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Sequence-Specific Inhibition of Gene Expression by a Novel Antisense Oligodeoxynucleotide Phosphorothioate Directed against a Nonregulatory Region of the Human Immunodeficiency Virus Type 1 Genome

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Previous studies have demonstrated that oligodeoxynucleotide phosphorothioates complementary to human immunodeficiency virus type 1 (HIV-1) RNA are more nuclease resistant and are effective inhibitors of HIV-1 replication than their unmodified counterpart. In this study, antisense oligodeoxynucleotide sequences were evaluated for therapeutic potential in the treatment of HIV infections. The use of HIV-infected lymphocytes to test the efficacy of a drug is very complex, and therefore it is difficult to draw conclusions about the mechanism. We used a COS-like Monkey kidney cell line (CMT3) stably transfected with plasmids pCMVgagpol-rre-r (containing *gag* and *pol* genes) and pCMVrev (containing the *rev* gene of HIV-1), derived from cDNA clone BH10, as a model. A biologically active provirus that transcribes and translates their nucleotide sequences into viral proteins p24, p39/41, p55, and p160 was generated. Sequence-specific and dose-dependent inhibition of HIV-1 viral protein synthesis and significant inhibition at the mRNA level were demonstrated by antisense construct GPI2A, directed against a nonregulatory region of the HIV-1 genome. Also, our studies demonstrated enhancement of the antisense effect through encapsulation in a cationic lipid preparation. The observed attenuation of HIV-1 mRNA levels suggests that, at least in part, the mechanism of action of GPI2A was at the transcript level. Further studies have also shown antiviral activity of this construct as determined by the reverse transcriptase assay using acutely and chronically infected cells of lymphoid origin (H9 cells). Toxicological studies involving cell growth characteristics, colony-forming ability, effects on cellular proteins, specific activities of labeled proteins, and DNA synthesis in cell culture showed no cytotoxic effects of GPI2A.

Antisense oligodeoxynucleotide (ODN) technology is an exciting new avenue for the therapy of many diseases, including viral infections and cancer (35). Prior attempts at antisense targeting of human immunodeficiency virus (HIV) have been focused on inhibition of the synthesis of some particular viral proteins essential to the success of the infection by using de novo infection of susceptible cells in culture (2, 3, 5, 21, 32, 33, 47). By its very nature, such an assay allows a test compound to potentially interfere with any critical process within the virus life cycle. Consequently, de novo infection assays are efficient for initial screening of compounds for antiviral activity but are complex systems for establishing a mechanism(s) of action, including whether the antiviral activity exhibited by an intended antisense oligomer actually derives from an antisense process or is the result of an action operating an early stage of the infection process (42).

Infection of T lymphocytes with HIV results in a cytopathic effect and cell death that has been linked to selective loss of the helper T-lymphocyte function of the immune system (8). In addition to acute infection, which leads to cell death through accumulation of HIV type 1 (HIV-1) antigens within an infected cell (43), a chronic or persistent infection also occurs. The persistence of these viral reservoirs has been implicated in the progression of HIV infection and AIDS (36). Rational drug discovery targeted to late-stage events in HIV replication has the potential to yield antiviral agents capable of blocking virus spread by inhibiting infectious virion production from this chronic reservoir (36). The finding that complementary interactions between nucleotide sequences exist in nature to regulate cellular functions led to the concept of using antisense ODNs as tools to alter specific metabolic functions of therapeutic importance (1, 25). The problem of ODN sensitivity to nuclease digestion (6, 44, 48) has been addressed by the development of chemically modified forms, including phosphorothioates and methylphosphonates, which have been shown to be more resistant to nuclease digestion (6, 15). A second barrier to the therapeutic application of ODNs relates to poor cellular uptake; thus, large quantities of ODNs must be present at the cell exterior to achieve even modest concentrations at the target site (28). Previous studies suggest that the antiviral activity of an ODN depends, in principle, on binding of the ODN to the target nucleic acid, thus disrupting the function of the target, either by hybridization arrest or by disruption of the target (RNA) via RNase H activity (4). However, very little is understood about the roles of various mechanisms or the factors that may determine which mechanisms are involved after an ODN binds to receptor sequences. By using a stably transfected COS-like Monkey kidney cell line, B4.14, we demonstrated sequence-specific and concentration-dependent suppression of HIV-1 viral protein synthesis, a significant inhibitory effect at the viral mRNA level, and inhibition of

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HIV-1 replication in virus-infected cells. Our results therefore show a combined feature of sequence specificity in inhibition of translational events and the possible activation of RNase H in cell culture experiments which may be linked to the inhibition of viral replication.

MATERIALS AND METHODS

Expression plasmid. pCMVrev contains the coding sequence from a HIV-1 cDNA fragment under the control of the promoter-enhancer region from the simian cytomegalovirus IE94 gene (−650 to +30) (27). This plasmid was constructed by inserting the *Bsu*361 fragment from pCV1 (8) into vector pCMV. In pCMV, the simian virus 40 sequences contained within pBABY (24, 38) have been exchanged for the cytomegalovirus promoter region (31).

