JOURNAL OF VIROLOGY, May 2000, p. 4621–4633 Vol. 74, No. 10
0022-538X/00/$04.00+0

Identification of a Key Target Sequence To Block Human Immunodeficiency Virus Type 1 Replication within the *gag-pol* Transframe Domain

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Received 10 November 1999/Accepted 17 February 2000

Although the full sequence of the human immunodeficiency virus type 1 (HIV-1) genome has been known for more than a decade, effective genetic antivirals have yet to be developed. Here we show that, of 22 regions examined, one highly conserved sequence (ACTCTTTGGCAACGA) near the 3′ end of the HIV-1 *gag-pol* transframe region, encoding viral protease residues 4 to 8 and a C-terminal Vpr-binding motif of $p6^{Gag}$ protein in two different reading frames, can be successfully targeted by an antisense peptide nucleic acid oligomer named PNA_{PR2}. A disrupted translation of *gag-pol* mRNA induced at the PNA_{PR2}-annealing site resulted in a decreased synthesis of $Pr160^{Gag\text{-}Pol}$ polyprotein, hence the viral protease, a predominant expression of $Pr55^{Gag}$ devoid of a fully functional $p6^{Gag}$ protein, and the excessive intracellular cleavage of Gag precursor proteins, hindering the processes of virion assembly. Treatment with PNA_{PR2} abolished virion production by up to 99% in chronically HIV-1-infected H9 cells and in peripheral blood mononuclear cells infected with clinical HIV-1 isolates with the multidrug-resistant phenotype. This particular segment of the *gag-pol* transframe gene appears to offer a distinctive advantage over other regions in invading viral structural genes and restraining HIV-1 replication in infected cells and may potentially be exploited as a novel antiviral genetic target.

The persistence of integrated proviruses in host cells presents formidable challenges to the treatment of human immunodeficiency virus type 1 (HIV-1) infection. Replication-competent HIV-1 can be recovered from resting $CD4^+$ T lymphocytes even from individuals receiving potent combination antiretroviral therapy, whose plasma virus levels have remained below the detection limit for a prolonged period (19, 33, 36, 147, 153). The discovery of a long-lived viral reservoir, which is established early in the infection (18, 33, 147), has suggested that HIV-1 cannot be easily eradicated from infected individuals with the current treatments. Another impediment to controlling HIV-1 infection is the emergence of drug-resistant viral strains (21, 47, 50, 61, 62, 67, 78, 90, 107, 110, 111, 130). HIV-1 protease, in particular, seemingly tolerates extensive sequence variations (8, 76, 79), contributing to a rapid emergence of protease inhibitor-resistant strains, which can be cross-resistant to multiple protease inhibitors (21, 47, 90, 107, 110, 111). Indeed, multidrug-resistant (MDR) HIV-1 strains are isolated increasingly from patients who have been extensively treated with various antiretroviral agents of similar classes (151), and the spread of these MDR strains may become a serious threat to the containment of the AIDS epidemic in the future. The identification of novel viral targets is clearly needed to empower anti-HIV-1 therapeutic strategies. In recent years, viral coreceptors (25, 26, 39, 99, 105, 132), integrase (7, 29, 31, 93, 101, 115, 154, 155), and the viral nucleocapsid protein zinc finger motif (94, 117–120, 137) have emerged as novel antiviral targets. However, substantial progress has yet to be made before any of the candidate compounds can be brought to practical applications.

Another possible antiviral target pursued over the years is the HIV-1 genome itself. Antisense reagents, in particular, have been extensively investigated primarily in the form of nuclease-resistant phosphorothioate oligodeoxynucleotides (PsODN). However, these previous attempts targeting various regions of the HIV-1 genome with antisense PsODN have produced inconsistent results in chronically infected cells (2, 4, 5, 64, 66, 83–86, 92, 141, 148). The reasons for a lack of consistency may vary. Selected target sequences may have been less critical for virion production in chronically HIV-1-infected cells or less accessible to the PsODN molecules because of the RNA-binding proteins or intracellular folding of the target RNA. It is also possible that antisense PsODN molecules were simply ineffective in abating ribosome elongation (9, 14, 139) or that available antisense molecule numbers were insufficient to overcome the enormous amount of viral transcripts expressed in the chronically infected cells (140).

