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Inhibition of Human Immunodeficiency Virus Type-1 *env* Expression by C-5 Propyne Oligonucleotides Specific for Rev-Response Element Stem–Loop V†

Steven D. Fenster,‡ Richard W. Wagner,§ Brian C. Froehler,§ and Daniel J. Chin*,‡

The Agouron Institute, 505 Coast Boulevard South, La Jolla, California 92037, and Gilead Sciences, 353 Lakeside Drive, Foster City, California 94404

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ABSTRACT: The binding of Rev to the Rev-response element (RRE) of the human immunodeficiency virus (HIV) is essential for RNA transport and expression of structural proteins such as gp160 encoded by *env*. To determine if *env* expression could be disrupted by complementary oligodeoxynucleotides (ODNs), band-shift studies were used to identify RRE sites that are essential for the formation of Rev–RRE complexes [Chin, D. J. (1992) *J. Virol. 66*, 600–607] or the stability of preformed complexes. In this report, we describe complete disruption of preformed Rev–RRE complexes by a subset of 15 ODNs complementary to stem–loop V. The most potent ODN complementary to bases CUGGGGCAUCAAGC disrupted 50% of preformed complexes at 1.2 μM, a 400-fold molar excess over the RNA. Expression of *env* in COS7 cells was blocked by nuclear microinjection of ODNs with C-5 propyne-modified pyrimidines and phosphorothioate linkages. Inhibition was highly dependent upon RNA target position, internucleotide chemistry, ODN sequence, and concentration. Unmodified phosphodiester or phosphorothioate ODNs were inactive. For the most potent ODN, 50% of the injected cells' *env* expression (I_{50}) was blocked with 0.1 μM. A translational block is unlikely since these ODNs blocked expression of a luciferase vector in which the RRE was placed downstream of the termination codon. Consistent with their *in vitro* effects upon Rev–RRE complexes, stem–loop V ODNs were 9-fold more active than stem–loop II ODNs in blocking *env* expression while having a reduced (I_{50} = 0.27 μM) but equivalent potency against luciferase–RRE. These results suggest that disruption of Rev–RRE complexes may assist in blocking *env* expression.

The use of antisense oligodeoxynucleotides (ODNs)[1] (Ghosh et al., 1992) is a gene-specific strategy to treat neoplasms and viral infections. The rapid therapeutic deployment of such reagents must overcome questions of RNA-binding specificity, stability to nucleases, and efficient delivery into cells (Bennett et al., 1992; Stein et al., 1993; Milligan et al., 1993). The cell membrane remains a major barrier to effective delivery of exogenous nucleic acids which are typically added or transfected at high extracellular concentrations to achieve an effect. Numerous studies have described the antiviral activity of antisense ODNs targeted to the translation initiation region, 5′-untranslated regions, coding regions, or splice junctions of RNA (Matsukura et al., 1989; Lisziewicz et al., 1992; Ropert et al., 1992; Li et al., 1993). Such studies often use phosphorothioate (PS) ODNs due to their nuclease resistance (Zon, 1988). However, the efficacy of such compounds is usually low, and nonspecific effects (Zon, 1988; Cazenave et al., 1989; Gao et al., 1990; Ho et al., 1991; Kinchington et al., 1992) may account for reports of both efficacy and inactivity of PS ODNs (Kirkland et al., 1993).

RNA target selection for antisense ODNs remains largely empirical. Often based upon translational mechanisms established in *Xenopus* (Harland et al., 1985; Kawasaki, 1985), few antisense reports in mammalian cells have optimized target selection and shown specific reductions in the target RNA or protein. Improved efficacy and high-affinity interactions with target RNA may be achieved by a combination of novel oligonucleotide modifications (Egholm et al., 1993; Wagner et al., 1993) and selection of an essential RNA target(s) with conserved sequence, structure, or function (Bordier et al., 1992; Li et al., 1993). Such a strategy might utilize multiple, sequence-specific mechanisms for inhibiting gene expression: from cleavage of the hybrid by endogenous RNase H activity to disruption of splicesome or nuclear protein–RNA interactions or blocking mRNA translation. In this report, we have addressed the issues of potency, target selection, and ODN chemistry in our continuing optimization of human immunodeficiency virus (HIV) mRNA targets.

We focused on a conserved mRNA region essential for the function of the HIV-encoded regulatory protein, Rev, that facilitates the cytoplasmic appearance of viral mRNAs that encode structural proteins essential for viral propagation (Sodroski et al., 1986; Felber et al., 1989; Malim et al., 1989). Rev binds to a 234-nt Rev-response element (RRE) within viral RNAs containing *env* and may either disrupt aberrant splicesome–HIV RNA interactions or directly facilitate RNA transport (Malim et al., 1988). A predicted RRE structure of 4–5 minor stem–loops attached to a major double-stranded stem (Malim et al., 1989; Le et al., 1990) is consistent with RNA mapping studies (Kjems et al., 1991; Chin, 1992). A region in stem–loop II, nt 7818–7824 and 7840–7848, designated IID (Bartel et al., 1991), was identified as the primary Rev binding site (Bartel et al., 1991; Holland et al., 1990; Olsen et al., 1991; Tiley et al., 1992). In contrast, stem–loops III–V play only a minor role in supporting Rev-mediated RNA transport (Benko et al., 1990). While deletion of stem–loop V resulted in either weak, 35–58% of unmodified RRE

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* To whom correspondence should be addressed at The Agouron Institute, 505 Coast Blvd S., La Jolla, CA 92037; (619) 456-5315 (tel); (619) 456-5339 (Fax); djchin@dsrna.agi.org (Internet).

‡ The Agouron Institute.

§ Gilead Sciences.

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[1] Abbreviations: HIV, human immunodeficiency virus; RRE, Rev-response element; ODN, oligodeoxynucleotide; stem–loop II, SLII; stem–loop V, SLV; nt, nucleotide.

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activity (Benko et al., 1990; Holland et al., 1990; Malim et al., 1990; Dayton et al., 1992), or little effect, 90–100% of unmodified RRE activity (Dayton et al., 1989; Olsen et al., 1990), upon RRE function, mutations that were predicted to disrupt stem–loop V led to functional inactivation (Dayton et al., 1989). Collectively, these results suggest that an undisturbed stem–loop V is important for full and efficient RRE function.

