from the SP6 promoter (mMessage machine kit; Ambion, Austin, TX). These non-polyadenylated RNAs were injected into the nucleus at a needle concentration of 500 ng/μL.

To generate spliced TAg RNA for *in vitro* translation and microinjection, total RNA was isolated from Cos7 cells (which express TAg, from ATCC). A 460-base region of the TAg RNA, containing the 5′-end and the exon/exon splice junction, was reverse transcribed and PCR amplified using standard procedures (the 5′-primer was 5′-TCTACAGTCG-TATACGCTATTCCAGAAGTAGTG-3′ and the 3′-primer was 5′-CTTCTACCTTTCTCTTCTTTTTTGGAGG-3′). The *Avr*II/*Ear*I fragment of p3ZTAg (see above) was replaced with the *Avr*II/*Ear*I-cleaved PCR fragment (p3ZTAg.SPL). The integrity of the new insert was confirmed by restriction analysis and DNA sequencing through the splice junction region. RNA was generated from *Bam*HI-cleaved p3ZTAg.SPL as described above. Polyadenylation was carried out using poly A polymerase (5 units; Pharmacia, Piscataway, NJ) in a reaction (50 μL; 30 min at 37 °C) containing RNA (~10 μg) and buffer [50 mM Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 50 μg/mL BSA]. The reaction was stopped by phenol/chloroform extraction and ethanol precipitation.

In Vitro Translation of RNA Prebound with Antisense Oligonucleotides. Spliced, polyadenylated TAg and polyadenylated CAT RNAs were hybridized to ONs as follows: TAg and CAT RNAs (1 μL in H_2O; 0.5 and 0.25 μg/μL, respectively) were mixed with ON [1 μL in 180 mM KCl, 100 mM Hepes (pH 7.3); 0.32 μM], heated (65 °C for 5 min), and allowed to hybridize at 25 °C for 1 h. A rabbit reticulocyte lysate (Promega) mix contained the following: lysate (8.8 μL), [^{35}S]methionine (0.25 μL, 50 μCi/μl), amino acid mix (0.25 μL, without methionine), and H_2O (to a final volume of 12.5 μL). The lysate mixture was added to the TAg and CAT RNAs, and the reaction mixture was incubated (1 h at 30 °C). After 45 min, complete amino acid mix (1 μL of 500 μM stock; Promega; cat. no. L4461) was added to aid completion of TAg protein synthesis. In some cases, poly(rA)·poly(dT) was added to the mix to titrate out any contaminating RNase H in the lysate [Walder & Walder, 1988; 0.25 μL of 2.6 μM stock; although we determined that RNase H activity was not detectable either with or without the poly(rA)·poly(dT)]. For RNase H studies, *E. coli* RNase H was added to the lysate mix (5 units; Promega). For ON/RNA dissociation assays, following ON/RNA hybridization, the 20-nt 5′-17 sense RNA was added to compete out unbound and dissociating 5′-17 antisense ONs (1 μL; 8 μM). Samples were loaded onto precast SDS/10% PAGE gels (Bio-Rad), electrophoresed (100 V, 1 h), fixed [15 min in 50% methanol/10% acetic acid (v/v)], stained with sodium salicylate (0.5 M; 20−30 min), dried, and autoradiographed.

Microinjection of ON/RNA Complexes in Situ. ONs were hybridized to the TAg RNA as follows: TAg (spliced or unspliced) and CAT RNAs [500 ng/μL each, in 90 mM KCl, 50 mM Hepes (pH 7.3)] were heated to 95 °C in the presence of ONs. Samples were then incubated at room temperature for 2 h prior to microinjection. TAg and CAT RNAs with or without ONs were co-microinjected into the nuclei of CV1 cells, incubated for 6 h at 37 °C, fixed, and immunolabeled. Immunolabeling was carried out as described (Hanvey et al., 1992), except that a rabbit polyclonal anti-CAT antibody (5′-3′, Inc., Boulder, CO) was used at 1:100 dilution instead of the anti-β-gal antibody. Cells were scored visually by fluorescence microscopy for TAg and CAT expression. Unspliced TAg and CAT RNAs did not contain poly(A) tails and injection of 500, 250, or 50 ng/μL each of the RNAs resulted in cotranslation in 85%, 40%, and 10% of the injected cells, respectively, after 6 h postinjection. Similar results were obtained at 4 h using the CAT and spliced TAg RNAs which had poly(A) tails. These results indicated a linear range of dose−response for RNA expression which was dependent on the quantity of injected RNA.

RESULTS

The 5′-Untranslated Region and the 5′-Splice Junction of TAg Are Effective Targets for Steric Blocking ONs. We targeted several regions of TAg RNA using ONs which contained varied phosphate, sugar and pyrimidine modifications. ON modifications which were permissive for RNase H cleavage of ON/RNA duplexes consisted of 5-(1-propynyl)-2′-deoxypyrimidine (C-5 propyne) and phosphorothioate backbone modifications; modifications which were non-permissive for RNAse H cleavage contained 5-(1-propynyl)-2′-*O*-allylpyrimidine, 2′-*O*-allyladenosine, and 2′-*O*-allylinosine nucleoside modifications with phosphodiester linkages. The RNA sites which were targeted using these two classes of ONs were the following regions: 5′-untranslated, translation initiation codon, 5′-splice junction, and polyadenylation signal (Table 1).