Selection of optimal genetic targets and the use of potent gene-intervening reagents are equally critical elements of a successful antigene or antisense strategy. Peptide nucleic acid (PNA) (27, 45, 103, 145), initially developed as a reagent for strand invasion of the duplex DNA, is a DNA mimic, consisting of a peptide backbone of *N*-(2-aminoethyl)glycine units in place of a deoxyribose backbone. Although unmodified PNA has a relatively poor cellular uptake compared to that of ODN (104, 144), it has unique molecular characteristics which may enhance its utility as a genome-intervening tool, such as resis-

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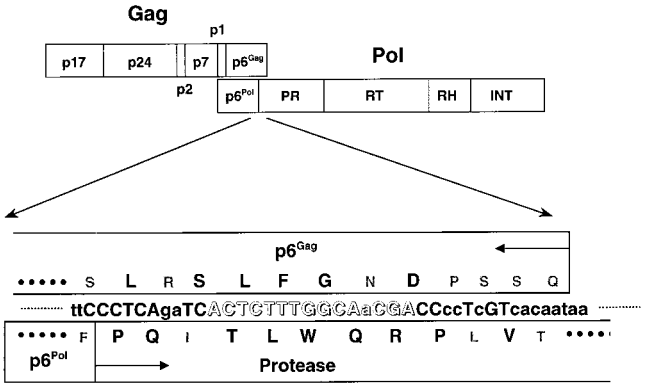


FIG. 1. Schematic representation of the *gag* and *pol* regions of HIV-1 (top) and the amino acid sequences of portions of $p6^{Gag}$ protein and viral protease encoded by two different reading frames (bottom). The amino acid residues conserved in >98% of the majority HIV-1 substrains (group M), which include subtypes A, B, C, D, F, G, H, and J and circulating recombinant forms (AE, AG, AGI, and AB) (HIV Sequence Database [http://hiv-web.lanl.gov]), are shown in large capital letters. The B subtype consensus sequences are shown in small capital letters. The nucleotide sequence of our interest is shown in open letters. PR, protease; RH, RNase H; INT, integrase.

tance to nucleases and proteases (24) and sequence-specific hybridization to DNA or RNA targets using Watson-Crick base pair formation (27) with much higher thermal stability than that of ODN (27, 28, 51).

Using a PNA oligomer as a prototype molecular tool, we explored viral sequences that were susceptible to PNA-mediated inhibition of gene expression and asked whether such sequences might be considered potential anti-HIV-1 genetic targets in the current study. Target sequences were selected from the previously less explored *gag-pol* gene, with a particular focus on the *pol* gene, as few studies have tried to directly block the expression of viral enzymes. In particular, we were interested in the protease-encoding sequence that begins upstream of the 3′ end of the *gag-pol* transframe gene. Despite its extensive sequence variations, a short segment of sequence toward the 5′ end of the viral protease-encoding gene, ACTC TTTGGCAACGA, which also encodes the C-terminal Vpr-binding motif of $p6^{Gag}$ protein, LXXLFG, by a different reading frame (17, 68, 88), is highly conserved among various HIV-1 subtypes (Fig. 1). We hypothesized that nucleotide sequences of certain segments of the transframe domain must be invariably conserved if a single transcript has to encode critical amino acid sequences of two different proteins by a ribosomal frameshift, thus reducing the probability of escape mutants, and considered this particular sequence as one of the prime targets.

MATERIALS AND METHODS

PNA oligomers. Because of the propensity of PNA molecules to be confined within the cytoplasm (104) (also see below), we decided to focus on the antisense intervention, primarily targeting the translation of viral RNA, and designed 22 PNA oligomers, 14- to 15-mers in length, complementary to the site of our interest within the *gag-pol* transframe domain or other highly conserved regions within the *gag* and *pol* genes for the initial screening (Table 1). PNA oligomers were synthesized on the Expedite Nucleic Acid Synthesis System (PerSeptive Biosystems, Inc., Framingham, Mass.) by Research Genetics, Inc., Huntsville, Ala. Although all these PNA oligomers were easily dissolved in water, some oligomers, including PNA_{GAG1}, PNA_{GAG2}, PNA_{PR1}, PNA_{PR2}, PNA_{RT1}, PNA_{RT5}, PNA_{RT8}, and PNA_{INT3}, formed fine precipitates when added to the culture medium at higher concentrations, as has previously been reported (104). There was no discernible relationship between the precipitate formation and effects on cell growth or HIV-1 production (see below).