We previously described *in vitro* inhibition of the Rev–RRE complex by complementary oligodeoxynucleotides (ODNs); certain ODNs encompassing stem–loops IIA–IID prevented Rev–RRE binding, and one stem–loop V (SLV) ODN altered the migration pattern of preformed Rev–RRE complexes (Chin, 1992). Thus, an appropriate SLV ODN might prevent Rev-mediated RNA transport in cells as efficiently as an inhibitor of Rev–RRE binding. In contrast to the weak effects of an initial stem–loop V ODN in our earlier report (Chin, 1992), herein we (i) identify SLV ODNs that completely disrupt preformed Rev–RRE complexes *in vitro* and (ii) correlate this activity to strong inhibition of RRE-containing mRNAs in COS7 cells. To ensure traversal of the membrane barrier and high-affinity interactions of ODNs with the target RNA, we microinjected COS7 nuclei with C-5 propyne-modified ODNs with phosphorothioate linkages (Wagner et al., 1993) and observed potent, complete inhibition of *env* expression. We discuss possible explanations for the site- and chemistry-dependent efficacy of oligos targeted to the RRE.

MATERIALS AND METHODS

Materials. Unlabeled ribonucleoside 5′-triphosphoates and ribonuclease H were purchased from Pharmacia (Piscataway, NJ), and [α-^{32}P]GTP was obtained from DuPont NEN (Boston, MA). Purified Rev was obtained from either Z. Hostomsky of Agouron Pharmaceuticals (La Jolla, CA) or C. Nalin at Roche (Nutley, NJ). The former preparation was used for all ODN optimization studies reported here. Inhibit-Ace was purchased from Five-Prime Three-Prime (Boulder, CO), and T7 RNA polymerase was obtained from US Biochemical (Cleveland, OH). An anti-β-galactosidase monoclonal antibody was purchased from Boehringer Mannheim (Indianapolis, IN), and fluorescent secondary antibodies were obtained from Accurate (New York, NY). A goat anti-HIV-1 SF2 gp120 antibody was obtained from Ogden BioServices (Gathersburg, MD), and a rabbit anti-luciferase antibody was purchased from Cortex (San Leandro, CA). Opti-MEM and lipofectin were purchased from Life Technologies (Gaithersburg, MD). COS7 cells were obtained from the American Type Culture Collection (Bethesda, MD). Cab-o-sil was obtained from Kodak (Rochester, NY). C-5 propyne-modified pyrimidine ODNs were prepared and purified as described (Wagner et al., 1993).

Plasmids. A chimeric luciferase–RRE vector was constructed by inserting the HIV1-SF2 RRE downstream of the translation termination codon of RSV luciferase (de Wet et al., 1987). Briefly, the 3′ untranslated region of RSV luciferase DNA was deleted and mutagenized with primer oligonucleotides[2] using the polymerase chain reaction (Mullis et al., 1987) to introduce *Bgl*II and *Kpn*I sites 15 nt 3′ of the luciferase translation termination codon. A 364 *Eco*RV–*Kpn*I fragment was isolated and subcloned into an *Eco*RV–*Kpn*I-digested RSV luciferase vector to yield pL3m. Subsequently, the *Bgl*II–*Kpn*I RRE fragment of pR1 (Chin 1992) was subcloned into *Bgl*II–*Kpn*I-digested pL3m to yield pLR.

Rev–RRE Gel-Shift and RNase H Assays. ^{32}P-Labeled RRE RNA was prepared, gel-purified, and used in band-shift and RNase H assays as previously described (Chin, 1992). For the gel-shift assay, 12–25 fmol of ^{32}P-labeled RRE RNA was incubated with 1 pmol of Rev in 10 μL of 20 mM Hepes, pH 7.3, 80 mM KCl, and 0.5 unit of Inhibit-Ace for 20 min at 37 °C prior to the addition of 10–600 pmol of ODN. Incubations were continued for 20 min at 37 °C and subjected to electrophoresis in nondenaturing gels.

Microinjection. Culture conditions for COS7 cells were described (Chin et al., 1991). All C-5 propyne ODN stocks were heated 20 min at 70 °C and renatured 15 min at 22 °C prior to serial dilution and addition to plasmid mixtures. The nuclei of COS7 cells were microinjected (Chin et al., 1991) with 0.1 mg/mL pCMV-Rev and pUCSV-9 (Peterlin et al., 1986) and 0.02 mg/mL pCMV-β-galactosidase in the absence or presence of ODNS and cultured for 24 h prior to processing for immunofluorescence (Chin et al., 1991). In luciferase–RRE expression assays, COS7 cells were injected with 0.02 mg/mL pCMV-β-galactosidase and 0.1 mg/mL pLR in the presence or absence of ODNs.

Lipofection Assays. To compare ODN delivery methods, COS7 cells were carefully washed with 2 × 5 mL in Opti-MEM, incubated 30 min prior to a second set of washes, and transfected with ODNs in 1 mL of 10 μg/mL lipofectin for 4 h at 37 °C. Lipid-depleted newborn calf serum (Weinstein, 1979) was added to 3% in a final 1.5-mL volume, and cells were grown for 15 h prior to microinjection with the mixture of *env*, Rev, and β-galactosidase plasmids described above. Under these conditions, 50–75% of COS7 cells showed nuclear accumulation of a cotransfected, rhodamine-labeled PS ODN at 0.1 μM with the sequence CATCGCGGTTGTTCC which is absent from both the β-galactosidase and HIV1-SF2 sequences. The intensity of the marker rhodamine ODN in the nucleus varied between 3- and 4-fold. After 48 h, the bulk of the nuclear rhodamine PS oligo represents less than ~1–10% of the total cell-associated rhodamine oligo after 4 h of lipofection as determined by diminishing nuclear fluorescence.