Microinjection of each of the antisense C-5 propyne phosphorothioate ONs, together with the TAg and β-gal plasmids, resulted in both gene- and sequence-specific inhibition of TAg at each of the targeted sites (Table 2). Four base mismatch ONs to each of the sites were >20-fold less active. These results confirmed our previous findings that multiple RNA sites could be targeted using C-5 propyne phosphorothioate ONs (Wagner et al., 1993). They also confirmed that each of the sites was accessible to antisense inhibition, since some highly structured RNA regions are less susceptible to C-5 propyne phosphorothioate ON inhibition (Fenster et al., 1994).

In contrast, the only 2′-*O*-allyl ONs which were effective at inhibiting TAg were the ones targeted to the 5′-untranslated region (5′-17; Table 2) and the 5′-splice junction (5′-SPL; Table 2). The 2′-*O*-allyl 5′-17 and 5′-SPL ONs were 20-fold less active than their 2′-deoxy phosphorothioate analogs (Table 2), but microinjection of either ON resulted in complete and gene-specific inhibition of TAg at higher concentrations (IC_{90} = 30 and 40 μM, respectively). In addition, microinjection of either 2′-*O*-allyl compound up to 40 μM showed no evidence of non-specific inhibition of β-gal. When either 2′-*O*-allyl ON was injected at 40 μM, inhibition of TAg was observed for greater than 20 h which indicated that there was little nucleolytic breakdown of the ONs, confirming earlier findings (Fisher et al., 1993). The assay was repeated in three other cell lines, Rat2, SK-BR-3, and HUV-EC-C, using the 5′-17 2′-*O*-allyl ON (40 μM), and gene-specific inhibition (>90%) of TAg was observed in each case.

Table 2: Inhibition of TAg vs β-Gal by Co-microinjection with Expression Plasmids

ON	length (nt)	modifications[a]	T_m[b] (°C)	IC_{50} (μM)[c] TAg	β-gal
5′-17a	17	T-mC, diester	67.0	≥40[d]	≥40
5′-17b	17	T-mC, thioate	58.0	7	7
5′-17c	17	T-mC, 2′-*O*-allyl	81.0	10	>40
5′-12A	12	T-mC, 2′-*O*-allyl	72.0	30	>40
5′-17d	17	pU-pC, diester	85.5	1[d]	30
5′-17e	17	pU-pC, thioate	79.0	0.25	20
5′-17m2	17	pU-pC, thioate	67.5	2.5	20
5′-17m4	17	pU-pC, thioate	51.5	5	20
5′-17f	17	pU-pC, 2′-*O*-allyl	>90.0	5	>40
5′-17m2	17	pU-pC, 2′-*O*-allyl	80.5	≥40	>40
5′-17m4	17	pU-pC, 2′-*O*-allyl	65.5	>40	>40
5′-12B	12	pU-pC, 2′-*O*-allyl	89.0	15	>40
AUG-a	17	T-mC, thioate	31.0	10	10
AUG-b	17	pU-pC, thioate	56.5	0.35	20
AUG-m4	17	pU-pC, thioate	25.0	8	20
AUG-c	17	pU-pC, 2′-*O*-allyl	72.5	~40	>40
SPL-a	17	T-mC, thioate	44.0	2	5
SPL-b	17	pU-pC, thioate	72.0	0.35	20
SPL-m4	17	pU-pC, thioate	33.5	5	20
SPL-c	17	pU-pC, 2′-*O*-allyl	90.0	7	>40
SPL-m4	17	pU-pC, 2′-*O*-allyl	32.5	>40	>40
pA-a[e]	17	T-mC, thioate	25.0	5	5
pA-b[e]	17	pU-pC, thioate	45.5	0.25	20
pA-m4[e]	17	pU-pC, thioate	<25.0	7	20
pA-c[e]	17	pU-pC, 2′-*O*-allyl	57.5	>40	>40

[a] T-mC represents T and 5-methyl C pyrimidines; pU-pC represents C-5 propyne U and C-5 propyne C; diester refers to phosphodiester backbone linkage; thioate refers to phosphorothioate linkage; 2′-*O*-allyl is a ribose sugar modification, and these ONs were synthesized with phosphodiester linkages. [b] T_ms were performed using 0.1× T_m buffer. [c] IC_{50} determination was performed as described (Wagner et al., 1993). [d] The activity of the ON was measured at 4.5 h postinjection. [e] pA ONs were tested using CMVβgal, since RSVβgal contains the SV40 TAg polyadenylation site.

The C-5 Propyne and Phosphorothioate Modifications Are Required for Maximum Potency. The T-mC (C-5 C) 2′-deoxy phosphodiester version of 5′-17 was inactive up to 40 μM, at which concentration it partially inhibited both β-gal and TAg (Table 2). In contrast, the C-5 propyne phosphodiester ON specifically inhibited TAg at 4.5 h postinjection with an IC_{50} of 1 μM. A time course analysis of inhibition using the C-5 propyne phosphodiester and phosphorothioate 5′-17 ONs showed that the phosphodiester was not active 24 h postinjection when injected at 20 μM whereas the phosphorothioate ON showed nearly complete TAg inhibition at 24 h (injected at IC_{90} = 2.5 μM). Thus, the decreased potency of the phosphodiester ON relative to its phosphorothioate homolog was likely due to nuclease susceptibility of the phosphodiester linkage.