pGAGPOL contains a cDNA fragment from the proviral BH10 clone of HIV-1 (corresponding to HXB2 nucleotides 679 to 5785) ligated into the *Xho*I site of pBABY. This fragment contained the open reading frames for *gag*, *pol*, and *vif* and was derived from a plasmid with the entire BH10 proviral clone as a *Sal*I fragment in vector pSP64 (Promega Biotec, Madison, Wis.). The HIV-1 sequences were excised as a *Sal*I fragment between the *Sal*I site in the pSP64 polylinker and the *Sal*I site at nucleotide 5785 in the HIV-1 DNA. After ligation into pBABY, a small deletion (*Ava*I-*Xba*I) was made in the remaining pSP64 polylinker; this was followed by T4 DNA polymerase repair and religation, resulting in deletion of a *Bam*HI site (polylinker). A second *Bam*HI site at the boundary of the simian virus 40 and pBR322 (24, 40) DNA sequences was also removed by restriction enzyme digestion and repair. These manipulations were performed to construct pGAGPOL-RRE-r. pGAGPOL-RRE-r was generated by ligating the *Bgl*II-*Bam*HI fragment derived from the HIV-1 BH10 clone (corresponding to HXB2 nucleotides 7620 to 8474) into the unique *Bam*HI site present in pGAGPOL in the sense orientation with respect to *gag*, *pol*, and *vif*, as in the viral genome.

Cells and cell culture. A COS-like Monkey kidney cell line, CMT3 (19), was transfected by the standard $CaPO_4$ method (23) with plasmids pCMVgagpol-rre-r and pCMVrev and a selective marker plasmid that expresses the hygromycin resistance gene to generate cell line B4.14. The pCMVrev gene is required for efficient expression of the *gag* gene (40) (David Rekosh, Ham-Rek Laboratory, State University of New York at Buffalo). The cells were maintained in Iscove's modified Dulbecco's medium with 10% fetal calf serum, 50 μg of gentamicin per ml, and 200 μg of hygromycin B per ml.

Umbilical cord blood mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. The cells were collected, washed, and phytohemagglutinin stimulated. The cells were added to culture flasks and maintained for 3 days in complete RPMI 1640 culture medium (Gibco Laboratories, Toronto, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Toronto, Ontario, Canada), 2 mM L-glutamine, and 200 U of penicillin per ml (12).

Synthesis of ODN sequences. By using a computer model program (OLIGO; Primer Analysis Software, Version 3.4), which calculates the free energy (ΔG), hybridization temperature, and secondary structure of DNA and RNA on the basis of highly accurate measurement of nearest-neighbor ΔG values, an ODN that is highly selective to the intended target sequence was generated. The ODN thus generated was expected to have minimal genetic variability (9), to form stable duplexes, and to be non-self-complementary. GPI2A is an antisense construct made of the following base composition, spanning nucleotides +1189 to +1208 according to the nomenclature of Ratner and coworkers (37): 5′-G(s)GT TC(s)T TTT G(s)G(s)T CC(s)T TG(s)T C(s)T-3′. The GPI2A sequence, with two point mutations (underlined), is 5′-G(s)GT TC(s)T TTT G(s)TG(s) CC(s)T TG(s)T C(s)T-3′, and the inverse of GPI2A has the sequence 5′-T(s)CT G(s)TT C(s)CT G(s)G(s)T TTT C(s)TT G(s)G-3′. This nucleotide sequence falls within a relatively conserved region (Fig. 1) in many retroviruses (9, 18, 20). Within this stretch, bovine immunodeficiency virus shared 8 of 10 residues with equine infectious anemia virus, 6 of 10 with simian immunodeficiency virus of macaques, 3 of 10 with visna virus, and 7 of 10 with simian immunodeficiency virus of African green monkeys, HIV-2, and HIV-1 (18). GPI2A, the mutated sequence, and the inverse of the antisense construct were chemically modified at seven different base positions by substitution of the naturally occurring nonbridging oxygen atoms of the phosphodiester backbone with sulfur atoms as shown above to form the corresponding relatively nuclease-resistant phosphorothioate derivative (15, 48). Modification of seven instead of all available base positions in the sequence was performed to reduce possible nonspecific cytotoxic effects (41).

ODNs were synthesized on Applied Biosystems 380B automated DNA and 392 DNA-RNA synthesizers by using standard phosphoramidite chemistry. The oligomers and their phosphorothioate analogs were purified by using oligonucleotide purification cartridges (Applied Biosystems), 20% denaturing polyacrylamide gel electrophoresis (16), or high-performance liquid chromatography (49).

Pretreatment of cells with an ODN-lipid formulation. The lipofectin reagent is a 1:1 (wt/wt) liposome formulation of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride and dioleoyl phosphatidylethanolamine in membrane-filtered water (GIBCO BRL, Research Products Life Sciences Inc.). Lipofectin reagent reacts spontaneously with DNA to form a lipid-DNA complex with complete entrapment of the DNA. Fusion of the complex with the cell membrane results in efficient uptake of the encapsulated DNA into the intracellular environment of the cell (17, 28).

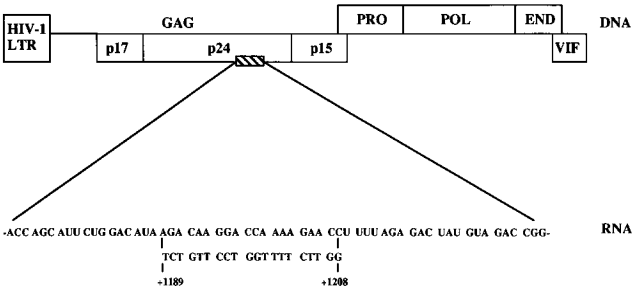

FIG. 1. mRNA sequence of the targeted region within the *gag* gene of the HIV-1 genome. The insert at the bottom shows the 20-mer sequence used to design the GPI2A antisense molecule described in this report. LTR, long terminal repeat.