Evaluation of cellular uptake of PNA oligomers. H9 cells chronically infected with $HIV\text{-}1_{LAI}$ ($H9_{LAI}$) were incubated with fluorescein-tagged PNA_{PR2}, PNA_{PR4}, or PNA_{RT2} at 10, 30, and 100 μM in fresh RPMI 1640 complete medium supplemented with 13% fetal bovine serum (HyClone, Logan, Utah), 4 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in 5% CO_2-containing humidified air overnight. The cells were fixed with 2% paraformaldehyde and examined by fluorescence microscopy or by fluorescence-activated cell sorter analysis.

Assessment of effects of PNA oligomers on HIV-1 infection in various cell culture systems. $H9_{LAI}$ cells or H9 cells chronically infected with another HIV-1 strain, $HIV\text{-}1_{RF}$ ($H9_{RF}$), were extensively washed to remove previously produced virions and incubated in a 96-well culture plate at 10^4 cells/well in 200 μl of RPMI 1640 complete medium in the absence or presence of PNA oligomers at various concentrations. After 4 days in culture, supernatant was collected from each well and the level of p24 Gag antigen was determined by radioimmunoassay (RIA) (HIV-1 p24 RIA kit; NEN Life Science Products, Boston, Mass.). To compare the antiviral effects of PNA_{PR2} and PNA oligomers targeting the sequences proximate to the PNA_{PR2}-annealing site, $H9_{LAI}$ cells were cultured as described above in the absence or presence of 100 μM PNA_{PR2} or other PNA oligomers tested. The relative antiviral effects were computed from the following formula: (% p24 antigen suppression by a given PNA oligomer)/(% p24 antigen suppression by PNA_{PR2}). Each PNA oligomer was tested in triplicate. $H9_{LAI}$ and $H9_{RF}$ cells were also cultured in a six-well culture plate at 10^5 cells/ml in the absence or presence of 30 to 100 μM PNA_{PR2}. After 4 days, cells were counted by the trypan blue dye exclusion method and lysed at 10^6 cells/ml in phosphate-buffered saline (PBS) containing 0.5% Triton X-100. The amounts of p24 antigen in the culture supernatant and cell lysate were measured by RIA. The experiments were repeated at least three times.

Phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) obtained from healthy blood bank donors (2×10^6 cells/ml) were infected with seven MDR HIV-1 clinical isolates (151) in RPMI 1640 complete medium supplemented with 2.5 ng of recombinant human interleukin-2 (R&D Systems, Minneapolis, Minn.) per ml and cultured for at least 2 weeks or until the p24 antigen levels in the culture medium persistently exceeded 30 ng/ml. The PNA_{PR2}-targeting nucleotide sequence of the transframe domain of each isolate was consistent with the consensus B sequence. Freshly PHA-stimulated PBMC from healthy donors (2×10^6 cells/ml) were added to each culture flask every 3 to 4 days. PBMC (10^6 cells/ml) infected with each MDR isolate were vigorously washed and cocultured with PHA-stimulated PBMC (10^6 cells/ml) in 200 μl of recombinant human interleukin-2-containing complete medium in the absence or presence of 10 to 60 μM PNA_{PR2}. The number of infectious virions released after 7 days in culture was determined by an assay with multinuclear activation of a galactosidase indicator (MAGI assay) (see below) using an indicator cell line, MAGI-CCR5 cells (16) (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, contributed by Julie Overbaugh). All assays were performed in triplicate.