In a previous result, a 20-nt T-mC phosphorothioate ON targeted to the 5′-untranslated region (completely overlapping the 5′-17 site) showed specific inhibition of TAg over a very narrow dose−response range: 100% inhibition of TAg at 5 μM, nonspecific inhibition of β-gal at 20 μM, and no inhibition of either protein at 1 μM (Wagner et al., 1993). In contrast, the 17-nt T-mC phosphorothioate ON targeted to the same site only showed nonspecific inhibition of TAg and β-gal with no evidence of specific inhibition at the lower concentrations (Table 2).

It was not clear why the T-mC ON did not show gene-specific activity at lower concentrations since (i) the T_m of the T-mC phosphorothioate ON was relatively high when bound to RNA (58 °C in reduced salt buffer) and (ii) the RNA target site was accessible to ONs, as evidenced by the activity of the C-5 propyne ONs. One possible explanation was that the T-mC ON was not efficiently cleaved by RNase H when bound to RNA. We conducted careful *in vitro* analysis of the RNase H susceptibility of the T-mC ON/RNA duplex relative to the C-5 propyne ONs. The results showed that each of the ON/RNA duplexes was efficiently cleaved (Figure 1). This assay was conducted such that >70% of the ON-bound RNA was still intact following cleavage by RNAse H, indicating single-hit cleavage kinetics of the duplex and allowing for fine mapping of the cleavage sites. While subtle differences in cleavage patterns were observed for the different ONs, it appeared that the duplexes were cleaved similarly. Thus, it was unlikely that RNase H susceptibility of the T-mC ON/RNA duplex was related to its low potency.

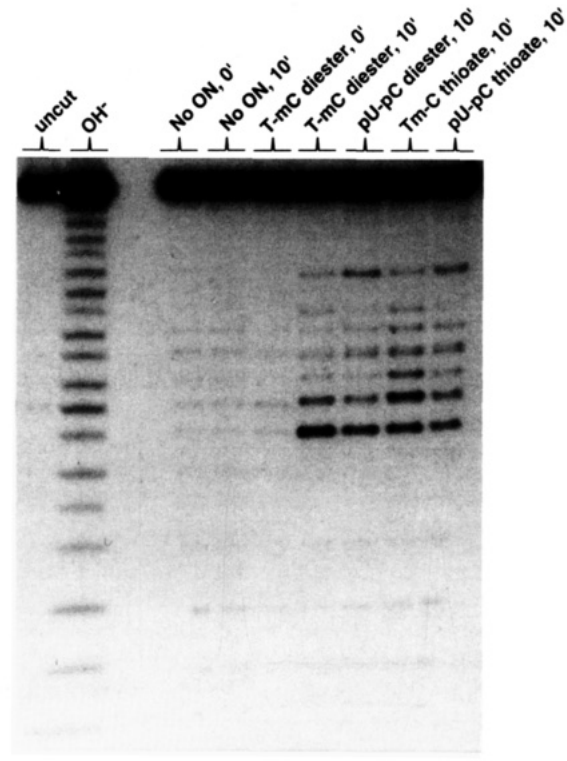

FIGURE 1: RNase H cleavage of ON/RNA duplexes. [^{32}P]RNA was bound to 5 nM 5′-17 ONs for 1 h in RNase H buffer, and subsequently, HeLa nuclear extract was added. Times (0′ and 10′) refer to reaction times following hybridization. A small amount of hydrolysis of the RNA was observed in each of the samples containing extract, as seen in the "No ON" lane. This experiment was repeated in duplicate, and identical results were obtained.

2′-O-Allyl ONs Show a High Degree of Specificity. Sequence-specific inhibition of the 5′-17 2′-*O*-allyl ON was examined by mismatch analysis. ONs containing two or four mismatches were inactive at concentrations up to 40 μM (Table 2), as compared to the C-5 propyne 2′-deoxy phosphorothioate mismatch homologs which showed 50% inhibition of TAg at 5 μM concentration. In addition, a four base mismatch of the 5′-splice junction 2′-*O*-allyl ON was inactive at 40 μM. These data suggest that the 2′-*O*-allyl ONs have high mismatch sensitivity.

Specificity of the 5′-17 2′-*O*-allyl ON was also evaluated by cloning the target sequence into a different gene, a luciferase construct, followed by evaluating antisense inhibition at this target site. In this gene system, luciferase was expressed by a cytomegalovirus (CMV) minimal promoter

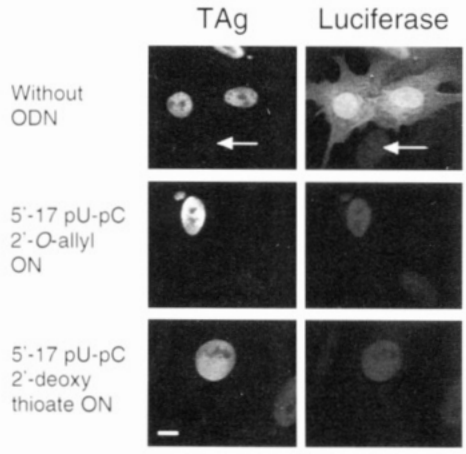

FIGURE 2: Inhibition of gene expression by targeting the +1 start site of luciferase in pTAL13-3. CV1 cells were microinjected with or without ONs, together with TAg (p5080.mut2) and luciferase (pTAL13-3) plasmids, incubated for 4.5 h, and analyzed as described in Materials and Methods. Upper fields (without ON) show 2 of 140 cells coexpressing TAg and luciferase; arrows indicate noninjected cell and show nonspecific background staining. Middle fields (5′-17f, 1 μM) show 1 of 155 TAg positive cells which showed barely detectable or no luciferase expression; 10 TAg positive cells showed >75% reduced luciferase expression, relative to without ON, and were scored as positive. Lower fields (5′-17e, 5 μM) show 2 of 160 TAg positive cells which had barely detectable or no luciferase expression; 15 TAg positive cells showed >75% reduced luciferase expression, relative to without ON, and were scored as positive. This experiment was repeated in duplicate, and similar results were obtained.