Appropriate amounts of DNA and lipofectin reagent were diluted separately in sterile distilled water, subsequently combined in a polystyrene tube, and mixed gently (no vortexing). The mixture was then allowed to stand for at least 15 min at room temperature. Approximately 50 to 70% confluent plates were washed with serum-free medium. One milliliter of medium was then layered over the cells, and the complex was added dropwise as uniformly as possible. Depending on the experimental conditions, cells were incubated for 0.5 min to 24 h at 37°C in a humidified 5 to 10% CO_2 environment. Either the cells were labeled for immunoprecipitation or the medium and ODN were subsequently replaced and the cells were incubated for the desired length of time in the presence of oligomer prior to radiolabeling and immunoprecipitation was performed by a modification of a previously described procedure (14).

Determination of cell-associated ODN. Cells were incubated with ^{32}P-labeled (5′ DNA terminus labeling system; GIBCO BRL) ODNs in the presence or absence of lipofectin reagent, and the cell-associated radioactivity was determined by direct suspension of the labeled cells in liquid scintillation medium (Universol; ICN Biomedicals Inc., Irvine, Calif.).

Antisense oligomer and viral gene expression. To determine the effect of the antisense oligomer on viral gene expression, B4.14 cells were added at the desired cell density to six-well plastic tissue culture plates containing Iscove's modified Delbecco's medium with 10% calf serum–50 μg of gentamicin per ml–200 μg of hygromycin B per ml and kept at 37°C in a humidified incubator with a 5 to 10% CO_2 environment. The cells were then washed and incubated with medium containing various oligomer concentrations and heat-inactivated serum. The control oligomer sequences either contained two point mutations or were the inverse of the antisense construct.

Viral antigen assay. The pretreated cells and the controls were labeled with 120 to 250 μCi of [^{35}S]methionine per ml (70% L-methionine, 15% L-cysteine; the [^{35}S]methionine concentration was 185 MBq, and the specific activity was 1,057 Ci/mmol) in the presence of methionine-free medium. Labeled samples were washed with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 1.6 mM K_2PO_4, 8.1 mM Na_2HPO_4) and resuspended in 200 μl of lysis buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% deoxycholic acid).

The samples were precleared with protein A-Sepharose beads for at least 30 min at 4°C. [^{35}S]methionine-labeled viral proteins were immunoprecipitated for 2 h or overnight at 4°C by using protein A-Sepharose beads and 2.5 μl of rabbit polyclonal antiserum directed against HIV-1 p24 (National Institute of Allergy and Infectious Diseases [AIDS Research and Reference Reagent Program] and MicroGeneSys, Inc.). The resulting pellets were washed four times with lysis buffer, once with lysis buffer containing 500 mM NaCl, and once with TNE buffer (10 mM Tris-HCl [pH 7.2], 25 mM NaCl, 1 mM EDTA). Samples were then resuspended in 20 to 30 μl of 2× sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 100 mM dithiothreitol, 60 mM Tris-HCl [pH 6.8], 0.001% bromophenol blue), boiled for 5 to 10 min, and then electrophoresed on a sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis gel. The gel was dried and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, N.Y.).

Northern (RNA) analysis. Northern analyses of the isolated RNA samples were done by a modification of a previously described procedure (7). Briefly, cells were pretreated with 1 μM ODN in the presence of 10 μg of lipofectin reagent per ml. mRNA was extracted from the cells by the rapid RNA preparation method (22). Electrophoresis was performed on 1% formaldehyde agarose gels that were then blotted onto nylon membranes (Nytran$^+$; 0.45-μm pore size; Schleicher & Schuell) overnight, dried, and then baked at 80°C for 2 h. The filters were prehybridized at 42°C overnight in a hybridization oven (Turbo Speed hybridization oven; Bio/Can Scientific). The filters were then probed overnight at 42°C with a ^{32}P-labeled probe (Oligolabeling kit; Pharmacia) corresponding to bases 334 to 2037 of the BH10 clone (37). The membranes were

washed appropriately after hybridization and subsequently exposed to film (Kodak X-Omat AR).

RT assay. Reverse transcriptase (RT) assays were carried out by a modification of a previously described procedure (11, 29). Briefly, 50 μl of clarified culture supernatant was added to 50 μl of reaction cocktail containing 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 150 mM KCl, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.5% Triton X-100, 2% ethylene glycol, 5 mM dithiothreitol, 0.3 mM reduced glutathione, 20 μCi of titrated thymidine triphosphate, and 50 μg of template primer [poly(rA)-oligo(dT)] per ml in polypropylene tubes. The tubes were agitated and then incubated at 30°C for 22 h. The reaction was stopped by addition of 1 ml of cold trichloroacetic acid. Newly synthesized DNA was precipitated on ice for at least 2 h and then collected on Whatman GF/C glass filters and rinsed twice with cold 10% trichloroacetic acid and absolute ethanol. Filters were dried for 20 min and counted for incorporated radioactivity.

Immunoprecipitation. Immunoprecipitation of the ribonucleotide reductase R1 protein was performed by a modification of the method of Chan et al. (14). Briefly, labeled cells were lysed with solubilizing buffer SB150 (25 mM Tris-HCl [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride [PMSF]). The lysates were preabsorbed with heat-killed, formalin-fixed *Staphylococcus aureus* cells (Pansorbin) for 10 min at room temperature. For immunoprecipitation of the R1 protein, 1 μl of anti-R1 mouse monoclonal antibody (InRo Biomedtek, Umea, Sweden) was added to the sample and incubated at room temperature for 15 min, and then 25 μl of a 10% suspension of Pansorbin coated with rabbit anti-mouse immunoglobulin G antibody (Sigma Chemical Co., St. Louis, Mo.) was added.

