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An assessment of the antisense properties of RNase H-competent and steric-blocking oligomers

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ABSTRACT

The antisense activity and gene specificity of two classes of oligonucleotides (ONs) were directly compared in a highly controlled assay. One class of ONs has been proposed to act by targeting the degradation of specific RNAs through an RNase H-mediated mechanism and consists of C-5 propynyl pyrimidine phosphorothioate ONs (propyne-S-ON). The second class of antisense agents has been proposed to function by sterically blocking target RNA formation, transport or translation and includes sugar modified (2′-*O*-allyl) ONs and peptide nucleic acids (PNAs). Using a CV-1 cell based microinjection assay, we targeted antisense agents representing both classes to various cloned sequences localized within the SV40 large T antigen RNA. We determined the propyne-S-ON was the most potent and gene-specific agent of the two classes which likely reflected its ability to allow RNase H cleavage of its target. The PNA oligomer inhibited T Ag expression via an antisense mechanism, but was less effective than the propyne-S-ON; the lack of potency may have been due in part to the PNAs slow kinetics of RNA association. Interestingly, unlike the 2′-*O*-allyl ON, the antisense activity of the PNA was not restricted to the 5′ untranslated region of the T Ag RNA. Based on these findings we conclude that PNAs could be effective antisense agents with additional chemical modification that will lead to more rapid association with their RNA target.

INTRODUCTION

The inhibition of gene expression mediated by RNA-binding oligonucleotides (ONs) offers great promise for therapeutic intervention in many human diseases and as an important research tool for dissecting complex biochemical pathways. While conceptually simple, the barriers precluding the routine use of antisense technology are many. These include considerations of agent stability, bioavailability, subcellular localization and RNA affinity [for a review see (1)]. While novel chemical modifications have solved many of the problems associated with oligomer stability, including phosphorothioate (S-), methylphosphonate (MP-) and 2′-*O*-allyl ONs (2–4), progress in the areas of agent bioavailability and affinity have only recently been addressed.

When incubated with primary, immortalized or transformed human or rodent cells in culture, fluorochrome-tagged oligomers are localized to punctate perinuclear vesicles, resembling endosomes and lysosomes (5). Since no visible fluorescence is observed in the nucleus with prolonged incubation, ON release from these vesicles must be very inefficient. It has been suggested that due to their lack of charge, methylphosphonates (MPs) may enter cells by passive diffusion (3). Shoji *et al.* (6), have shown that while MP-ONs may enter cells by a fluid-phase enodcytosis process, the MP-ONs, like S-ONs and phosphodiester (P-) ONs, are trapped in vacuoles.

Cationic liposomes such as DOTMA, have been reported to successfully deliver ONs to the cell nucleus, and strong antisense activity for a variety of S-ONs has been reported (7–9). Alternatively, the cell membrane barrier can be by-passed by mechanical microinjection of antisense ONs into the cell (9–12). Using this approach, we and others have been able to quantitatively assess the relative potency of chemically modified antisense agents (9,11,12).

From a mechanistic consideration, two classes of ONs have emerged: RNase H-competent and steric-acting. First, following heteroduplex formation with RNA, the ON•RNA complex can serve as a substrate for RNase H, which leads to cleavage of the RNA component (13,14). While not yet validated, it has been postulated that once the RNA is cleaved, the ON can target multiple copies of each mRNA (reviewed in 15). Both P-ONs and S-ONs have been proposed to function by an RNase H-mediated mechanism (1). More recently, replacement of the C-5 methyl

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group of thymidine and C-5 hydrogen of cytidine with a propyne moiety has been shown to provide the most potent and specific antisense effects (in the context of an S-ON; propyne-S-ON) and to likely function through an RNase H-mediated mechanism (9,12).

For the steric-acting mechanism, ON heteroduplex formation does not lead to RNA turnover, but in theory results in the hindrance of RNA processing, nucleocytoplasmic transport or translation (16). MP-ONs, 2′-*O*-methyl ONs, 2′-*O*-allyl ONs and peptide nucleic acids (PNAs) are all members of this class. Based on this mode of action, it has been suggested that these agents can only be targeted to limited regions of the RNA [see (1) for review]. To date, steric blocking ONs have been shown to be active at several RNA target sites including the 5′ untranslated region (17; BF and RW, unpublished), the AUG translation initiation region ({18}) and the 5′ splice site (BF and RW, unpublished). However, since potency is a function of both target location and the life-time of the ON•RNA complex, steric-acting ONs which form more stable duplexes may be functional against many RNA target sites.