coupled to seven VP16-tetracyline repressor binding sites (Gossen & Bujard, 1992). In contrast to the SV40 promoter which directs transcription at multiple start sites (Pauly et al., 1992), the CMV minimal promoter directs transcription from a unique start site. Thus, the 5′-17 2′-*O*-allyl ON target site was cloned into the transcription start position, +1−+17, allowing us to examine whether an ON targeted to the 5′-cap region of the RNA would be more active. To demonstrate specificity, the same site was mutated out of TAg, and this mutant vector was used as the control gene. Gene-specific inhibition by the 5′-17 C-5 propyne 2′-*O*-allyl phosphodiester ON was observed using the luciferase construct containing the target site, but no inhibition was observed when the construct lacking the site was used (Figure 2). The IC_{50} for inhibition of luciferase by the 5′-17 C-5 propyne 2′-*O*-allyl phosphodiester ON was 5-fold lower (1 μM) compared to that for TAg (5 μM). In contrast, the IC_{50} for inhibition by the 5′-17 C-5 propyne 2′-deoxy phosphorothioate was unchanged (0.25 μM). The results from this strategy showed the specificity of inhibition by the 5′-17 2′-*O*-allyl ON and demonstrated 5-fold enhanced potency of this ON by targeting the +1 start site of transcription. The enhanced potency was specific for the 2′-*O*-allyl ON and, thus, was likely a reflection of an improved steric block at the 5′-cap region of the transcript.

Dependence of Inhibition by 2′-O-Allyl ONs on Base Modification and Length. C-5 propyne modification of pyrimidines is critical for potent antisense inhibition using 2′-deoxy phosphorothioate ONs (Wagner et al., 1993). For the 5′-17 2′-*O*-allyl phosphodiester ON, the T- and 5-methyl C-substituted ON was only 2-fold less active at inhibiting TAg than the C-5 propyne analog (Table 2). Given the high melting temperature of the 17-mer ONs, with or without the C-5 propyne modification, we reasoned that there would be a greater influence of the C-5 propyne substitution using shorter ONs targeted to the same site. Surprisingly, we found only a 2-fold difference in activity between the 12-mer C-5 propyne 2′-*O*-allyl ON and the corresponding T-mC ON, even though the difference in T_m between the two ONs was substantial (89.0 vs 72.0 °C, respectively; Table 2). Thus, it appears that there is a minor 2-fold contribution to potency imparted by C-5 propyne modifications for 2′-*O*-allyl ONs as compared to the dramatic (10−400-fold) increase in activity for 2′-deoxy phosphorothioate ONs.

Table 3: Kinetics of Hybridization of 5′-17 ONs to Synthetic cRNA: Rate of Association and Percent Dissociation (24 h) at 37 °C Using Near-Physiological Salt Conditions

modifications	K_{on} ($\times$ M^{-1} s^{-1})	percent dissociation (24 h)
T-mC, diester	6.4	23.3
T-mC, thioate	1.2	35.5
T-mC, 2′-*O*-allyl	3.4	0
pU-pC, diester	8.8	2.3
pU-pC, thioate	3.1	2.3
pU-pC, 2′-*O*-allyl	3.2	0

In Vitro Analysis of 5′-17 ON/RNA Duplexes. In the studies described above, C-5 propyne 2′-*O*-allyl ONs were significantly less active as antisense inhibitors of TAg gene expression than C-5 propyne 2′-deoxy phosphorothioate ONs, despite the fact that they were predicted to have enhanced affinity for RNA on the basis of T_m analysis. T_m analysis is not always an accurate indicator of binding affinity in cells since it measures the equilibrium of duplex formation at elevated temperatures (Young & Wagner, 1991). To further investigate the hybridization properties of ONs with RNA using near-physiological salt conditions, a gel-shift assay was developed to measure the rates of association and dissociation of 5′-17 ONs to RNA at 37 °C *in vitro* (see the methods section). In this assay, C-5 propyne ONs had increased hybridization rates compared to T-mC ONs (Table 3). For the phosphorothioate 2′-deoxy ONs, the effect was greater than 2.5-fold. As anticipated, a significant effect of the C-5 propyne substitution was observed in the dissociation rate of the ON/RNA complexes (Table 3). For the phosphorothioate 2′-deoxy ONs, the T-mC ON/RNA complex was 35% dissociated at 24 h, while the C-5 propyne ON/RNA complex was <5% dissociated. In the case of the 2′-*O*-allyl ONs, the dissociation rates of the ON/RNA complexes were not detectable at 24 h. We would predict, on the basis of these results, that C-5 propyne 2′-*O*-allyl ONs should bind to RNA as well as C-5 propyne 2′-deoxy phosphorothioate ONs and that they should remain bound during the time course of the previously described microinjection gene inhibition assay (4.5 h).