Indirect Immunofluorescence. For *env* expression assays, cells were fixed with either 3.7% formaldehyde or 2% paraformaldehyde, quenched with 50 mM glycine and 0.1 μg/mL sodium borohydride, blocked with 5% bovine serum albumin in TBS-TX (10 mM Tris-HCl, pH 8, 0.12 M NaCl, 0.25% Triton X-100), and incubated sequentially (with appropriate washes) with an anti-β-galactosidase monoclonal antibody, goat anti-HIV-1 SF2 gp120, fluorescein-labeled rabbit anti-goat antibody, 10% normal goat serum, and Texas Red-labeled goat or rabbit anti-mouse antibody. Injected cells were counted for β-galactosidase-expressing cells that coexpressed *env*. Fluorophore combinations that reversed the color-labeling scheme gave identical results. The nonspecific crossover of fluorescein (*env*) or Texas Red (β-galactosidase) emission was less than 0.1%.

For luciferase expression assays, cells were incubated with an anti-β-galactosidase monoclonal antibody, rabbit anti-luciferase and fluorescein-labeled sheep anti-rabbit, and Texas Red-labeled sheep anti-mouse antibodies.

Microscopy. Representative samples were imaged with a Noran Odyssey (Middleton, WI) confocal laser-scanning module on an inverted Nikon Diaphot (New York, NY) microscope with 60× NA 1.4 lens controlled by custom software on an Apple Macintosh IIfx (Cupertino, CA) that

[2] Primer sequences were as follows: AAAGGATATCAGGTGGCCCCCGCTGAATTG and TGCAGGTACCACGTAGATCTAAGAATTTCGTCATCGCTG.

Table 1: Essential Region of SLV Phosphodiester Anti-REE ODNs[a]

	ODNs from Stem-loop V (- strand)		
5'-	TCTTTCCACAGCCAGGACTCTTGCCTGGAGCTGCTTGATGCCCCAGACTGTG	-3'	
		10 uM*	I_{50}#
SLV-90.1	TCTTTCCACAGCCAGGAC	-	>60 uM
SLV-90.2	....TCCACAGCCAGGACTCTT	-	
SLV-90.3	.....CAGCCAGGACTCTTGCCT	-	
SLV-90.4	.....CAGGACTCTTGCCTGGAG	-	
SLV-90g	.....ACTCTTGCCTGGAGCTGC	-	30
SLV-90.5	.....TTGCCTGGAGCTGCTTGA	-	
SLV-90.6	.....CTGGAGCTGCTTGATGCC	±	
SLV-90.61	.....TGGAGCTGCTTGATGCCC	+	
SLV-90.62	.....GGAGCTGCTTGATGCCCC	++	
SLV-90.63	.....GAGCTGCTTGATGCCCCA	++	2.5
SLV-90.7	.....AGCTGCTTGATGCCCCAG	+++	1.2
SLV-90.71	.....GCTGCTTGATGCCCCAGA	+++	
SLV-90.72	.....CTGCTTGATGCCCCAGAC	++	
SLV-90.73	.....TGCTTGATGCCCCAGACT	++	
SLV-90.8	.....GCTTGATGCCCCAGACTG	++	~7.5
SLIV-79b	.....TTGATGCCCCAGACTGTG	-	>60

[a] Bases 7925–7976 of HIV-1 SF2 RRE SLV are shown at top. The listed ODNs overlapped SLV-90g, the prototype SLV anti-RRE ODN that mildly disrupted preformed Rev–RRE complexes (Chin, 1992). Results of gel-shift assays are summarized in two columns: (*) Inhibition of preformed Rev–^{32}P-RRE complex gel shifts in the presence of 10 μM ODN was scored as follows: (+++), (++), (+), and (±) correspond respectively to <10%, <25%, <50%, and 75% ^{32}P-RNA comigrating with the Rev–RRE complex in the absence of ODN. (#) ODN concentration required to redistribute ^{32}P-RRE RNA equally between the gel migration of free ^{32}P-RRE and Rev–^{32}P-RRE complexes. Missense ODNs SLV-90.7MS (GGCTCG-GCAGTCTCCAAT) and SLV-90.63MS (CCAGCTTCGGTACGACGT) had no effect upon the migration of Rev–RRE complexes (not shown). None of the ODNs had any specific binding to Rev alone (^{32}P-labeled ODNs did not bind to Rev in either nondenaturing gels or solid-phase "dot" blot assays, not shown).

integrated control of the Odyssey with a Perceptics PixelPipeline (Knoxville, TN) frame-grabber board. Routine scoring of injected cells was performed on a Nikon Microphot (New York, NY) microscope with epi-fluorescence at 40× NA 1.3 and an intensified CCD camera. Digital capture of images was performed with Nuvision hardware and software from Perceptics (Knoxville, TN). Calibrated neutral-density filters from Omega Optical (Brattleboro, VT) were used to assess partial inhibition at constant illumination and camera gain and black levels (Chin et al., 1989). The public domain software NIH Image, written by Wayne Rasband (NIH, MD), was used to annotate images and perform densitometry for subsequent calculation of intensity ratios.

RESULTS

To optimize disruption of Rev–RRE complexes by SLV ODNs (Chin, 1992), a series of unmodified 18-mer SLV ODNs were screened (Table 1). ODNs containing bases complementary to nt 7951–7958 of SLV showed complete Rev–RRE complex disruption in gel-shift studies (Table 1 and Figure 1B). Missense ODNs with a randomized order were ineffective in both the gel-shift assay and RNase H assays (not shown). In Table 1, dose–response curves with selected ODNs showed 50% disruption of preformed complexes at 1.2 μM (for ODN SLV-90.7, which represents a 400-fold molar excess over the ^{32}P-RRE RNA) and 8 μM (for SLV-90.8). However, both ODNs were indistinguishable in their ability to support *Escherichia coli* RNase H digestion of ^{32}P-RRE RNA (not shown); hence differences in target accessibility cannot explain the potency variation in the gel-shift assay. It is possible that the 5′-flanking nt of SLV-90.7 are more important for maintaining the stability of preformed Rev–RRE complexes.

Disruption of preformed Rev–RRE complexes by SLV ODNs was temperature dependent. ODNs SLV-90g and SLV-90.7 were more inhibitory at 37 °C than at 4 °C (Figure 1C). Thus, Rev–RRE complexes formed at different temperatures show similar gel shifts but vary in their susceptibility to disruption by ODNs (Figure 1C) as was found with SLII ODNs (Chin, 1992).