Recently, a novel ON analog was described in which the phosphodiester backbone was replaced with a polyamide and termed a peptide nucleic acid (PNA; 18). PNAs have the unique ability to strand invade duplex DNA at a specific target site (11,18,19), and they can also form a heteroduplex with RNA. The latter property was exploited in an *in vitro* translation assay and resulted in the loss of RNA function via steric blockade of SV40 large T antigen (T Ag) RNA by targeting the coding region with a 20mer PNA (11). In microinjection assays designed to target T Ag expressed in cells, the same PNA showed 50% inhibition of T Ag expression at 1 μM. In that study it was not assessed whether PNAs could show more potent inhibition by targeting other regions of RNA or how their potency compared to modified ONs.

In this paper we have compared the relative potency/affinity of the most active antisense ONs, propyne-S-ONs, to that of PNAs using a highly controlled T Ag microinjection assay. We determined that the propyne-S-ONs showed superior antisense effects by giving rise to the most persistent and gene-specific inhibition. In comparison, the PNA showed a narrow range of target specificity and poor potency which may be explained by its slow kinetics of RNA association. However, when compared to the 2′-*O*-allyl ON, the PNA was the more effective steric-blocking agent and was able to block T Ag expression when targeted to a variety of regions of the T Ag hn- and mRNA.

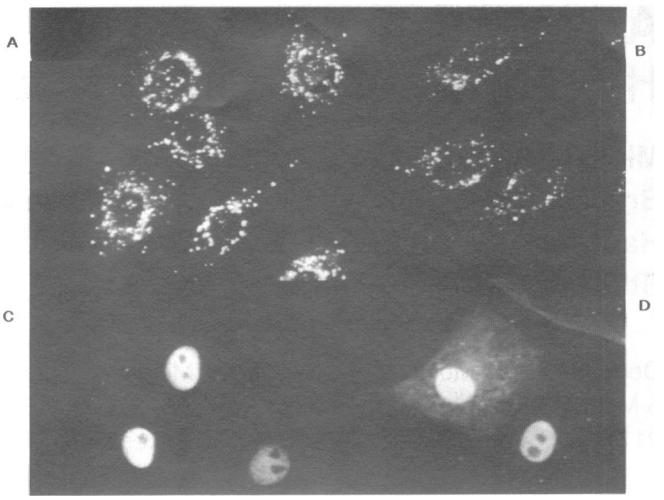


Figure 1. Intracellular localization of flurochrome-tagged propyne-S-ONs and PNAs (TAg-15 sequence) in CV-1 cells. CV-1 cells were incubated with 1 μM of fluorochrome-conjugated oligomers: PNA (**A**) and propyne-S-ON (**B**), for 5 h in DMEM containing 10% fetal bovine serum, prior to visualization of oligomers in live cells by confocal microscopy. Approximately 10 μM of a solution containing flurochrome-tagged PNA (**C**) or propyne-S-ON (**D**) was injected into the cytoplasm of CV-1 cells (estimated intracellular dilution is 1:10 for cytoplasmic injections) and localized within seconds to the cell nucleus.

MATERIALS AND METHODS

ON, RNA and PNA synthesis

PNAs were synthesized as described (11), propyne-S-ONs and propyne 2′-*O*-allyl ON were synthesized as described (9), custom-synthesis DNA was obtained from Synthecell (Columbia, MD), and RNA from Integrated DNA Technologies (Coralville, IA). DNA and RNA purity was checked by capillary zone electrophoresis (CZE), and PNA purity was checked by RP-HPLC. ON and PNA identities were performed by electrospray mass spectral analysis.