Antiviral activity assay. To test the antiviral activity of the antisense ODN phosphorothioate in acutely infected cells, laboratory strain IIIB of HIV-1, isolated from chronically infected H9 cells, was used to infect phytohemagglutinin-stimulated umbilical cord blood mononuclear cells at a viral titer of 2,000 50% tissue culture-infective doses (the viral stock was 5×10^3 50% tissue culture-infective doses per ml). After 2 h of infection at 37°C, cells were washed free of unattached virus and resuspended in fresh medium containing 10 U of interleukin 2 per ml. Cells were plated at 4×10^5 per well, and the appropriate concentrations of insulin (Sigma) or IGF-1 (Gibco) and ODN were added. After 4 days of incubation, half of the incubation medium was withdrawn and replaced with fresh medium containing the corresponding amount of ODN. The HIV-1 activity in the cell culture supernatant was determined by the RT assay as described above after an additional 3 days in culture.

To test the effect of ODN on chronically infected H9/IIIB cells, these cells were added at 4×10^5 per well with heat-inactivated serum and a 1 μM concentration of the ODN-lipid formulation. The cells were washed with PBS after 24 h of incubation, and fresh medium containing 10% heat-inactivated serum plus ODN was added. The cells were kept in culture for 5 to 7 days. Inhibition of HIV-1 activity in the cell culture supernatant was determined by the RT assay with 50 μl of the supernatant.

[^{3}H]thymidine incorporation. Briefly, cells were pretreated with ODN for 3 days and [^{3}H]thymidine incorporation into DNA was measured as previously described (26). The cells were washed with PBS, pulsed with 1 μCi of [^{3}H]thymidine per well for 16 h, and harvested. Cells were lysed with lysis buffer, and the protein concentration was determined. The radioactivity in the rest of the lysate was counted in a gamma counter for assessment of the cytotoxicity of the antisense ODN and the controls.

RESULTS

Antisense activity of GPI2A. We used an ODN (GPI2A) complementary to a nonregulatory region of the HIV-1 mRNA coding region for the Gag proteins in our cell culture model (Fig. 1). The immunoprecipitable translation products of the HIV-1 gene derived from untreated B4.14 cells are shown in Fig. 2 and include p160, p55, p39/41, and p24. We demonstrated some inhibitory effect of the antisense construct on HIV-1 viral protein synthesis. Under the assay conditions used, densitometric analysis indicated that the antisense oligomer exerted modest (20%) inhibitory activity on viral protein synthesis (e.g., p39/41) at an oligomer concentration of 5 μM, relative to the control, the inverse of GPI2A, and appeared to be ineffective at 1 μM (Fig. 3).

Effect of lipofectin reagent on cell-associated ODN. To optimize the effect of the lipofectin reagent on cell-associated ODN, a cationic lipid delivery system was used to determine if it would increase the effectiveness of the ODN and, in the process, offset the high cost of ODN synthesis. B4.14 cells were incubated with ^{32}P-labeled ODNs in the presence or absence of lipofectin reagent, and the cell-associated radioactivity was determined by direct suspension of the labeled cells in liquid scintillation medium. The result showed markedly increased (up to approximately fourfold) cell-associated radioactivity in the presence of lipofectin reagent relative to that of cells incubated in its absence (Table 1).

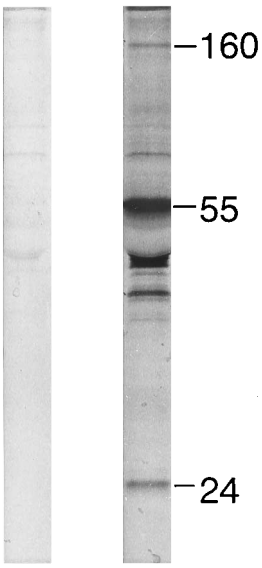

FIG. 2. Immunoprecipitable viral proteins after radiolabeling with [^{35}S]methionine. CMT3 (left lane) and B4.14 (right lane) cell lysates (200 μl) following 16 h of [^{35}S]methionine labeling were immunoprecipitated with p24-specific rabbit polyclonal antibody. Equal protein concentrations were loaded. The positions of the p160, p55, and p24 viral proteins are visible in the B4.14 cell lysate but not the control CMT3 (wild-type) cell lysate. Other viral protein processing intermediates are also visible in the B4.14 lane.

Potentiation of ODN activity by cationic lipid formulation. A mixture of ODN and lipofectin reagent was prepared (see Materials and Methods). Cells were washed with PBS and subsequently layered with the preincubation medium. The

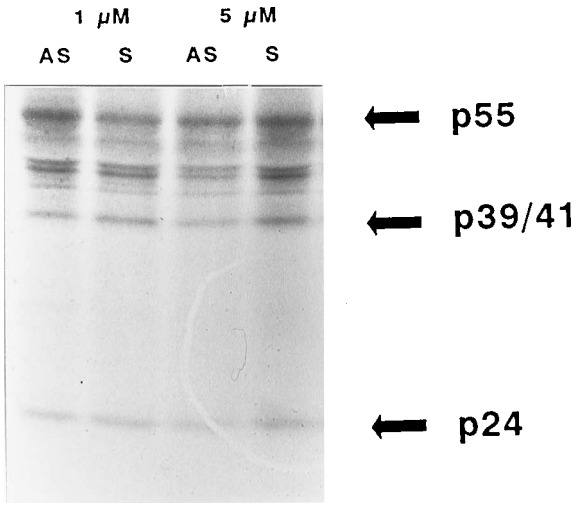

FIG. 3. Antisense activity of GPI2A on HIV-1 viral protein synthesis in the absence of lipofectin reagent. Cells were incubated for 3 days in the presence of 1 and 5 μM GPI2A or the inverse sequence of the antisense (AS) molecule as the control strand (S). The cells were subsequently washed with PBS and labeled for 16 h with 120 μCi of [^{35}S]methionine per ml. The viral proteins were immunoprecipitated by using a polyclonal antibody against p24.