MT2 cells (10^4 cells/ml) were exposed to 64 50% tissue culture infectious doses of $HIV\text{-}1_{NL4\text{-}3}$ at 37°C for 1 h. After a vigorous virus washout, the cells were cultured in 200 μl of RPMI 1640 complete medium at 37°C in 5% CO_2-containing humidified air in the absence or presence of various PNA oligomers at 100

TABLE 1. Sequences of PNA oligomers and locations of targeted genes relative to the HXB2 numbering system

PNA oligomer	Sequence (5′-3′)	Position in HIV-1 genome
PNA_{Gag1}	CGCTCTCGCACCCAT	790–804
PNA_{Gag2}	TCCCTGCTTGCCCAT	894–908
PNA_{Gag3}	TCATCATTTCTTCT	1818–1831
PNA_{PR1}	CCAAAGAGTGATTTT	2256–2270
PNA_{PR2}	TCGTTGCCAAAGAGT	2262–2276
PNA_{PR3}	ATCATCTGCTCCTGT	2328–2342
PNA_{PR4}	CCCCCTATCATTTTT	2384–2398
PNA_{RT1}	TTTTACTGGTACAGT	2568–2582
PNA_{RT2}	CTGTCAATGGCCATT	2617–2631
PNA_{RT3}	ATTTTCAGGCCCAA	2698–2711
PNA_{RT4}	CTGTCTTTTTTCTTT	2738–2752
PNA_{RT5}	ATTGTACTGATATCT	2976–2990
PNA_{RT6}	AATCATCCATGTATT	3094–3108
PNA_{RT7}	TGCTGCCCTATTTCT	3128–3142
PNA_{RT8}	ATTGACAGTCCAGCT	3300–3314
PNA_{RH1}	TGTGCTGGTACCCAT	4151–4165
PNA_{INT1}	TAGCCATTGCTCTCC	4285–4299
PNA_{INT2}	TCTACTTGTCCATGC	4379–4393
PNA_{INT3}	CTTCCTGCTAATTTT	4535–4549
PNA_{INT4}	CCCCCAATCCCCCCT	4793–4807
PNA_{INT5}	AATTTTGAATTTTT	4883–4896
PNA_{INT6}	TTGCTGGTCCTTTCC	4933–4947

μM. The amount of p24 antigen produced by the MT2 cells was determined by RIA on day 7. All assays were performed in triplicate.

COS-7 cells were transfected with 1 μg of pNL4-3 by FuGENE 6 transfection reagent (Boehringer Mannheim, Indianapolis, Ind.) in a six-well culture plate (35-mm diameter) for 5 h, immediately followed by incubation in Dulbecco's modified Eagle medium (DMEM) supplemented with 13% fetal bovine serum (HyClone), 4 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in the absence or presence of 100 μM PNA_{PR2}. After 48 h, cells and supernatants were harvested from each sample and evaluated for viral protein expression and virion production. HLtat cells (AIDS Research and Reference Reagent Program; contributed by Barbara K. Felber and George Pavlakis) were transfected with 1 μg of p55M1-10 (kindly provided by George Pavlakis, National Cancer Institute-Frederick Cancer Research and Development Center) by FuGENE 6 transfection reagent (Boehringer Mannheim) for 5 h, immediately followed by incubation in complete DMEM in the absence or presence of 100 μM PNA_{PR2}. After 24 h, HLtat cells were lysed for Western blot analysis.

MAGI assay. The MAGI assay was employed to determine the number of newly produced virion particles in the culture supernatant of HIV-1-infected cells as described previously (65). Briefly, the HeLa–CD4–long terminal repeat–β-galactosidase indicator cells were plated in a 96-well tissue culture plate at 10^4 cells per well, each well containing 125 μl of complete DMEM, 24 h prior to the assay. On the following day, the cells were generally 20 to 30% confluent. The cells were washed with 200 μl of Opti-MEM (Life Technologies, Inc., Rockville, Md.) twice and then exposed to serially diluted infectious culture supernatants in a total volume of 30 μl per well in the presence of 20 μg of DEAE-dextran (Sigma, St. Louis, Mo.) per ml. The infectious titers of the supernatants from PHA-PBMC infected with MDR isolates were examined with the MAGI-CCR5 indicator cell line (16), using 30 or 60 μl of inoculum. After the plates were incubated at 37°C in 5% CO_2-containing humidified air for 2 h, 140 μl of complete DMEM was added to each well. The plates were incubated for another 46 h, followed by fixation at room temperature with 1% formaldehyde and 0.2% glutaraldehyde in PBS for 5 min. The cells were then washed with PBS and incubated in 100 μl (per well) of staining solution containing 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. The blue cells were counted under a microscope.