Cells, plasmids and microinjection assay

CV-1 cells (ATCC, Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal calf serum. The SV40 T Ag expression vector plasmid has been previously described (9). Antisense target sequences were cloned into the sites indicated in Figure 3 by using duplex oligomers with the appropriate restriction enzyme compatible ends. DNA sequence analysis was performed to confirm faithful incorporation of the target sequences into the T Ag gene. The CV-1 cell microinjection assay has been previously described (9,11) and was slightly modified [pRSV ß-gal was replaced with pCMVβ expression vector (Clontech Laboratories, Palo Alto, CA) and used at 5–10 copies per cell]. CV-1 cells were plated at a density of 4×10^4 cells per well [2-well glass chamber slide (Nunc Inc., Naperville, IL)] 48 h prior to injection. Following injections, cells were incubated 4.5–6 h, fixed, stained and analyzed as previously described (9,11). For detection of ß-gal and T Ag protein, as well as the subcellular localization of fluorochrome-conjugated ONs/PNAs, we used a Zeiss Axiovert 35M microscope (32×/0.4 phase) equipped with an epifluorescence illumination system [flourescein filters (450–490/FT 510/LP 520), Texas red filters (BP 546/FT 580/LP 590)]. Photomicroscopy was performed using a Leica CLSM (40×/1.3 NPL fluotar). Photomicrographs were obtained using Kodak Ektachrome 100 film. A statistical analysis was performed to determine the optimal conditions for T Ag and β-gal gene expression, considering both the onset of expression and plasmid copy number per cell. Sixteen experiments were performed without antisense oligomers, varying incubation time after injections, ratio of T Ag to β-gal plasmid copy number, and number of cells injected. β-gal expressing cells were scored as either positive, +/–, or negative for T Ag expression. Percent inhibition was calculated as follows: $\%I = 100 - 100 \times \{ [\#\,T\,Ag\,(+) + 0.5 \times \#\,T\,Ag\,(+/-)]/\#\,ß\text{-}gal\,(+)\}$. The results of these experiments showed that 15 +/– 8% of the cells expressing ß-gal were not co-expressing T Ag, and the optimal incubation time was 4.5–6 h.

Kinetic studies

All T_m measurements were done at 5 μM total strand concentration (e.g. 3.33 μM PNA + 1.66 μM DNA for 2:1 complexes), in 20 mM NaH_2PO_4, 140 mM KCl, 1.0 mM $MgCl_2$, pH 7.40. The measurements were done on a Gilford Response II spectrophotometer with a six-position water-heated microcuvette thermal accessory unit, at 0.1°/min setting in three successive steps; 10–90°, 90–10° and 10–90° after equilibrium had been attained at each step, as monitored by no further changes in optical density. Samples were 200 μl volumes in 1 cm pathlength microcuvettes (Helma 110-QS, Jamaica, NY) that were tightly sealed. Buffer salts were from Sigma (St Louis, MO).

RESULTS

Peptide nucleic acids have similar cell permeation characteristics to propyne-S-ONs

We analyzed the cell permeation characteristics of a fluorescein derivatized 15mer propyne-S-ON, [5′-$(TC)_5T_5$-FITC-3′] (T Ag 15) and a FITC-tagged 15mer PNA [N-FITC-$(TC)_5T_5$-K#-C (where N and C refer to the terminal ends of the peptide and K# is an abbreviation for a terminal lysine cap)]. CV-1 cells were incubated with 1 μM of each oligomer for 5 h. Following incubation, the subcellular distribution of these compounds was assessed by viewing live cells using a laser-based confocal microscope. As shown in Figure 1A and B, both compounds were localized in cytoplasmic vesicles indicative of endosomes and lysosomes, and were not detectable in the nucleus. With extended incubation times, increasing the concentrations of the oligomers, or using multiple cell types (WI38, Rat1, HAL35 and CREF cells, for example), no change in this distribution pattern was observed (data not shown). Using these cell-feeding conditions we have never observed gene-specific antisense effects using either PNAs or modified ONs (data not shown). Based on these results, it has been our assessment that antisense compounds are not freely available for binding to RNA once they have been internalized by endocytosis.

In contrast to these findings, when CV-1 cells were cytoplasmically injected with FL-S-ON or FITC-PNA, we observed a rapid (within seconds) redistribution of these compounds into the cell nucleus (Fig. 1C and D). It is interesting to note that both PNAs and ONs localize to specific compartments in the cell nucleus, with complete nucleoli exclusion. We confirmed that for the cell feeding and microinjection studies, the molecular integrity of the PNA and ON were retained when these compounds were internalized, based on analysis by capillary zone electrophoresis (data not shown). Therefore, these findings cannot be explained by an uncoupling of the fluorochrome-tag and the ON or PNA.

Peptide nucleic acids are less potent than propyne-S-ONs, and their antisense effect is not as prolonged

Based on the results of the cell-feeding studies, we used a cell-based microinjection assay to assess the relative antisense potency of both classes of oligomers. A novel antisense target sequence was cloned into the SV40 T Ag gene, such that it was localized to the 5′ untranslated region (5′ UT) of the T Ag mRNA. CV-1 cell nuclei were microinjected with a mixture containing five copies of the T Ag expression vector which encoded the target sequence, five copies of a CMV-ß-gal expression vector [to identify successfully injected cells and to assess antisense specificity (9)] and various concentrations of the appropriate antisense agent. Following injection, the cells were incubated for 6 h, then fixed and stained for T Ag and ß-gal expression, as previously described (9,11). Following an assessment of both ß-gal and T Ag expressing cells (at least 100–200 per experiment), we calculated the percentage of T Ag inhibition based on a ratio of T Ag to ß-gal expressing cells. As shown in Figure 2A, the propyne-S-ON was able to effectively inhibit T Ag expression, with 70% inhibition at 100 nM. Likewise, the PNA was able to specifically inhibit T Ag expression, although it was less effective (57% inhibition at 1 μM). Finally, a C-5 propynyl pyrimidine 2′-*O*-allyl modified antisense ON (propyne 2′-*O*-allyl ON) was the least effective in this assay (55% inhibition at 5 μM).