The disruption of preformed Rev–RRE complexes by SLV ODNs *in vitro* suggested a potential HIV-1 target to test in cultured cells expressing *env*. We microinjected[3] the nuclei of COS7 cells with mixtures of expression vectors for HIV-1 *env*, Rev, and β-galactosidase, an internal control, in the absence or presence of SLV ODNs (Figure 2), and determined the number of injected cells expressing *env*. With plasmids alone, ~90% of injected cells expressed both β-galactosidase and HIV-1 *env*. Gene-specific and dose-dependent inhibition of *env* expression was found when antisense RRE ODNs were coinjected (Figure 3).

Inhibition of HIV-1 *env* expression by anti-RRE ODNs was dependent upon ODN modifications. Phosphodiester SLV ODNs were inactive (not shown) while phosphorothioate backbone ODNs with unmodified pyrimidines (18-mer SLV-90.7 and SLV-90.63) weakly reduced *env* expression, even when microinjected at 30 μM (Figure 4). In contrast, SLV ODNs (18-mer SLV-90.7 and SLV-90.8) synthesized with C-5 propyne-modified pyrimidines and PS backbones were potent, I_{50} ~0.1 μM intracellular, inhibitors of *env* expression (Figure 3). Similar potency values of injected C-5 propyne-modified ODNs were found for other targets (Wagner et al., 1993). Since ^{32}P-labeled propyne SLV ODNs did not bind Rev *in vitro* (data not shown), the inhibitory effect of SLV ODNs upon *env* expression is not an aptamer effect, i.e., a result of ODN binding to Rev.

[3] Transfection by nuclear injection was performed since $CaPO_4$ or cationic lipid transfection of plasmid mixtures gave heterogeneous expression; subpopulations expressed only β-galactosidase or HIV-1 *env*, and only ~33% of cells expressed both *env* and β-galactosidase in control transfections.

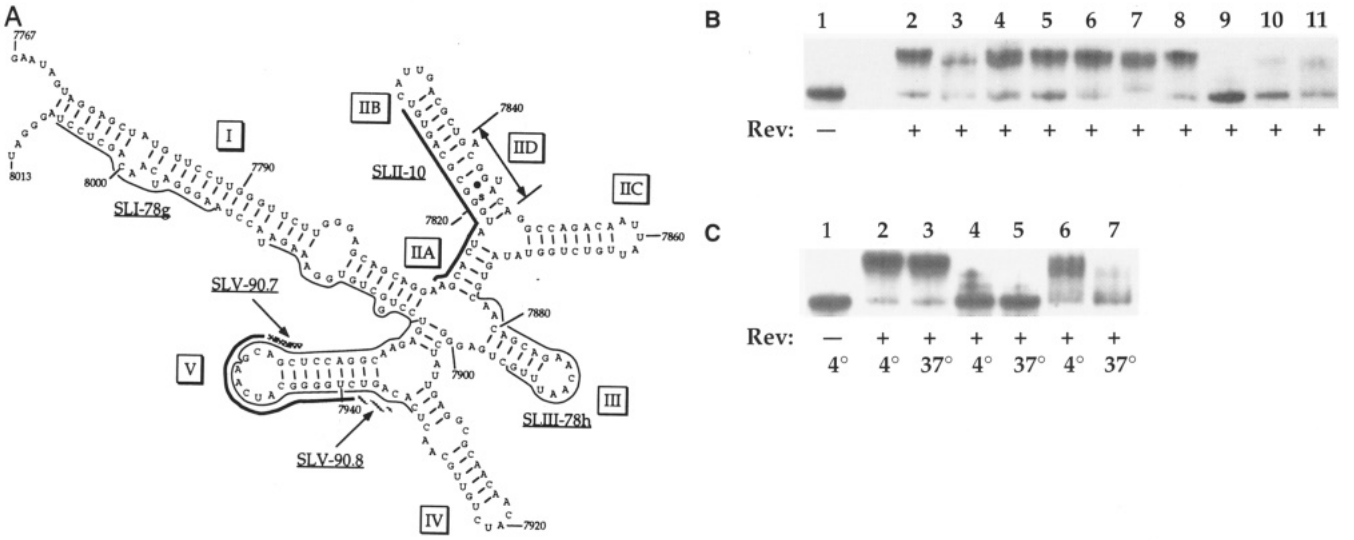

FIGURE 1: (A) RRE targets of C-5 propyne–pyrimidine ODNs. The thick solid line between stem–loop IIA and stem–loop IIB indicates the position of the SLII-10 ODN. The thick solid line along stem–loop V indicates the core of ODNs SLV-90.7 and SLV-90.8. Stippled regions flanking this line show the boundaries within either SLV-90.7 or SLV-90.8. The thin line running from stem–loop I to the base of stem–loop IV is the region over which ODNs from panel B were derived. Positions of ODNs SLI-78g (adjacent to stem–loop I; Chin, 1992) and SLIII-78h (adjacent to stem–loop III; Chin, 1992) are indicated. The depicted HIV1 SF2 RRE structure was slightly modified from the predicted structure resulting from M. Zuker's Mufold program run on a Cray Y-MP, San Diego Supercomputer Center (Chin, 1992). The mutagenesis results of (Bartel et al., 1991) were incorporated to constrain G7821·G7844 base-pairing (indicated by ●) and possible G7820·A7846 base-pairing (indicated by ▲). (B) Gel-shift assay of the SLV-90g family of phosphodiester ODNs. Preformed Rev–RRE complexes were incubated with 10 μM ODNs: SLV-90.1 (lane 3), SLV-90.2 (lane 4), SLV-90.3 (lane 5), SLV-90.4 (lane 6), SLV-90.5 (lane 7), SLV-90.6 (lane 8), SLV-90.7 (lane 9), SLV-90.8 (lane 10), and SLV-90.g (lane 11). RRE RNA is shown in lane 1. Similar results were found with C-5 propyne-modified ODNs (not shown). (C) Rev–RRE complex susceptibility to disruption by ODNs SLV-90.7 and SLV-90g: effect of temperature. Rev was either thawed at 4 °C (lanes 2, 4, and 6) or thawed at 20 °C and preheated for 20 min at 37 °C (lanes 3, 5, and 7) prior to a 20-min incubation with renatured ^{32}P-labeled RRE at the indicated temperature. Unmodified 18-mer ODNs SLV-90.7 (lanes 4 and 5) and SLV-90g (lanes 6 and 7) were added to these preformed complexes, and the incubation was continued at 4 or 37 °C for 20 min prior to electrophoresis.