Electron microscopy. Electron microscopic examination was performed as previously described (38). Briefly, the harvested cells were centrifuged at 1,500 × *g* for 5 min. The cell pellets were fixed in 1.25% glutaraldehyde and then in 1% osmium, dehydrated in graded alcohol, and embedded in pure epoxy resins. Thin sections (60 nm) were stained with uranyl acetate and lead citrate and stabilized by carbon evaporation for an examination.

RNA analysis. The culture supernatant was subjected to microcentrifugation at 32,800 × *g* for 2 h to pellet virions (35, 142). Pelleted virion particles were subjected to RNA extraction as previously described (6), followed by reverse transcriptase PCR (RT-PCR) with a primer pair, SK38-SK39 (128, 129), to estimate the amount of virion-derived RNA. The harvested cells were subjected to RNA extraction as previously described (129) followed by RT-PCR using two primer pairs, SK38-SK39 (128, 129) and BSS-KPNA (102, 124), in order to evaluate the levels of unspliced and singly spliced HIV-1 RNA, respectively.

Western blot analysis. The virion particles pelleted from the culture supernatant (see above) were lysed in viral lysis buffer (10 mM Tris [pH 7.4], 1 μM EDTA, 0.02% NP-40). The harvested cells were washed in PBS and lysed in cell lysis buffer (10 mM Tris [pH 7.4], 50 mM NaCl, 100 mM KCl, 1 mM EDTA, 1% NP-40, 1 mM phenylmethanesulfonyl fluoride) at 2 × 10^7 cells/ml at 4°C for 30 min, followed by centrifugation at 13,800 × *g* to remove cell debris. Protein concentrations of the cell lysates were determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). Virion-associated protein derived from 100 μl of supernatant and 2 to 25 μg of cellular protein were resolved by electrophoresis on sodium dodecyl sulfate–4 to 12% polyacrylamide gradient gels (Novex, San Diego, Calif.) under reducing conditions, followed by electroblotting onto a polyvinylidene difluoride membrane (Novex). The HIV-1 Gag proteins were visualized by a chemiluminescence detection system (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) using anti-$p24^{Gag}$ antiserum (Advanced Biotechnologies, Inc., Columbia, Md.) (2 μg of cellular protein per lane loaded for this antibody), anti-$p17^{Gag}$ monoclonal antibody (Advanced Biotechnologies, Inc.) (2 μg of protein per lane), and anti-$p6^{Gag}$ antiserum (kindly provided by Louis E. Henderson, National Cancer Institute-Frederick Cancer Research and Development Center) (5 μg of protein per lane). For the evaluations of *pol* gene products, antiprotease antiserum (kindly provided by Louis E. Henderson), (25 μg of protein loaded per lane) and anti-$p66^{RT}$ monoclonal antibody (AIDS Research and Reference Reagent Program; contributed by Paul Yoshihara) (15 μg of protein per lane) were employed. For a resolution of anti-$p66^{RT}$ antibody-reactive proteins, sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis was used instead of a 4 to 12% gradient gel. The anti-$p66^{RT}$ antibody is reactive with the $p66^{RT}$ but not the $p51^{RT}$ band by Western blot analysis (NIH AIDS Research and Reference Reagent Program Catalog). Anti-gp120 antiserum (AIDS Research and Reference Reagent Program; contributed by Margarita Quiroga) (10 μg of protein loaded per lane) was used to evaluate the amount of *env* gene product expressed in HIV-1-infected cells.