To begin to understand the molecular basis for this variation in antisense agent potency, we derived dose–response curves for the propyne-S-ON and the PNA (Fig. 2A). While the PNA was effective at inhibiting T Ag expression, the specific dose range of activity was narrow. At doses >5 μM (7 μM for example), non-specific inhibition of ß-gal expression was observed. At doses <0.5 μM, no antisense effects were seen. In contrast, the propyne-S-ON had a much broader range of dose-dependent activity and did not show non-specific inhibition of ß-gal up to 5 μM.

We next asked how long the antisense effect could persist following a single dose of the antisense agent. For this study, CV-1 cell nuclei were injected, and at the times indicated in Figure 2B, the cells were fixed, stained and quantitated for T Ag and ß-gal expression. When a 2 μM dose of the PNA was used, the antisense effect persisted for 48 h, but had declined by 2-fold. A similar time course was observed when the propyne-S-ON was used at a 0.2 μM dose. However, when the ON dose was increased to 0.5 μM, complete inhibition of T Ag expression persisted for 48 h. While these differences could reflect variations in persistence of the PNA versus the propyne-S-ON in the cell, we found that both persisted for similar times using a fluorescence assay similar to that previously described (4) which measured the disappearance of fluorescent molecules from cells (data not shown).

From the above studies we concluded that the propyne-S-ON had superior gene inhibitory properties as compared to the PNA. We envision that the enhanced activity of the propyne-S-ON may be a result of one of the following mechanistic hypotheses. (i) RNase H may cleave the ON/RNA duplex and liberate the ON to rehybridize to T Ag RNA, thus allowing the ON to act in a 'catalytic' fashion and therefore at lower concentrations than the PNA. (ii) The PNA may rapidly dissociate from the RNA in the cell to permit translation, whereas the propyne-S-ON forms a stable enough complex to allow RNase H cleavage to occur prior to its dissociation. (iii) The PNA may associate with the RNA at a decreased rate relative to the propyne-S-ON. In previous studies we showed that PNAs were incapable of forming a substrate with RNA for RNase H cleavage (11). To address the association rate of PNAs with RNA, we conducted *in vitro* binding studies as described below.

PNAs display much slower on-rate kinetics than ONs

We measured the kinetics of association of either the PNA or propyne-S-ON with RNA by monitoring the temperature dependence of UV absorbance changes at 260 nm. The PNA was shown to form a 2:1 complex with a complementary RNA, with slow