Inhibition of *env* expression was sequence specific and was dependent upon ODN length; missense C-5 propyne ODNs were ineffective *in situ* (Figure 3), and unlike complementary C-5 propyne ODNs, missense C-5 propyne ODNs were inactive in RNase H assays (not shown). Short SLV-90.8 versions (12- and 15-mers) were 14-fold less active than 18-mers, Figure 3.

The RNA target position is a major determinant of ODN efficacy; C-5 propyne ODNs targeted to noncritical RRE stem–loops (SLI and SLIII) were either ineffective (SLI-78g) or 30-fold less active (SLIII-78h) than 18-mer SLV ODNs (Figure 3). Consistent with the position dependency found in the gel-shift analysis, SLV-90g was 10-fold less active than SLV-90.8. However, the equivalent potency of SLV-90.7 and SLV-90.8 *in situ* contrasts with their 8-fold difference in activity *in vitro*; this may result from a different RRE RNA conformation *in situ*. The 18-mer SLV ODNs (SLV-90.7 and SLV-90.8) were 9-fold more potent than the 18-mer SLII-10 ODN which is specific for the known, primary Rev binding site on SLIID (Bartel et al., 1991).

Since the RRE lies within the *env* coding region, we asked whether the effect of C-5 propyne ODNs upon *env* expression in COS7 cells resulted from a block in gp160 translation. For these experiments, we tested the effects of anti-RRE ODNs against pLR, a Rev-independent luciferase–RRE vector in which the HIV1-SF2 RRE was attached downstream of the firefly luciferase translation termination codon. When SLV-90.818 or SLII-10 were coinjected with pLR into the nuclei of COS7 cells, specific inhibition of luciferase expression was observed (Figures 5B and 6). This result suggests that translational inhibition is a minor mechanism for inhibition of *env* expression by anti-RRE ODNs because the RRE in pLR is downstream of the luciferase termination codon. Since luciferase–RRE is Rev-independent, RNase H cleavage of

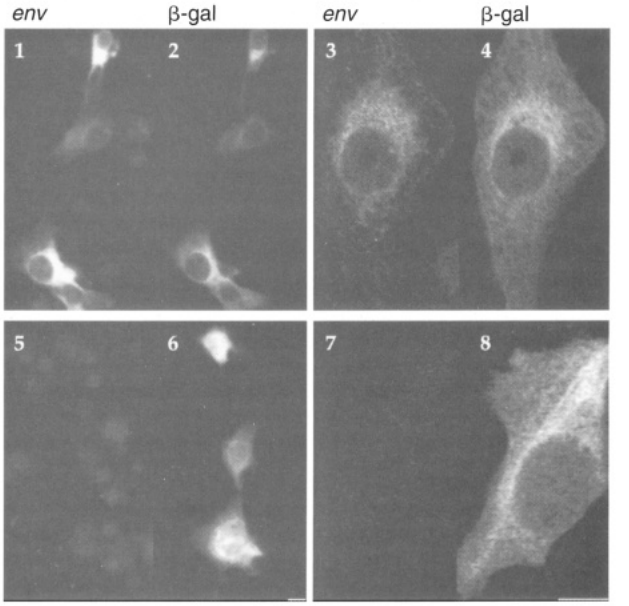

FIGURE 2: Potent inhibition of *env* expression with C-5 propyne–pyrimidine PS anti-RRE ODNs in COS7 cells. After nuclear injection of plasmids encoding β-galactosidase, Rev, and *env*, cells were processed for indirect immunofluorescence. Panels show cells injected with plasmids in the absence (panels 1–4) or presence of SLV-90.8 (panels 5–8) at 40× magnification with standard epi-fluorescence (panels 1, 2, 5, and 6) or confocal microscopy (panels 3, 4, 7, and 8). β-Galactosidase (even-numbered panels) and *env* (odd-numbered panels) expression is shown. Cells were injected with plasmids and 20 μM C-5 propyne-modified SLV-90.818 ODN (panels 5–8). The estimated intracellular concentration is 2 μM. Note that inhibition of *env* expression is complete; the residual fluorescein of injected cells in panel 5 is the same level of the background fluorescence of noninjected cells. Bar at lower right = 10 μm.

hybrids likely accounts for the bulk of C-5 propyne ODN activity against pLR. In contrast, other mechanisms including