TABLE 1. Potentiation of the cell-associated radiolabeled antisense ODN phosphorothioate GPI2A through formulation with lipofectin reagent

Incubation time (min)	Mean cpm ± SEM[a]	
	Minus lipofectin reagent	Plus lipofectin reagent
60	4,605 ± 1,179[b]	17,100 ± 3,344[c]
240	6,306 ± 1,344[d]	20,727 ± 3,250[e]

[a] Statistical analysis was done by Student's *t* test for unpaired data *b* versus *c*, $P < 0.01$; *d* versus *e*, $P < 0.005$; *b* versus *d*, $P < 0.02$; *c* versus *e*, $P < 0.002$. These data are from four independent experiments done in duplicate.

ODN-lipid formulation was added to the cells in a dropwise fashion. The cells were incubated at 37°C in a humidified incubator with 5 to 10% CO_2 environment for various lengths of time. Under these experimental conditions, a significant inhibitory effect of GPI2A on levels of both p55 and its cleavage product p39/41 was observed, even after only 4 h of pretreatment (Fig. 4). We also observed sequence-specific inhibition of viral protein synthesis, as determined by the use of a longer labeling period to allow the biosynthesis of a detectable amount of the viral core antigen, which is the breakdown product of the p55 precursor (Fig. 5).

The 50% inhibitory concentration of GPI2A for p24 viral core antigen biosynthesis after pretreatment of cells with different concentrations of the ODN was estimated by densitometric analysis to be approximately 0.40 μM (Fig. 6).

Inhibitory effect of GPI2A on HIV-1 message levels. To determine whether the observed inhibitory effect of GPI2A on viral protein synthesis was due to changes in mRNA levels, B4.14 cells were pretreated with the ODN-lipid formulation for various lengths of time and total mRNA was extracted from the cells by the rapid preparation method (22). Northern analyses of the isolated samples showed a significant reduction in the mRNA levels obtained from the cells pretreated with GPI2A compared with the controls (Fig. 7). The inhibition pattern agrees with the data obtained at the protein level, since the same effects were also observed after 4 h of pretreatment.

Antiviral activity of GPI2A. The results reported so far were obtained from a transfected-cell model. Further studies using acutely infected, phytohemagglutinin-stimulated umbilical cord blood mononuclear cells showed a dose-dependent antiviral activity (RT activity) of the GPI2A ODN under our experimental conditions (Table 2). The construct also showed statistically significant inhibition of viral replication as determined by the RT activity in chronically infected H9/IIIB cells

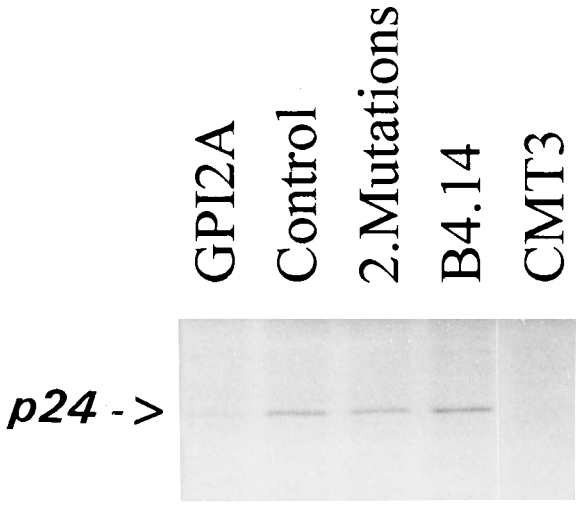

FIG. 5. Sequence-specific inhibitory activity of 1 μM GPI2A on the biosynthesis of HIV-1 core antigen p24. Cells were pretreated with the ODN-lipid formulation. They were then washed with PBS and radiolabeled for 16 h with methionine-free medium and 120 μCi of [^{35}S]methionine per ml. The viral protein was immunoprecipitated by using a rabbit polyclonal antibody directed against viral core antigen p24. The controls were the inverse sequence of the antisense molecule and GPI2A with two point mutations.

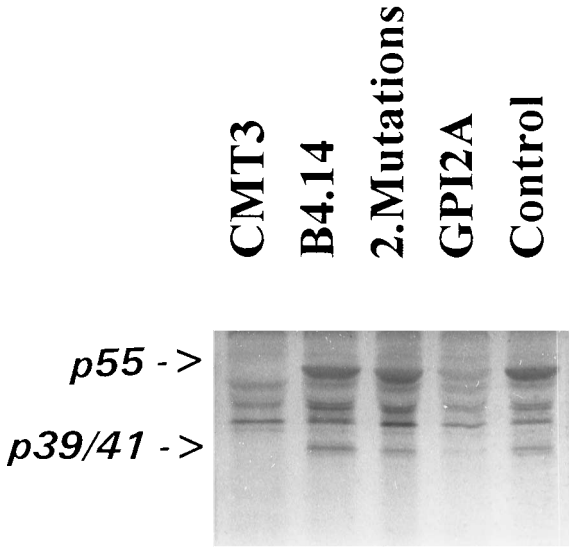

FIG. 4. Inhibitory activity of 1 μM GPI2A on the biosynthesis of HIV-1 Gag protein precursor p55 and its cleavage product p39/41. Cells were pretreated with the ODN-lipid formulation for 4 h. The cells were then washed with PBS and radiolabeled for 30 min with methionine-free medium and 250 μCi of [^{35}S]methionine per ml. The viral proteins were immunoprecipitated by using rabbit polyclonal antibody directed against viral core antigen p24. The controls were the inverse sequence of the antisense molecule and GPI2A with two point mutations.