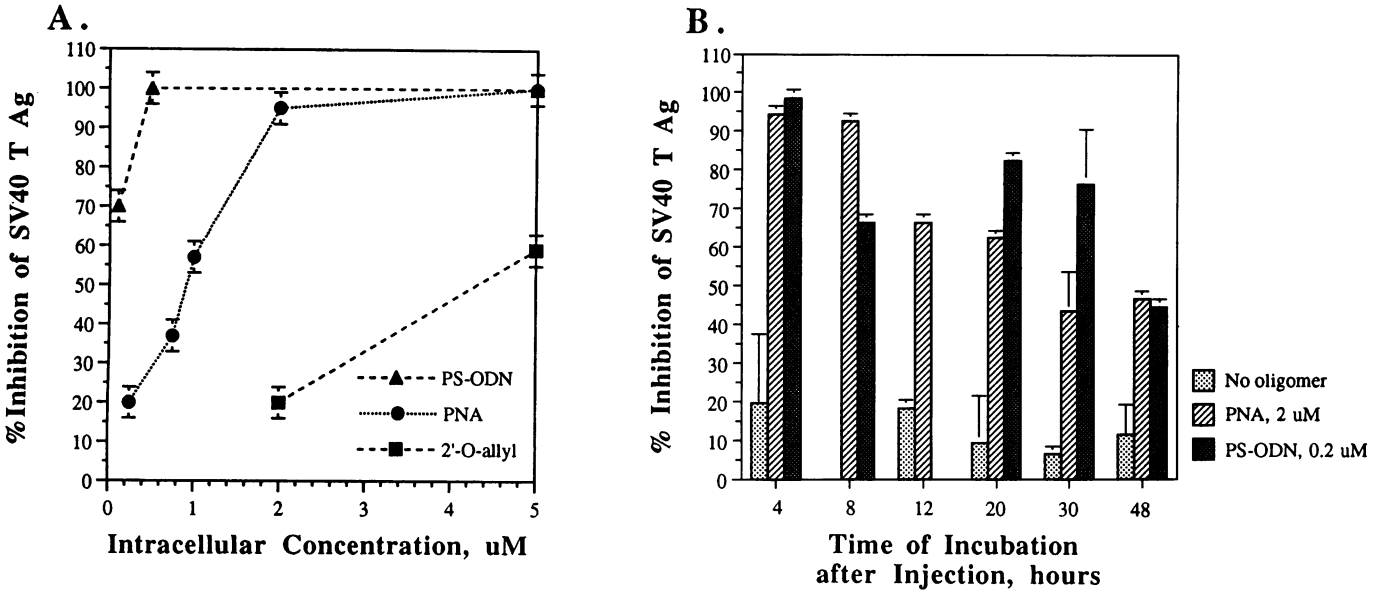


Figure 2. (A) CV-1 cells were microinjected in the nucleus with five copies of the T Ag gene containing the antisense target sequence, five copies of a CMV β-galactosidase expression vector as an indicator of injected cells, and several concentrations of antisense oligonucleotides (estimated intracellular concentration is 1/20 of needle concentration, for nuclear injections). Cells were then incubated for 6 h, formaldehyde fixed, and immunostained for T Antigen and β-gal expression. Inhibition of T Ag was calculated as a ratio of β-gal expressing cells. (**B**) Persistence of antisense inhibition effect: CV-1 cells were microinjected as in (A), but were allowed to incubate for the indicated length of time before formaldehyde fixation. Immunostaining and calculation of inhibition of T Ag expression is as in (A).

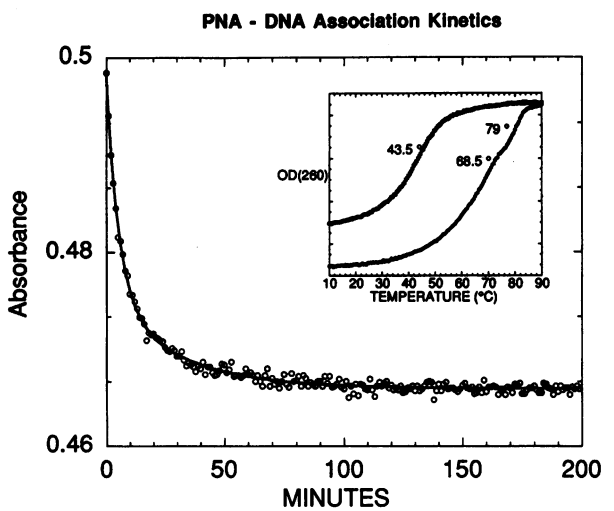


Figure 3. T_m analysis and rate of association of PNA•DNA heteroduplexes. The T Ag-15mer PNA (5 μM) and d(AAAAAGAGAGAGAGA) (5 μM) were hand-mixed at a volume ratio of 2:1 for a 5 μM final concentration of total strands, at 25°C. Complex formation was monitored by UV absorbance (260 nm) and fit by non-linear least-squares (R^2 = 0.995, line drawn through data points) to a two-exponential sum with rates of 0.192 and 0.033/min. Insert: UV melting curves of the same sample. The lower curve records UV trace upon heating (T_m = 68.5, 79°C) and the upper curve records the cooling trace (T_m = 43.5°C) of the same sample. The temperature program rate (0.5°/min) is not sufficiently slow to track the equilibrium of the PNA–DNA association–dissociation processes. Several hours are required (at 10°C) for the UV absorbance to return to its original value, and subsequent heating–cooling cycles were reproducible, indicating complete reversibility.

binding kinetics leading to profound hysteresis in the melting curve (Fig. 3). In fact it required >150 min for the PNA reaction to reach equilibrium at 25°C. In contrast, using a propyne-S-ON, the reaction was complete in <30 s at the same concentration. Under these conditions the propyne-S-ON formed a 1:1 complex with a complementary RNA.