ON/RNA Duplexes Dissociate in Rabbit Reticulocyte Lysate. 5′-17 ONs were next evaluated for their ability to inhibit TAg protein production in an *in vitro* translation assay by an RNase H-independent mechanism. 5′-Capped and polyadenylated TAg cRNA and CAT RNA were prepared and efficiently translated in a rabbit reticulocyte extract (30 nM TAg and 20 nM CAT RNA concentrations in the lysate). To eliminate any contaminating amounts of RNase H in the extract, poly(rA)·poly(dT) was added, although in *in vitro* assays RNase H could not be detected in the lysate in the presence or absence of poly(rA)·poly(dT). Pre-incubation of either the 5′-17 C-5 propyne 2′-*O*-allyl phosphodiester ON or the C-5 propyne 2′-deoxy phosphorothioate to the

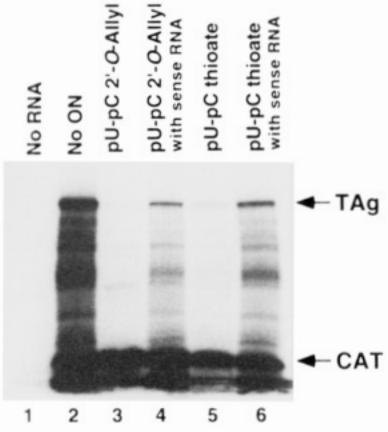

FIGURE 3: Inhibition of TAg and CAT translation in rabbit reticulocyte lysate. Spliced, polyadenylated TAg and polyadenylated CAT RNAs, with or without prebound ONs, were incubated (60 min, 30 °C) in rabbit reticulocyte lysates as described in Materials and Methods. Poly(rA)•poly(dT) was added to each sample to compete RNase H activity. In two translation samples, a 20-nt sense RNA was added (indicated) as a 25-fold mole excess over the ON concentration in order to bind unbound and dissociating ON. This experiment was repeated in triplicate, and the autoradiogram shows one of the three identical results. Lane 1, extract incubated in the absence of RNA; lane 2, translation of TAg and CAT RNAs; lane 3, translation of TAg prebound with 5′17 C-5 propyne 2′-*O*-allyl phosphodiester ON in the absence of the 20-nt sense RNA; lane 4, same as lane 3 but in the presence of the 20-nt sense RNA; lane 5, translation of TAg prebound with 5′-17 C-5 propyne 2′-deoxy phosphorothioate ON in the absence of the 20-nt sense RNA; lane 6, same as lane 5 but in the presence of the 20-nt sense RNA. The bands that migrate between TAg and CAT are derived from TAg RNA and occur because of incomplete translation.

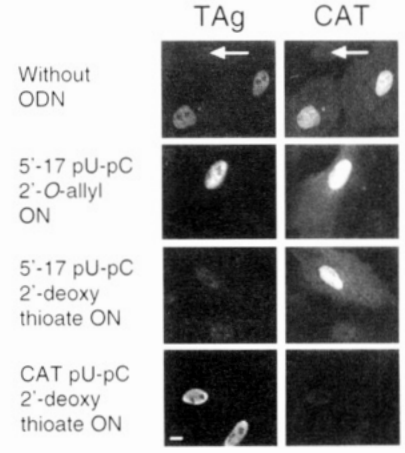

FIGURE 4: Inhibition of either TAg or CAT RNA expression in cells using injected ON/RNA complexes. CV1 cells were nuclear microinjected with or without ONs which had been previously preincubated for 2 h with capped, unspliced TAg RNA and capped CAT RNA (needle concentration of RNA, 500 ng/μL each). The injected cells were incubated for 6 h and analyzed by indirect immunofluorescence for both TAg and CAT expression as described in Materials and Methods. Upper fields (without ON) show 2 of 155 injected cells coexpressing TAg and CAT; arrows indicate a noninjected cell and show nonspecific background staining. Upper middle fields (5′-17f, C-5 propyne 2′-*O*-allyl phosphodiester ON; needle concentration, 2.5 μM) show 1 of 154 injected cells which coexpressed TAg and CAT. Lower middle fields (5′-17e, C-5 propyne 2′-deoxy phosphorothioate ON; needle concentration, 2.5 μM) show 1 of 161 CAT positive cells which showed no TAg protein. Bottom fields (CAT-a, C-5 propyne 2′-deoxy phosphorothioate ON; needle concentration, 2.5 μM) show 2 of 150 TAg positive cells which showed no CAT protein. Bar indicates 10 μm. This experiment was repeated in duplicate and similar results were obtained.

TAg and CAT RNAs (10-fold excess over TAg RNA, 300 nM) resulted in complete inhibition of TAg translation relative to CAT (Figure 3, lanes 3 and 5). These results demonstrate that the 5′-17 C-5 propyne 2′-*O*-allyl ON is an effective inhibitor of translation when bound to the RNA.

An assay was next developed to evaluate the stability of ON/RNA complexes in the translation assay. The design of the experiment was such that either the 5′-17 C-5 propyne 2′-*O*-allyl phosphodiester ON or the C-5 propyne 2′-deoxy phosphorothioate ONs were preincubated with the TAg and CAT RNAs and subsequently added to the reticulocyte lysate which contained a 20-nt RNA complementary to the 5′-17 target (sense) RNA sequence (25-fold mole excess over the antisense ON). The sense RNA was added so that it could bind any 5′-17 antisense ON which was unbound or which dissociated during the assay. When the sense RNA was added to the translation mix, translation of TAg was partially restored (Figure 3, lanes 4 and 6). As a control, addition of a 20-nt nonspecific RNA to the mix, in place of the sense RNA, did not restore TAg expression. Also, elimination of poly(rA)•poly(dT) from the lysate (but containing the sense RNA) did not alter the restoration of TAg translation; however, when RNase H was added to the lysate [without poly(rA)•poly(dT) but containing the sense RNA], complete inhibition of TAg was observed for the 5′-17 C-5 propyne 2′-deoxy phosphorothioate ON (data not shown).