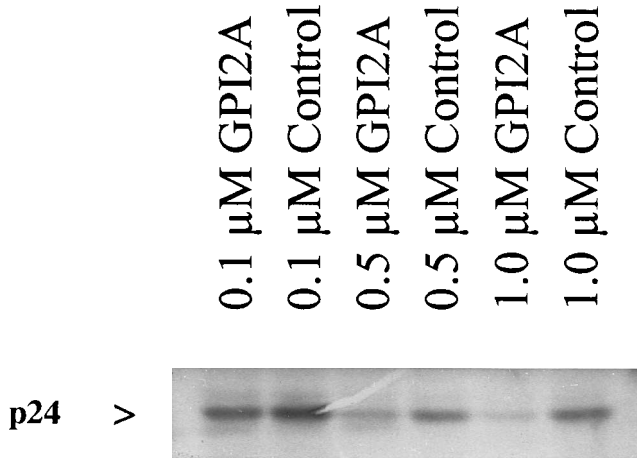

FIG. 6. Autoradiograph showing a concentration-dependent inhibitory effect of GPI2A on the biosynthesis of HIV-1 Gag protein p24. Cells were pretreated with the ODN-lipid formulation. The cells were then washed with PBS and radiolabeled for 16 h with methionine-free medium and 120 μCi of [^{35}S]methionine per ml. The viral protein was immunoprecipitated by using a rabbit polyclonal antibody directed against viral core antigen p24. The control in this experiment was the inverse sequence of the antisense molecule.

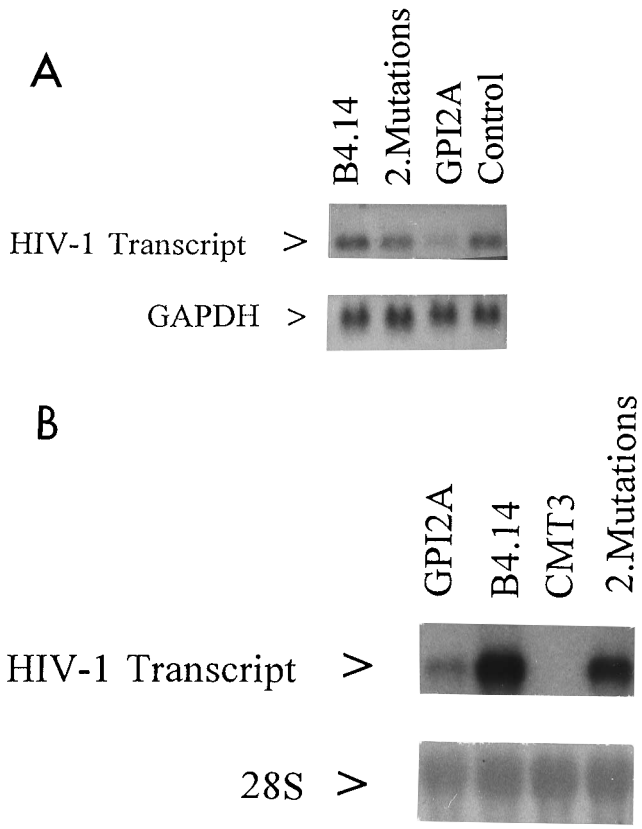

FIG. 7. Inhibitory activity of 1 μM GPI2A on HIV-1 mRNA. Cells were pretreated with the ODN-lipid formulation. Total RNA was extracted from the cells and probed by using the *gag* sequence corresponding to nucleotides 334 to 2037 of the cDNA fragment of the BH10 clone. (A) HIV-1 transcript after 4 h of pretreatment with the ODNs prior to isolation of total RNA, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) shown as a loading control. (B) HIV-1 transcript after 24 h of pretreatment prior to isolation of total RNA with 28S shown as a loading control. The controls were the inverse sequence of the antisense molecule and GPI2A with two point mutations.

in culture ($P < 0.02$) versus the effect observed with the control strand, the inverse of GPI2A (Table 3).

[^{3}H]thymidine incorporation. To determine whether the antisense and control (the inverse of the antisense sequence) oligomers have any significant effects on mammalian cell DNA synthesis, [^{3}H]thymidine incorporation was estimated after pretreatment of cells with ODN (Table 4). There was no statistically significant effect of the oligomer on the ability of the cells to incorporate nucleotide monomers into their DNA posttreatment with the ODNs.

TABLE 2. Concentration-dependent inhibition of HIV-1 strain IIIB replication in acutely infected phytohemagglutinin-stimulated umbilical cord blood mononuclear cells by antisense ODN phosphorothioate GPI2A

GPI2A concn (μM)	Mean RT activity[a] ± SEM
0.1	52.0 ± 6.4
1.0	43.0 ± 6.2
10.0	27.0 ± 6.7
50.0	15.25 ± 5.1

[a] Percentages of RT activity after pretreatment of infected cells compared with that of untreated infected cultures are shown. These data are from three independent experiments carried out in duplicate.