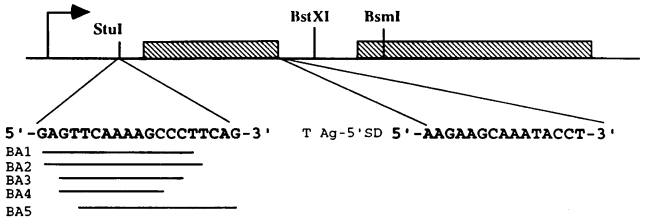
These *in vitro* findings suggested two insights into the mechanism of PNA antisense effects. One was that the active complex of the PNA with RNA may be 2:1 PNA to RNA. The second was that if these findings can be extrapolated to the situation in the cell, then the poor potency of the PNA compared to the propyne-S-ON may reflect its poor association kinetics.

Peptide nucleic acids show activity when targeting a variety of sites

Using the schemes outlined in Figure 4A, we next determined if the antisense activity of the PNA was restricted to the 5′ UT of the T Ag mRNA, as had been suggested for steric-blocking ONs (15). The T Ag-15 target sequence was cloned into three regions of the SV40 T antigen gene (*Stu*I, *Bst*XI and *Bsm*I sites). We first confirmed that introducing this sequence had no impact on the expression or half-life of the T Ag protein (data not shown). Using the cell microinjection assay, we found that both intronic, exonic and 5′UT sites were all equally susceptible to antisense inhibition by the complementary propyne-S-ON. In contrast, the 2′-*O*-allyl modified ON was active only when it was targeted to the 5′UT. Similarly, the PNA most effectively inhibited T Ag expression when it was targeted to the 5′ UT (see Fig. 4B). However, in contrast to the 2′-*O*-allyl ON, the PNA was found to partially inhibit T Ag expression when targeted to intronic and exonic regions. The intron result suggests that the steric blockade due to PNA•RNA heteroduplex formation could prevent RNA processing or subnuclear transport.

In another series of studies we were able to confirm that a PNA could block T Ag expression by targeting an RNA that is only present in the nucleus (hnRNA). In this study, a PNA was synthesized that was complementary (N-AGG-TAT-TTG-CTT-CTT-C) to the T Ag 5′ splice donor sequence (T Ag 5′ SD).

A. SV40 T Ag
Target Sequences:



B.

Oligomer	Target Site	% Inhibition
PS-ODN (0.1uM)	StuI	70
	BstXI	75
	BsmI	55
2'-O-allyl PS-ODN (5uM)	StuI	59
	BstXI	(-)
	BsmI	(-)
PNA (TAg-15, 2uM)	StuI	99
	BstXI	68
	BsmI	60
PNAs (5 uM)		
BA1	StuI	94
BA2	StuI	76
BA3	StuI	91
BA4	StuI	74
BA5	StuI	80
5' SD (0.5 uM)	5' Splice site	88

Figure 4. (A) Antisense target sequences were cloned into sites along the SV40 T Ag gene in the 5′ UT (*StuI*), the intron region (*BstXI*) or the second exon (*BsmI*). All target sites contained the sequence 5′-AAAAAGAGAGAGA-3′ (T Ag-15), [except as indicated for the BA sequences and the T Ag 5′ SD (splice donor) site]. (B) Microinjection assays were performed as in Figure 2A, using the T Ag plasmid containing the target sequence at the appropriate site.

Co-injection of 0.5 μM of this compound with the T Ag and ß-gal expression vectors into CV-1 cell nuclei resulted in 88% inhibition of T Ag expression. Interestingly, this PNA was shown to bind to its complimentary RNA sequence as a 1:1 complex (data not shown).

PNAs discriminate mismatches in the target sequence

The high affinity of PNAs for a complementary RNA raises the question of whether PNA•RNA heteroduplexing would be sensitive to mismatches. The PNA target sequence containing the single base pair mismatch shown in Figure 5, was cloned into the *StuI* site in the 5′ UT region of the T Ag gene. Cell microinjection studies were performed as before, and we assessed PNA antisense inhibition of T Ag expression at the dose-range shown. When one base pair mismatch was introduced, we observed a 40% decrease (at a 2 μM PNA dose) in antisense activity, when compared to the wild-type control (see Fig. 5). The T Ag-15 propyne-S-ON showed a depression in antisense activity at 0.1 μM (29% reduction), when compared to the wild-type control. Interestingly, at 0.5 μM of the propyne-S-ON, we no longer observed a discrimination between the mutant and wild-type target sequences.

DISCUSSION

The inactivation of gene expression mediated by antisense ONs and PNAs represent an important means by which one can modulate gene levels in a therapeutic setting. Chemical modifications of the ON base, sugar and backbone residues coupled with an assisted delivery vehicle, such as liposomes, have lead to significant improvements in agent stability, bioavailability and target recognition (1,15). Because of these improvements, we can now ask questions relating to the mechanism of the antisense effect.