Dissociation of ON/RNA Complexes Is Detected in Cells. 5′-Capped TAg (unspliced) and CAT RNAs were both prepared *in vitro*. Nuclear microinjection of both RNAs in the absence of ONs resulted in cotranslation of both proteins in approximately 85% of the injected cells (6 h postinjection; Figure 4). As in the *in vitro* translation experiments above, the 5′-17 C-5 propyne 2′-deoxy phosphorothioate and C-5 propyne 2′-*O*-allyl phosphodiester ONs were prebound to TAg RNA in the presence of CAT RNA [2.5- and 200-fold mole excesses over RNA concentration (needle concentrations, 2.5 and 200 μM, respectively; estimated intracellular concentrations, 0.125 and 10 μM)[1]]. Injection of either the 5′-17 or CAT C-5 propyne 2′-deoxy phosphorothioate ON/RNA complexes resulted in complete and gene-specific inhibition of their respective target RNAs at both the 2.5- and 200-fold mole excess concentrations (Figure 4 and Table 4). TAg protein expression was inhibited at both the 6 and 24 h time points. The 5′-17 C-5 propyne 2′-*O*-allyl ON, prebound to RNA, only showed inhibition of TAg at the 200-fold mole excess concentration (data not shown) and no inhibition at 2.5-fold (Table 4). At the 2.5-fold concentration, TAg protein was not inhibited at 6 or 24 h (Table 4 and Figure 4). This latter result suggested that the 2′-*O*-allyl ON/RNA complex dissociated in the cell in less than 6 h to allow TAg translation. By comparison, the C-5 propyne 2′-deoxy phosphorothioate ONs blocked translation of their targeted RNAs and inactivated the RNAs for greater than 24 h. This result is in contrast to the *in vitro* translation result using the C-5 propyne 2′-deoxy phosphorothioate ON in the absence of RNase H (above), since in that experiment the ON dissociated from TAg RNA in the lysate.

[1] At 0.125 and 10 μM intracellular concentrations, there would be 25% and 100% inhibition, respectively, of TAg protein expression from injected plasmids for the C-5 propyne 2′-deoxy phosphorothioate ON and 0% and >75% inhibition, respectively, for the C-5 propyne 2′-*O*-allyl phosphodiester ON.

Table 4: Percent Inhibition of TAg vs CAT by Microinjection of Preformed DNA/RNA Complexes (Using a 2.5-fold Mole Excess of ON to RNA)

		unspliced[a] nuclear[c]		spliced, poly(A)[b] nuclear		spliced, poly(A)[b] cytoplasmic[d]	
site	modifications	6 h	24 h	4 h[e]	24 h	4 h	24 h
5′-17b	T-mC, thioate	0[f]	0	0	0	0	0
5′-17e	pU-pC, thioate	100	100	100	30	100	10
5′-17f	pU-pC, 2′-*O*-allyl	0	0	0	0	0	0
5′-20	T-mC, thioate	30	30	30	0	30	0
SPL-a	T-mC, thioate	0	0				
SPL-b	pU-pC, thioate	100	100				
SPL-c	pU-pC, 2′-*O*-allyl	0	0				

[a] ONs were prehybridized to unspliced TAg RNA. [b] ONs were prehybridized to spliced, polyadenylated TAg RNA. [c] Nuclear refers to the injection site. [d] Cytoplasmic refers to the injection site. [e] Nuclear injected, spliced, poly(A) TAg RNA expressed protein approximately 4−5-fold faster compared to injection of the unspliced RNA, which is reflected in the time of the experiment. At 4 h, the same percentage of cells expressed TAg for the spliced RNA as compared to 6 h for unspliced, and in the cells which expressed the spliced, poly(A) TAg, there was approximately 2−3-fold more protein than for unspliced. [f] Refers to percent inhibition of TAg positive cells relative to CAT positive cells. Each data point was evaluated for 150 CAT positive cells; each experiment was repeated in duplicate (see Figure 2 and the methods section).

T-mC ON/RNA Complexes Dissociate in Situ. Next, we investigated whether the 5′-17 T-mC 2′-deoxy phosphorothioate ON also dissociated in cells after being prebound to RNA. Surprisingly, microinjection of this ON/RNA complex resulted in 100% TAg expression 6 h later (2.5-fold mole excess over RNA; Table 4). This result suggested that the ON/RNA complex dissociated in the cell before RNase H cleavage (or any other RNA-degrading mechanism) could occur, despite the fact that *in vitro* dissociation occurred slowly (35% dissociated at 24 h; Table 3), and that RNase H can cleave the duplex (Figure 1). We evaluated the previously described T-mC 20-nt ON targeted to the same 5′-untranslated region (Wagner et al., 1993). This ON partially inhibited TAg expression as a preformed complex with RNA (5′-20; Table 4). In addition, the T-mC phosphorothioate ONs targeted to the AUG initiation codon region, 5′-splice junction region, and polyadenylation region of TAg RNA were each inactive as preformed RNA complexes (2.5-fold mole excess over RNA; data not shown).