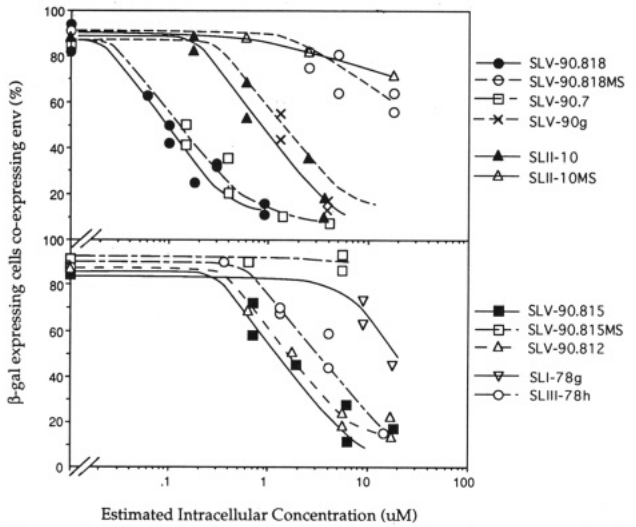

FIGURE 3: Concentration-dependent inhibition of *env* expression in COS7 cells by C-5 propyne ODN with phosphorothioate backbones. Each data point represents the fraction of cells coexpressing the marker β-galactosidase and *env* from 100–200 COS7 cells after nuclear injection of a mixture of plasmids in the absence or presence of ODNs as described in Figure 2. In the upper panel, the ODNs tested were as follows: ▲, SLII-10 GACACUGCGCCCAUAGUG (ODN 10 from SLII; Chin, 1992); △, SLII-10MS GAAACUGCACCUCUGGUA (mismatched SLII-10 control); ●, SLV-90.818 (SLV-90.8 18-mer, Table 1); ○, SLV-90.818MS AGGGAGCTGUUGAUCCCU (mismatch SLV-90.8 18-mer control); □, SLV-90.7 (18-mer SLV-90.7 shown in Table 1); and ×, SLV-90g ACUCUUGCCUGGGAGCUGC. In the lower panel: ■, SLV-90.815 GCUUGAUGCCCCAGA (a 15-mer version of SLV-90.8); □, SLV-90.815MS GCCAACUCCGCAGGUUUG (a mismatch control for SLV-90.815); △, SLV-90.812 GCUUGAUGCCCC (a 12-mer version of SLV-90.8); ▽, SL1-78g (an 18-mer ODN from SLI; Chin, 1992); and ○, SL3-78h (an 18-mer ODN from SL3; Chin, 1992). The intracellular concentration were estimated to be one-tenth the ODN concentration in the injection needle. At high ODN concentrations, limited nonspecific inhibition of gene expression was observed, as previously observed (Wagner et al., 1993).

steric disruption and RNase H cleavage may reduce expression when the RRE is in its native context within *env*. The 2.7-fold difference in the activity of C-5 propyne ODNs against luciferase–RRE versus *env* may be attributed to (i) a specific (or general) steric disruption of RRE structure resulting in the displacement of RRE-bound Rev, (ii) sequences flanking the RRE in the different vectors, or (iii) translational inhibition.

Common delivery methods were evaluated to determine their role in affecting C-5 propyne-modified ODN efficacy. C-5 propyne-modified ODNs were added to the medium or transfected by cationic lipids. Incorporation of ODNs into cells was assessed by the distribution of a marker, rhodamine-labeled unmodified PS ODN. Addition of 0.1–5 μM C-5 propyne-modified ODNs to the medium together with the rhodamine ODN resulted in a punctate, perinuclear ODN distribution of the fluorescent ODN (Chin et al., 1990; Bennett et al., 1992). However, ODN delivery by this endocytotic treatment was unable to mitigate *env* expression (not shown). In contrast, *env* expression in COS7 cells was blocked if the cells were transfected with 0.1 μM SLV-90.818 or SLII-10 prior to plasmid microinjection (Figure 7). Nuclear accumulation of the marker ODN occurred in only 50–75% of transfected cells; hence, in a line of plasmid-injected cells 25–50% of injected cells did not incorporate the marker rhodamine ODN, and hence C-5 propyne ODN, into their nuclei. Not surprisingly, *env* expression was unaltered in cells that were not transfected with ODNs as assessed by rhodamine marker in the nucleus (Figure 7A). We were unable to accurately determine the I_{50} levels for injected cells containing

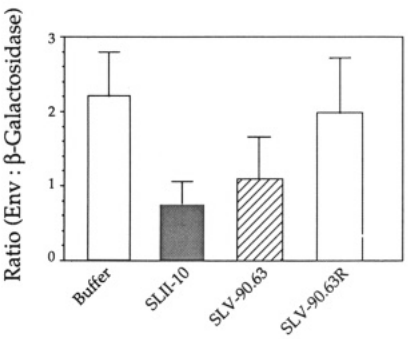

FIGURE 4: Nuclear microinjection of 30 μM unmodified phosphorothioate ODNs results in a weak suppression of *env* expression in COS7 cells. After nuclear injection of ODNs plasmids encoding β-galactosidase, Rev, and *env*, cells were processed for indirect immunofluorescence. The sequences for SLV-90.63 and SLII-10 are described in Table 1 and Chin (1992), respectively. SLV-90.63R is a randomized 90.63 sequence: GCTCAGTCTGGCTAGACC. Digital images were acquired with calibrated neutral-density filters to enable calculation of fluorescein:Texas Red intensity ratios in the absence or presence of ODNs. The means and standard errors of 4 experiments, 22–50 cells/experiment, are shown.

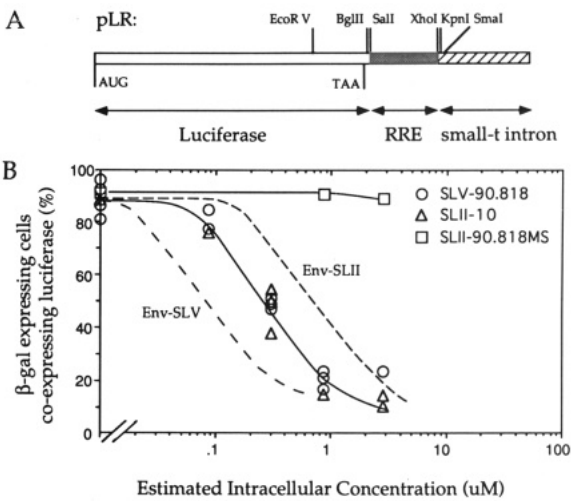

FIGURE 5: Inhibition of luciferase–RRE by C-5 propyne-modified SLV and SLII ODNs. (A) Diagram of pLR, a chimeric firefly luciferase–RRE vector. (B) Luciferase–RRE expression in the presence and absence of C-5 propyne-modified ODNs. The dashed lines show the dose–response profiles of ODNs against *env* expression; from Figure 2, SLV-90.818 is indicated by the "Env-SLV" label and SLVII-10 is indicated by the "Env-SLII" label.

transfected propyne-modified ODNs due to the highly variable (3–4-fold) and nonuniform, nuclear ODN loading [not shown and Sixou et al. (1994)]. However, similar potency values were previously reported for transfected C-5 propyne-modified ODNs (Wagner et al., 1993).

DISCUSSION

In this report, we systematically evaluated antisense inhibition of HIV *env* using both *in vitro* and *in situ* assays. Several conclusions which have important implications for antisense strategies can be drawn from these studies: (i) target site selection for antisense ODNs has profound effects on activity, (ii) a strong correlation was found between *in vitro* and *in situ* activity of ODNs targeted to a highly structured RNA, (iii) a complex mechanism of action of the active ODNs is likely responsible for the antisense activity, (iv) our results using C-5 propyne phosphorothioate ODNs differ from other HIV studies using conventional, unmodified phosphorothioate ODNs, and (v) the use of techniques which can traverse ODNs across membranes is absolutely required for antisense effects.