In this paper we have sought to determine whether ONs that have the potential ability to catalytically inactivate a targeted gene are more efficient/potent than PNAs which act in a steric-blocking manner. Like ONs, we found that PNAs are sequestered in cytoplasmic endosomes when incubated with many cell types in culture. This may be due to the high degree of polarity of the peptide bond in the PNA and likely reflects the inability of most peptides to freely enter cells. Furthermore, we determined that the cytoplasmic sequestering of both PNAs and ONs correlated with an inability of these agents to provide an antisense effect. Therefore, we chose to compare the antisense activities of both agents using a cell-based, SV40 T Ag microinjection assay (9). The virtues of this assay have been previously described (9) and principally include both a quantitative analysis of gene expression together with controls to show the specificity of the antisense effect. For the purpose of this study, we targeted artificial sequences cloned into the T Ag gene; however, it is possible that we would have observed even greater inhibition had we surveyed the T Ag gene with a series of antisense PNAs designed to pinpoint regions of the RNA which were most sensitive to antisense effects (12,21).

Based on an analysis of the data shown in Figure 2, the propyne-S-ON showed superior antisense effects compared to the PNA. These desirable antisense properties included a lower dose requirement for activity, a broader range of activity, a longer duration of action and a higher degree of target specificity. The latter is supported by the relative effects both agents have on an RNA encoding β-gal, which did not contain the antisense target. In addition, the propyne-S-ON showed antisense effects when the target sequence was placed in a variety of sites in T Ag, confirming earlier observations that the potency of the propyne-S-ONs is relatively independent of RNA target choice (9).

PNAs, while not showing the potency of the propyne-S-ON, clearly outperformed a steric blocking ON which also has high affinity for RNA (9; BF and RW, unpublished results), e.g. the propyne 2′-*O*-allyl ON. When compared with this ON, the PNA showed more potent inhibition and inhibited TAg at a greater variety of target sequences including exonic/intronic regions of the T Ag hnRNA or mRNA. We believe that the PNAs are unique among the steric block agents in their ability to interrupt translation of an RNA by targeting the intron and coding regions. This may be due to a property of the sequence which was used in this study, since it was shown that this PNA could form a 2:1 complex with RNA under *in vitro* conditions. While we do not have any direct evidence, it is possible that the active complex in the cell is a 2:1 complex with RNA which, in turn, forms a more stable complex than other steric blocking agents. However, we determined that a mixed sequence PNA targeted to the T Ag 5′ splice donor site (Fig. 4) forms a 1:1 complex with RNA, and was able to similarly inhibit T Ag expression.

The differences in the potency of the PNA versus the propyne-S-ON could be a reflection of their respective rates of dissociation