Site of Dissociation of C-5 Propyne 2′-O-Allyl ON/RNA Complexes. To investigate whether dissociation of the C-5 propyne 2′-*O*-allyl ON/RNA complexes occurred in the cytoplasm or nucleus, two experiments were conducted. In the first, the 5′-SPL 2′-*O*-allyl ON (targeted to the 5′-splice site) was evaluated for its ability to block splicing in the nucleus when prebound to RNA. Unspliced TAg RNA was bound *in vitro* with a 2.5-fold mole excess of the 5′-SPL 2′-*O*-allyl ON (needle concentration, 2.5 μM), and the complex was injected into the nucleus of CV1 cells. The 5′-SPL ON did not inhibit TAg expression, suggesting that rapid dissociation took place to allow splicing, RNA transport, and translation of TAg protein (Table 3). As a control, injection of the 5′-SPL C-5 propyne phosphorothioate ON/RNA complex (2.5-fold mole excess of ON; needle concentration, 2.5 μM) resulted in specific TAg inhibition (Table 4).

In the second experiment, a 5′-17 (targeted to the 5′-untranslated region) 2′-*O*-allyl ON/RNA complex was evaluated for its ability to interrupt translation of RNA in the cytoplasm. Spliced, polyadenylated TAg RNA was prepared for cytoplasmic microinjection.[2] Cytoplasmic or nuclear microinjection of the preformed 5′-17 2′-*O*-allyl ON/RNA complex (2.5-fold mole excess of ON; needle concentration, 2.5 μM) resulted in 100% TAg expression when evaluated 4 h postinjection (Table 4). Taking both experiments into account, it appears that 2′-*O*-allyl ON/RNA complexes are destabilized in both the nucleus and cytoplasm.

Site of Action for C-5 Propyne 2′-Deoxy Phosphorothioate ONs. To determine the site of action of C-5 propyne 2′-deoxy phosphorothioate ONs, capped, spliced, and polyadenylated TAg RNA/ON complexes (2.5-fold mole excess of ON; needle concentration, 2.5 μM) were injected into the cytoplasm and nucleus. The ON inhibited TAg expression at 4 h postinjection, regardless of where the complex was injected. However, at 24 h, only 30% and 10% inhibition of TAg expression was observed for the nuclear and cytoplasmic injections, respectively (Table 4). These results were surprising since they were in contrast to the results using nuclear injection of unspliced RNA/ON complexes (100% inhibition at 6 and 24 h).

This experiment demonstrated that C-5 propyne 2′-deoxy phosphorothioate ONs do not completely inactivate RNAs once they are bound to mRNAs which do not undergo splicing and polyadenylation. This strongly suggests that unspliced RNA without polyadenylation may be a primary site of action in the nucleus for inhibition by C-5 propyne ONs.

DISCUSSION

Several conclusions can be derived from the studies described in this report which have broad implications for how antisense ONs inhibit gene expression in mammalian cells. (i) RNase H-independent antisense inhibition occurred at relatively low potency when the 5′-untranslated or 5′-splice site regions of TAg were targeted with 2′-*O*-allyl-modified ONs. (ii) ON/RNA duplexes can dissociate relatively rapidly in cells which likely contributes to the low potency of some ON analogs including the 2′-*O*-allyl ONs. (iii) Unidentified dissociation factors which are present in the nucleus and cytoplasm of cells, and in reticulocyte lysate, are the likely cause of ON/RNA dissociation. (iv) In RNase H-free *in vitro* translation assays, C-5 propyne 2′-deoxy phosphorothioate ONs dissociate from RNA and allow translation using defined conditions. However, complete inhibition of translation can be observed when C-5 propyne 2′-deoxy phosphorothioate ON/unspliced RNA hybrids are injected into the nucleus of the cell. RNase H cleavage in the cell is the most likely explanation; however, other RNA-inactivating functionalities could exist. (iv) Heteronuclear (hn) RNA may be a primary target for C-5 propyne 2′-deoxy phosphorothioate ONs.

Dissociation of ON/RNA Hybrids. A recent report described gene inhibition by a steric block mechanism using

[2] A critical design feature of the experiment was that the RNA did not enter back into the nucleus following cytoplasmic injection. It was observed that splicing and polyadenylation were both critical for cytoplasmic expression of TAg RNA. Unspliced, nonadenylated TAg RNA and spliced, nonadenylated TAg RNA did not translate protein upon cytoplasmic injection, but both translated TAg protein when injected into the nucleus (data not shown). These results suggest that cytoplasmic microinjection did not result in RNA entry into the nucleus during the time course of the assay.

2′-*O*-allyl ONs, without the C-5 propyne modification, in *in vitro* translation assays (Johansson et al., 1994). This study showed that gene-specific inhibition occurred when the 5′-untranslated and AUG translation initiation codon regions were targeted. While our *in vitro* results correlate well with this study, our cellular studies show that the *in vitro* translation assay is not a good predictor of cellular potency since dissociation of ON/RNA hybrids occurs in cells.