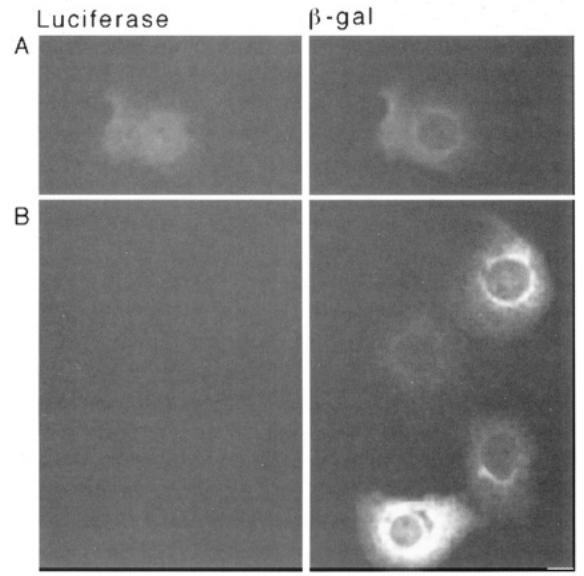

FIGURE 6: Luciferase–RRE expression. (A) COS7 cells injected with 2.7 μM SLV missense, C-5 propyne-modified ODN (SLV-90.818MS), pLR, and β-galactosidase plasmids. (B) COS7 cells injected with 0.9 μM SLV-90.818, pLR, and β-galactosidase plasmids.

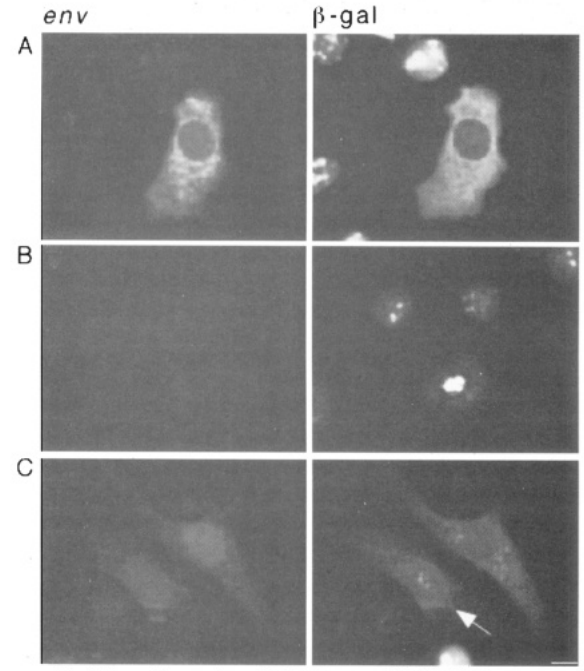

FIGURE 7: Effect of transfected ODNs upon HIV1-SF2 *env* expression in COS7 cells. (A–C) COS7 cells transfected with 0.1 μM SLV-90.8.18 and subsequently injected with Rev, *env*, and β-galactosidase plasmids. In panel A, a cell lacking nuclear ODNs expresses both β-galactosidase and gp160. Neighboring cells with the characteristic, punctate nuclear pattern of the marker rhodamine PS ODN, showing ODN delivery, were uninjected. In panel B, the injected cell, with intense nuclear ODN staining, was both transfected with ODNs and injected with plasmids. In panel C, the cells were transfected with 0.1 μM missense SLV-90.818MS ODN and injected with plasmids. Only the cell indicated by the arrow shows nuclear accumulation of ODNs, which represents between 1% and 10% of the total cell-associated ODN prior to fixation and processing for immuochemistry. The bulk of cell-associated ODN was in perinuclear vesicles prior to fixation.

In many antisense ODN studies, site selection is based upon anticipated translational mechanisms [reviewed by Cheng and Stein (1993) and Milligan et al. (1993)], and target sites including the AUG initation codon are chosen without optimization. Our iterative optimization strategy revealed that a structured, internal coding region can be a sensitive antisense target as demonstrated by potent and specific inhibition with ODNs targeted to either SLV or SLIID of HIV1 RRE. In contrast to a previous study with C-5 propyne ODNs (Wagner et al., 1993), site-dependent activity of ODNs was found in studies of the RRE. In addition, there was an extraordinary correlation between the ability of certain ODNs to disrupt purified Rev binding to labeled, synthetic RRE RNA *in vitro* and the antisense activity of the same ODNs *in situ*. While site dependency of antisense ODNs can be influenced by RNA structural effects upon the hybridization kinetics of unmodified PS ODNs (Lima et al., 1992), we observed that, even for highly optimized RRE sites, unmodified PS ODNs were ineffective *in situ*. This result suggests that the hybridization properties of C-5 propyne-modified ODNs confer significant biological activity (Wagner et al., 1993) against targets within a context of strong RNA structure. As discussed below, the site dependency of antisense ODNs was not simply the result of target accessibility within the highly structured RRE because (i) ODNs with weak antisense activity in cells (SLI and SLIII) can form hybrids *in vitro* that are sensitive to *E. coli* RNase H and (ii) the order of ODN potency was affected by the context in which the RRE was placed.

Target accessibility or the ability of an ODN to affect RRE structure is not sufficient to define an effective antisense target. Target accessiblity was addressed by comparing sites on different stem–loops or families of overlapping ODNs that spanned a single, core target sequence; i.e. SLV ODNs (90.7 and 90.8) offset by 4 nt had an 8-fold difference in their ability to disrupt Rev–RRE binding but had equivalent *in vitro* RNase H susceptibility. SLI appears relatively accessible within the limits of RNA structural predictions and footprinting data (Kjems et al., 1991; Chin, 1992). Also, *in vitro* the SLI-78g ODN both supported RNase H cleavage of RRE RNA and induced RRE structural alterations (Chin, 1992) but lacked *in situ* activity.

Three general mechanisms can account for the antisense activity of ODNs in cells: (i) translational inhibition in the cytoplasm, (ii) steric effects upon RNA binding proteins in the nucleus, and (iii) the activity of endogenous RNase H upon susceptible ODN–RNA hybrids. Within the limits of the reported assays, we explored the role of these mechanisms by direct analysis or by comparing ODNs in different contexts. Further analysis with alternative techniques will be required to definitively answer the exact contribution of each mechanism, but a number of conclusions can be deduced from these studies.