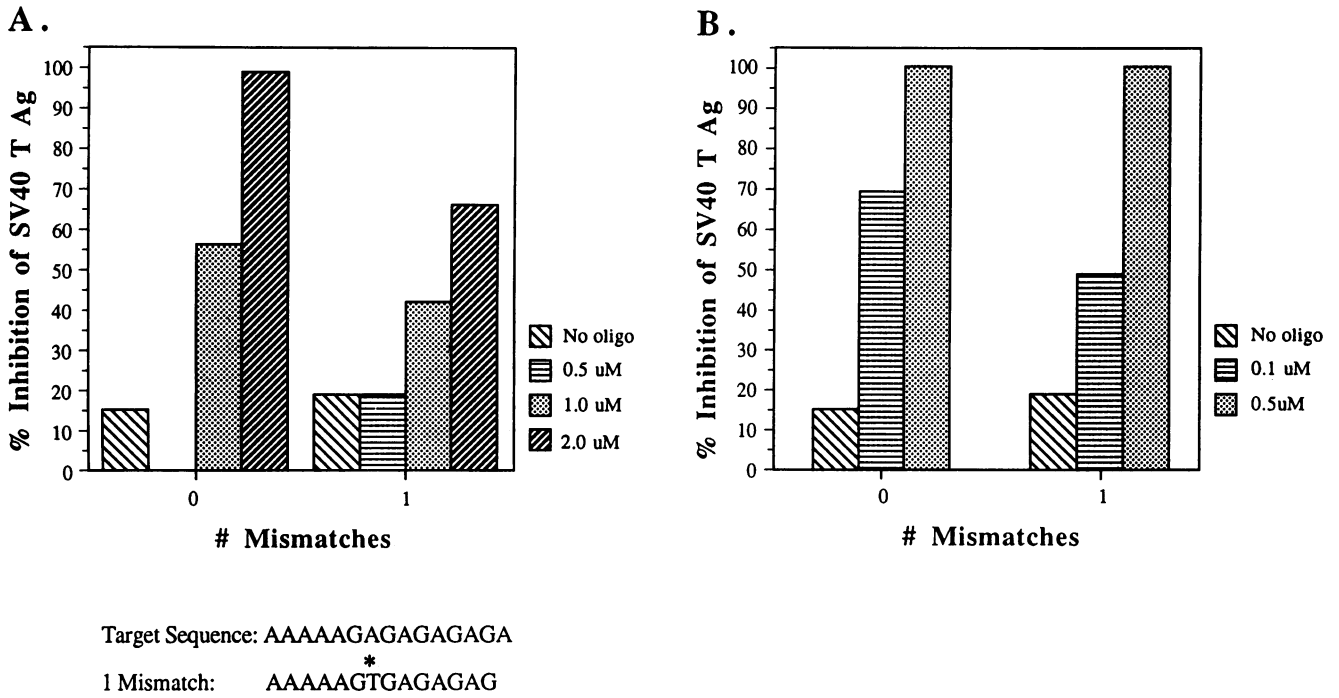


Figure 5. Oligonucleotides containing the wild-type or mutant versions of the antisense target sequence were cloned into the 5″ UT of the SV40 T Ag gene. Microinjection asssays were performed as in Figure 2A, using antisense PNAs (**A**) or propyne-S-ONs (**B**).

or association. It has recently been shown that when a propyne-S-ON forms a complex with RNA it results in complete suppression of the RNA in the cell, which likely reflects the susceptibility of the complex to RNase H. Hence, the dissociation rate in the cell cannot be measured (RW, unpublished results). We currently do not know the dissociation rate of the PNA/RNA duplex in the cell. If the rate is faster than that of the propyne-S-ON, we would predict that an RNase H-inactivating functionality is an important feature for the most potent antisense agents.

Our *in vitro* studies clearly showed that the rate of association for the PNA to RNA was significantly slower than that of the propyne-S-ON, and this may reflect the relative rate of association in the cell. In this light, it is interesting to note that when both PNAs and propyne-S-ONs are microinjected into CV-1 cells, rapid nuclear localization occurs (Fig. 1). It is likely that the bulk of the fluorescent signal in the nucleus is the result of the ON/PNA being in a bound state with non-specific receptors. As a consequence, the pool of available antisense compound is likely to be much less than would be predicted based on dose. In a preliminary series of experiments, we found that a PNA 11mer targeted to the 5′ UT of the T Ag mRNA was not active at 0.3 μM. However, when the same amount of antisense PNA was co-injected with 1.5 μM of a nonsense PNA oligomer, we observed >70% inhibition of T Ag protein expression. The nonsense PNA alone (1.5 μM) had no impact on T Ag expression. It may be that the target-binding concentration of a PNA is only achieved once non-specific intranuclear sites are saturated. We can conclude from our studies that the rate of association of a PNA to its complementary RNA sequence appears to be a limiting factor for its potency.

While PNAs were less effective than propyne-S-ONs at inhibiting T Ag protein expression, a non-propynylated-S-ON of the same sequence showed no activity in our assay (data not shown). Since the T_m of the S-ON versus the propyne-S-ON was reduced by ~23°C, this result was not surprising. However, we reasoned that substitution of the PNA bases with propyne might lead to a similar increase in agent potency. A fully-propynylated PNA of the sequence [$(TC)_5T_5$-K#] was synthesized and tested in the cell microinjection assay. While an antisense effect was observed, there was no increase in activity when compared to the unsubstituted PNA (data not shown). It is possible that in the RNA•propyne-S-ON heteroduplex, the propyne groups stack upon one another and this hydrophobic interaction stabilizes the duplex. In contrast, the helix formed in the 2:1 PNA•RNA complex may preclude these interactions from occurring.

In conclusion, we have assessed the antisense characteristics of ONs and PNAs. While both compounds were mechanistically capable of providing an antisense effect, the propyne-S-ON showed the most favorable antisense effects. PNAs showed superior antisense effects as steric blocking agents, which may be explained by their ability to form 2:1 complexes with RNA; however, their limited potency currently precludes their use as effective antisense agents. With continued efforts to constrain the PNA backbone to afford faster kinetics of RNA binding, the PNA has the potential to emerge as a significant antisense inhibitor of gene expression with potential therapeutic applications.

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