Although its identity is unknown, a cellular dissociating activity(s) may exist which recognizes ON/RNA duplexes. This activity(s) could account for dissociation of the high-affinity 2′-*O*-allyl ON/RNA complexes which occurred in cells in less than 4.5 h. Rapid dissociation occurred in both the nucleus and cytoplasm, as evidenced by the dissociation of both nuclear injected 5′-SPL 2′-*O*-allyl ON/RNA complexes and cytoplasmic injected 5′-17 2′-*O*-allyl ON/RNA complexes. The putative activity is likely unrelated to the previously described "dsRNA unwindase" activity (Bass & Weintraub, 1987; Rebagliati & Melton, 1987; Wagner & Nishikura, 1988), since this misnomered activity is actually a dsRNA-specific adenosine deaminase (Bass & Weintraub, 1988; Wagner et al., 1989) which has a specific length dependence of duplex RNA (>30 nt) for efficient modification (Nishikura et al., 1991). Cellular factors which reside in the nucleus and are reportedly capable of destabilizing RNA secondary structure have been described, for example A1 hnRNP (Pontius & Berg, 1992). Such an activity may be capable of dissociating ON/RNA duplexes. In addition, an RNA helicase was recently purified which unwinds RNA/DNA hybrids *in vitro* (Flores-Rozas & Hurwitz, 1993). It is possible that such factors are capable of dissociating ON/RNA complexes in the nucleus of cells.

Gene Inhibition by Antisense ONs. C-5 propyne 2′-deoxy phosphorothioate ONs have unique properties which allow them to be potent inhibitors of gene expression at sites throughout an RNA message. While T-C phosphorothioate ONs can be optimized to show specific gene inhibition at some RNA sites (Bennett et al., 1992, 1994), issues regarding potency and gene specificity remain (Kirkland et al., 1993; Milligan et al., 1993; Wagner et al., 1993; Fenster et al., 1994; Wagner, 1994).

The steric block ONs we have tested suffer from the same potency problems encountered by the T-mC phosphorothioate ONs; however, 2′-*O*-allyl ONs are more specific inhibitors. Of particular note was the result that the 5′-17 T-mC phosphorothioate ON was not more potent than the T-mC 2′-*O*-allyl ON and, in fact, was more nonspecific. Bennett et al. recently described inhibition of ICAM, vascular cell adhesion molecule (VCAM), and E-selectin using T-C phosphorothioate ONs (Bennett et al., 1994). We note that while the authors observed gene-specific inhibition at defined ON concentrations, the T-C phosphorothioate ONs which they identified to inhibit ICAM, VCAM, and E-selectin showed nonspecific inhibition of nontargeted proteins at 2−3-fold higher concentrations. It seems that these authors may be observing a similar phenomenon to our results using T-mC phosphorothioate ONs. Additionally, we showed that the T-mC ON/RNA complexes which we examined are destabilized in cells, and this phenomena was likely responsible for the low potency of these ONs. Thus, our results demonstrate that the unique ability of the C-5 propyne phosphorothioate ONs is to form a stable complex with RNA and presumably allow RNA inactivation, and this stability is a critical component for potent gene-selective inhibition over an extensive dose−response range.

Site of Action of C-5 Propyne 2′-Deoxy Phosphorothioate ONs. These studies revealed several interesting insights about the mode of action of the C-5 propyne 2′-deoxy phosphorothioate ONs. The primary site of action for C-5 propyne phosphorothioate ONs is likely to be newly transcribed RNA. Cytoplasmic injection of preformed C-5 propyne 2′-deoxy phosphorothioate ON/RNA complexes only partially inhibited TAg protein synthesis, suggesting that RNase H levels in the cytoplasm are low. In the cytoplasm, ON/RNA complexes were rapidly destabilized and allowed the observed translation. Complete inhibition of TAg occurred when C-5 propyne 2′-deoxy phosphorothioate ONs were bound to unspliced RNA and injected into the nucleus, thereby providing evidence for a nuclear site of action by these molecules. In addition, we previously showed that intron RNA could be effectively targeted with C-5 propyne 2′-deoxy phosphorothioate ONs (Wagner et al., 1993) which also pointed to a nuclear site of action. One possible explanation is that splicing complexes preferentially recruit RNase H. An alternative model is that spliced and polyadenylated RNA is targeted for rapid nuclear transport to the cytoplasm, where RNase H levels are low (Büsen, 1980) and dissociation of ON/RNA duplexes rapidly occurs. Although we do not have formal proof of this latter hypothesis, we note that expression of nuclear injected, spliced, polyadenylated TAg RNA occurred much faster than that of unspliced TAg RNA (Table 4), and this result suggests that nuclear/cytoplasmic transport of spliced RNA is relatively rapid compared to that of unspliced RNA. Our current hypothesis is that the most potent antisense inhibition using C-5 propyne 2′-deoxy phosphorothioate ONs occurs by inhibiting newly transcribed RNA presumably through an RNase H mechanism in the nucleus; however, non-RNase H mechanisms are possible (Dolnick, 1990). For example, ON-mediated disruption of RNA splicing might trigger RNA inactivation by an unknown mechanism.

SUMMARY

The studies described in this report demonstrate that C-5 propyne 2′-deoxy phosphorothioate ONs are effective inhibitors of gene expression in cells. A dramatic finding of this study is that once the C-5 propyne 2′-deoxy phosphorothioate ONs hybridize RNA in the nucleus, they are completely effective at preventing mRNA translation. The implication is that further increases in complex stability, coupled with effective RNase H cleavage, will not result in enhanced potency. We predict that enhancement of potency will only come from increasing the rate of ON/RNA complex formation within the nucleus. This important discovery may provide new insights into the design of future ON analogs.

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