TABLE 3. Inhibition of HIV-1 strain IIIB replication in chronically infected H9/IIIB cells by 1 μM antisense ODN phosphorothioate GPI2A

ODN	Mean % RT activity[a] ± SEM
Antisense (GPI2A)	31.80 ± 9.3[b]
Inverse of GPI2A	71.25 ± 19[c]
Control	100

[a] Percentages of RT activity after pretreatment of infected cells compared with that of untreated infected cultures and the control are shown. These data are from four independent experiments done in duplicate. *b* versus *c*, $P < 0.02$.

Effect of ODN on colony formation and growth characteristics of B4.14 cells. Colony-forming experiments were performed to determine if the ODN sequences could affect the proliferative ability of B4.14 cells. As shown in Fig. 8, the colony-forming efficiencies of GPI2A-treated and untreated cells were not significantly different. The effects of ODNs on the general growth characteristics of the cells were also compared (Fig. 9). The cells were grown in ODN-containing medium for various lengths of time. Cells were then harvested, tested for viability by the trypan blue exclusion test (39), and counted to determine the growth pattern in the presence of oligomer. Pretreatment of B4.14 cells with the ODN sequences did not appear to affect either their growth rate or the saturation density. Under the experimental conditions used, ODN-treated and untreated cells showed a doubling time of 18 to 20 h.

Effect of ODN on the cellular protein ribonucleotide reductase R1 subunit. Experiments were performed to determine if GPI2A treatment of cells affected the synthesis of a nontarget cellular protein. We chose to examine the level of the large subunit of ribonucleotide reductase, R1 (M_r, 88,000), which is required for the synthesis of deoxyribonucleotides and, therefore, DNA (27, 45). In keeping with the observation that GPI2A was not cytotoxic for cells (Fig. 8 and 9), we found that the level of R1 protein was not significantly affected by ODN treatment, although viral protein synthesis was markedly inhibited (Fig. 10). The specific activities of R1 protein from antisense (GPI2A)- and control ODN-pretreated cells were not significantly different at a 1 μM ODN concentration.

DISCUSSION

Matsukura and associates have demonstrated that HIV-1 gene expression can be inhibited in chronically infected cells without killing the host cells (34). However, in that study, only the amount of p24 secreted into the medium was measured. Our data indicate that it is also possible to monitor the inhibition of HIV-1 gene expression by assaying the levels of the viral core antigen in infected cells. This assay is convenient, and measurement of the cellular-peripheral p24 level is a good

TABLE 4. [^{3}H]thymidine incorporation by treated and untreated B4.14 cells

ODN	Mean cpm/mg of protein[a] ± SEM
Antisense (GPI2A)	1,800 ± 100
Inverse of GPI2A	1,833 ± 120
Control (B4.14 cells)	1,830 ± 88

[a] Statistical analysis was done by Student's *t* test for unpaired data, and no significant differences were found. These data were obtained from three independent experiments done in duplicate.

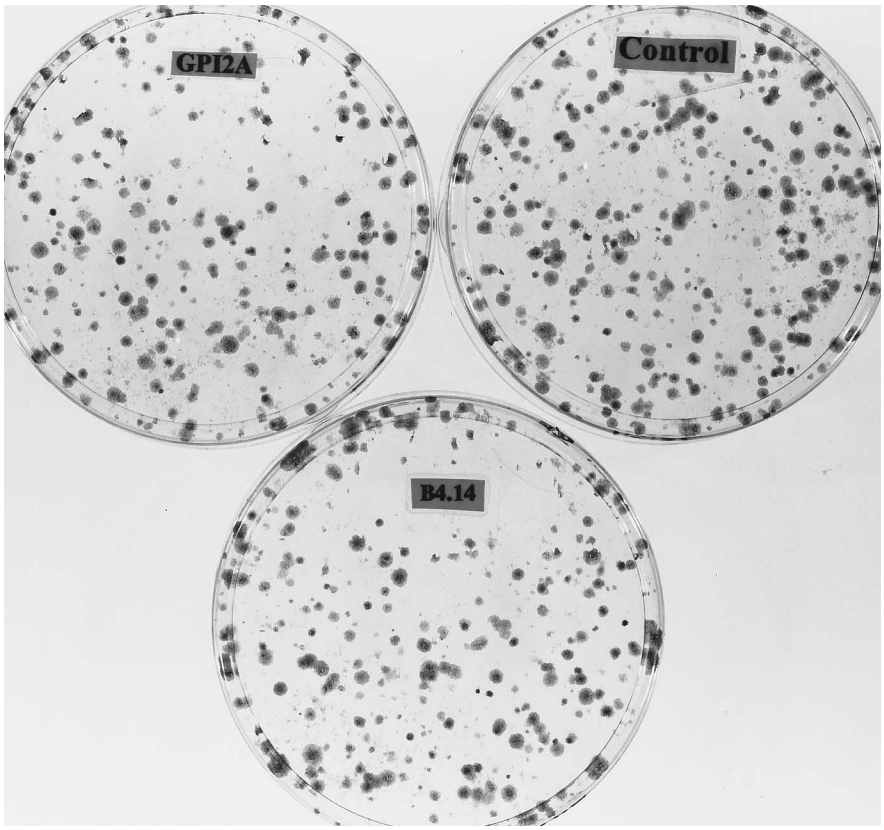

FIG. 8. Plates showing the colony-forming abilities of pretreated and untreated B4.14 cells. The cells were pretreated with 1 μM oligomer for 3 days. The cells were then replated, incubated for 11 days in the presence of 1 μM ODN, and stained. The control shown is a plate pretreated with the inverse sequence of the antisense molecule.