The default mechanism of antisense inhibition for both the *env* and luciferase studies is cellular RNase H activity since C-5 propyne ODNs support the cleavage of hybrids by RNase H (Wagner et al., 1993). However the *in vitro* results suggested that antisense inhibition by SLV ODNs should be Rev-dependent. By comparing the effects of SLV ODNs against *env* and the Rev-independent pLR, in which the RRE was fused downstream of the luciferase termination codon, we are able to conclude that (i) a translational mechanism is unlikely to explain the *env* antisense results and (ii) an additional steric mechanism is likely to explain the many differences in SLV ODN effects upon *env* and pLR. SLV and SLIID ODNs had equivalent potency when tested against pLR, but SLV was 9-fold more potent than SLIID when tested against *env*. Also, SLV ODNs were 3-fold less potent against pLR than *env*. Consistent with these Rev-dependent differences in activity is the unusual ability of SLV ODNs to disrupt preformed Rev–RRE complexes *in vitro*.

It is unknown if SLV ODNs disrupt preformed complexes by preventing Rev binding to secondary sites on SLV (Kjems et al., 1991) or via a generalized conformational change in RRE structure leading to destabilization of Rev bound to the high-affinity site on SLIID. In this light, it is surprising that SLV ODNs were more potent than SLIID ODNs, and the protoptype SLV-90g ODN induced enhanced duplex structure in SLIII without affecting SLI or SLIID structure (Chin, 1992). In addition, a weak Rev footprint was found in SLV (Kjems et al., 1991). Further studies will be required to clarify the relationship between low-affinity Rev binding sites on SLV and the mechanism of SLV ODNs.

In other antisense studies with HIV, translational initiation regions, coding regions, and splice sites were targeted with unmodified phosphorothioate ODNs (Matsukura et al., 1989; Lisziewicz et al., 1992). Since unmodified PS ODNs have many nonspecific effects (Zon, 1988; Cazenave et al., 1989; Ho et al., 1991), the index of sequence-specific activity is often narrow. Furthermore, nonspecific effects upon virus binding (Gao et al., 1990; Kinchington et al., 1992) are difficult to eliminate, particularly when PS ODNs are continually added to the culture medium (Li et al., 1993; Lisziewicz et al., 1992). The reduced potency and specificity of unmodified PS ODNs against HIV targets within the context of reporter genes (Li et al., 1993) are consistent with multiple mechanisms explaining the inhibition of viral propagation by unmodified PS ODNs.

Recently, 28-mer PS ODNs specific for the primary Rev binding site on the RRE were shown to be modestly effective in blocking the expression of reporter genes linked to the RRE after transfection of PS ODNs at 1 μM (Li et al., 1993). Our results are generally consistent with those of Li et al. (1993) yet distinguishable. (i) An 18-nt site on SLV was a more sensitive target than SLIID (although we used slightly different sequences). (ii) Unmodified PS ODNs were weakly active even at high, injected concentrations. (iii) In contrast, the 18-mer C-5 propyne ODN was 10-fold more potent than a previously described 28-mer unmodified PS ODN (Li et al., 1993), which may be explained by the enhanced hybridization properties of C-5 propyne ODNs (Wagner et al., 1993). (iv) Low nonspecific effects of C-5 propyne-modified ODNs were found even at 10 μM while toxicity of unmodified PS ODNs was reported at these levels (Li et al., 1993). (v) Inhibition by the anti-RRE C-5 propyne ODNs was attained only if the plasma membrane barrier was breeched either by microinjection or by permeabilizing agents such as cationic lipids. Endocytotic uptake of SLV and SLIID C-5 propyne ODNs was insufficient to cause inhibition of *env* expression. This result contrasts with a large number of antisense ODNs employing this route of entry, reviewed by Milligan et al. (1993). In contrast, we note that, in other studies of RRE-specific ODNs, transfection of unmodified PS ODNs was no more efficient than simple addition to the medium (Li et al., 1993; Lisziewicz et al., 1992).

While most antisense studies report the extracellular concentration at which biological effects are measured, the potency of microinjected anti-RRE ODNs were determined by an estimated intracellular concentration.[4] However, transfection results in a 50–500-fold concentration of the ODN in the medium[5] into the cytoplasm (Felgner et al., 1989; Legendre et al., 1992). Consistent with this calculation, transfection with cationic lipids improved the efficacy of an unmodified, endocytosed PS ODN by 1000-fold (Bennett et al., 1992). An even greater amplification of efficacy was found with SLV and SLIID C-5 propyne ODNs because they were inactive when endocytosed but potent 100 nM inhibitors when transfected into cells. Despite the concentrating effects of transfection, the potency of transfected C-5 propyne ODNs was equivalent to injected ODNs. This may be explained by a combination of factors. Injection of SLV and SLIID ODNs into the nucleus avoids the cell surface and, initially, the cytoplasm while the prolonged journey of transfected ODNs subjects them to a greater exposure to nucleases and nonspecific interactions with membrane proteins (Fisher et al., 1993; Kinchington et al., 1992). An additional complication is the relative toxicity of most cationic lipids (Felgner et al., 1989). Coupled with the 3–4-fold variability in intracellular ODN concentration after transfection, we believe direct nuclear microinjection of ODNs enables a more consistent and accurate means of assaying sequence-specific ODN potency.

In summary, C-5 propyne-modified stem–loop V ODNs will be useful in further studies of Rev–RRE interactions since these ODNs completely disrupt preformed Rev–RRE complexes and block *env* expression. These findings have important implications upon the design and assay of antisense strategies and particularly stress the importance optimizing target sites, delivery strategies, and ODN modification.

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[4] The cellular volume of somatic cells is about 1 pL, and the largest, tolerable nuclear injection volume is approximately 50–100 fL.

[5] About 10% of transfected ODNs becomes cell-associated after 15 h. From fluorescently-labeled phosphorothioates, we calculate that between 1% and 10% of the cell-associated ODN accumulates in the nucleus, with the remainder compartmentalized in perinuclear vesicles. Since the volume of 2×10^4 cells grown on a 25-mm diameter cover slip is approximately 2×10^{-8} L, the ODN that accumulates in a cell from 1 mL of medium containing 1 μM ODN ranges between 50 and 500 μM.

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