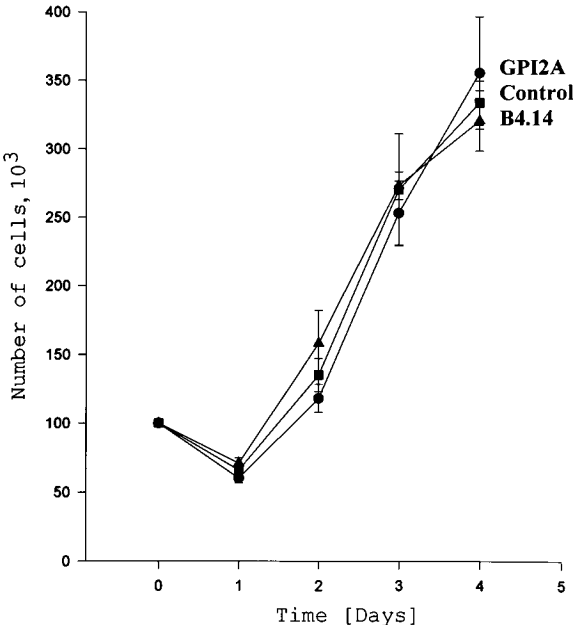

FIG. 9. Growth curves of pretreated and untreated B4.14 cells. Cells were pretreated with 1 μM oligomer for 3 days. Cells were added to 100-mm-diameter tissue culture plates and allowed to grow in the presence of 1 μM ODN for the lengths of time indicated and then counted. The cells had a doubling time of about 18 to 20 h. Control plates contained cells that were treated with the inverse sequence of the antisense molecule.

marker for disease progression in HIV-1-infected individuals (13).

Our studies suggest that translation of mRNA may be blocked by the binding of a complementary ODN to its target sequence. Several possible mechanisms could account for low levels of viral proteins and mRNA. For example, base-specific hybridization inhibits translation (i.e., hybridization arrest) and

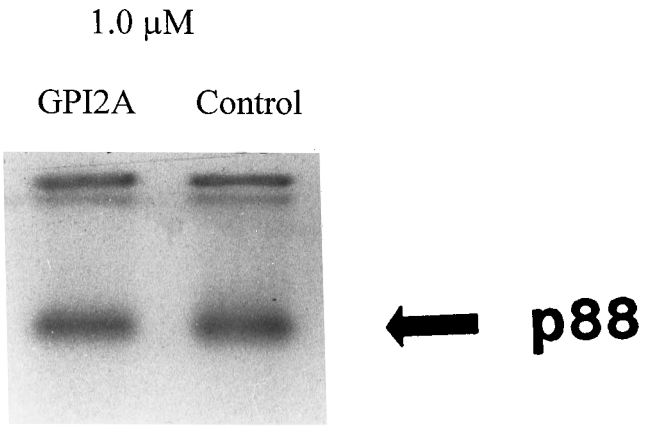

FIG. 10. Autoradiograph of an immunoprecipitated cellular protein, showing immunoprecipitation of cellular protein ribonucleotide reductase R1. Immunoprecipitation was carried out with supernatant by using a monoclonal antibody against the reductase large subunit after precipitation of viral proteins with a rabbit polyclonal antibody against p24.

may also accelerate degradation of the target mRNA. These observations are consistent with the proposed mechanism of action for phosphorothioate derivatives of ODNs (4), although it is still possible that the attenuated HIV-1 mRNA levels may be due, at least in part, to inhibition of transcription. The patterns of inhibition observed in this study were reasonably specific; however, we have not determined how other viral RNA species react to treatment with GPI2A. Furthermore, the results of trypan blue exclusion tests, cell growth and colony-forming ability studies, and immunoprecipitation analysis of the R1 subunit of ribonucleotide reductase as a nontarget cellular protein showed that pretreatment of cells with GPI2A did not result in cytotoxicity or changes in the normal proliferative abilities of the cells.

For ODNs to be useful antiviral agents, they should be taken up by cells in an efficient manner. Since the target sites for these agents lie in the cytoplasm and nucleus of a cell and these agents usually cross cell membranes rather poorly, significant quantities of oligomer must be presented to the cell to achieve even modest concentrations at the intracellular target sites (30). Phospholipid vesicles (liposomes) have been widely used as a drug delivery system for standard anticancer and anti-infection drugs (10, 28, 30, 35, 46). Therefore, we explored the potential of liposomes for delivery of GPI2A and its analogs with our model system. The goal was to maximize the amount of active drug at the intracellular target site, where therapeutic effects can be achieved, and to minimize the amount of drug (or duration of exposure) at other sites, where toxic effects or drug loss may occur. Results of these studies supported the effectiveness of DNA-lipofectin reagent for delivering ODNs to cultured cells (17), since in the presence of this DNA-cationic lipid formulation, the antisense activity of GPI2A was markedly increased. There have been a few previous attempts to use liposomes in the delivery of ODNs (10, 30). For example, by using fluorescent labeled probes, ODNs were localized to discrete structures in the cytoplasm in the absence of liposomes; however, in the presence of liposomes, markedly increased localization of ODNs was observed in the nucleus and cytoplasm (10). These observations demonstrated that cationic lipids may have brought about the increased antisense effect by increasing the amount of ODN associated with the cells and by altering its intracellular distribution.

In summary, we designed an ODN sequence corresponding to a nonregulatory region of the HIV-1 genome which inhibits viral gene expression and HIV-1 replication. This probably occurs at least partly via a translation arrest mechanism associated with RNase H activation (1, 4). The ODN sequence described here will be of benefit in investigations into the regulation of HIV-1 gene expression, especially in a liposome delivery system, and perhaps eventually as a therapeutic agent.

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