Peptide ELISA. The affinity of rabbit anti-$p6^{Gag}$ antiserum (kindly provided by Louis E. Henderson) for a series of HIV-1 HXB2 (subtype B) Gag synthetic peptides, 441–458 (YKGRPGNFLQSRPEPTAP), 451–470 (SRPEPTAPPEESF RSGVETT), 459–479 (PEESFRSGVETTTPPQKQEPI), 470–489 (TTPPQKQ EPIDKELYPLTSL), and 480–500 (DKELYPLTSLRSLFGNDPSSQ) (obtained through AIDS Research and Reference Reagent Program), which encompassed the entire $p6^{Gag}$ domain, was determined by enzyme-linked immunosorbent assay (peptide ELISA). Briefly, 96-well plates were coated with serially diluted Gag peptides (9.8 to 2,500 ng/well) in triplicate. Anti-$p6^{Gag}$ antiserum diluted 1:500 was incubated in the peptide-coated plates for 90 min at room temperature. After washing, horseradish peroxidase-labeled anti-rabbit secondary antibody was added to each well, followed by the peroxidase substrate. The antibody affinity for each peptide was determined by the optical density (OD) at 405 nm.

RESULTS

Cellular uptake of PNA and its intracellular localization. First, in order to determine concentrations of PNAs required to achieve a sufficient cellular delivery in tissue culture, $H9_{LAI}$ cells were incubated with fluorescein-tagged PNA at 10 to 100 μM. We elected to deliver PNA by simply adding it to the culture medium, thus relying on endocytosis (42, 104, 131) rather than the microinjection technique commonly adopted in the previous studies (10, 45, 104). After an overnight incubation, $H9_{LAI}$ cells were examined under a fluorescence microscope. A clear fluorescent signal was demonstrated in the majority of cells incubated with PNA at ≥30 μM compared to untreated $H9_{LAI}$ cells (Fig. 2A to C), albeit the signal was virtually confined to the cytoplasm, as has previously been reported (104) (Fig. 2D). The fluorescence intensity increased in a dose-dependent manner as demonstrated by fluorescence-activated cell sorter analysis regardless of the sequences tested (Fig. 2E).

PNA_{PR2}, targeting the 3′ end of the transframe domain, decreases extracellular virion production while increasing intracellular concentration of Gag protein in HIV-1-infected cells. Preliminary effects of various PNA oligomers were evaluated in chronically HIV-1-infected $H9_{LAI}$ cells. While the majority of PNA oligomers, all tested at 100 μM, exhibited virtually no antiviral effect, PNA_{PR2} reduced the HIV-1 p24 antigen production in the culture supernatant by up to 98.4% (Fig. 3A). This p24-inhibitory effect of PNA_{PR2} appeared to be dose dependent in $H9_{LAI}$ as well as in $H9_{RF}$ cells (Fig. 3B) and specific to the PNA_{PR2}-targeting region, as shifting the target sequence by two or more nucleotides upstream or downstream resulted in a more than 50% decrease in the inhibitory effect (Fig. 3C). Significant inhibition of p24 by PNA_{PR2} was also observed in MT2 cells acutely infected with $HIV\text{-}1_{NL4\text{-}3}$ (inhibition of [99.6 ± 0.4] % [mean ± standard deviation (SD) of triplicate values]) compared to untreated HIV-1-infected MT2 cells. Similarly, PNA_{PR2} reduced infectious virion production from PBMC infected with clinical MDR isolates, originally isolated from extensively pretreated patients (151), by 97.3 to 99.4% (Table 2). These data suggested a potential antiviral effect of PNA_{PR2} against a broad spectrum of HIV-1 strains.

In contrast to a marked decrease in the supernatant p24 antigen levels, the amount of intracellular p24 antigen was increased in $H9_{LAI}$ cells treated with 30 or 60 μM PNA_{PR2} (Fig. 3D) with moderately reduced cell numbers at the time of harvest. Comparable results were obtained in other experiments, in which PNA_{PR2} was tested up to 100 μM. Percent cell growth of $H9_{LAI}$ cells incubated with 60 to 100 μM PNA_{PR2} ranged from 68.3 to 78.1% (mean ± SD, [73.9 ± 5.1]%). Similar degrees of impaired cell growth were observed in $H9_{RF}$ as well as uninfected H9 cells incubated with up to 100 μM PNA_{PR2}, while other PNA oligomers had no effect on cell proliferation in $H9_{LAI}$, $H9_{RF}$, or uninfected H9 cells (data not shown). These findings suggested that the impaired cell growth

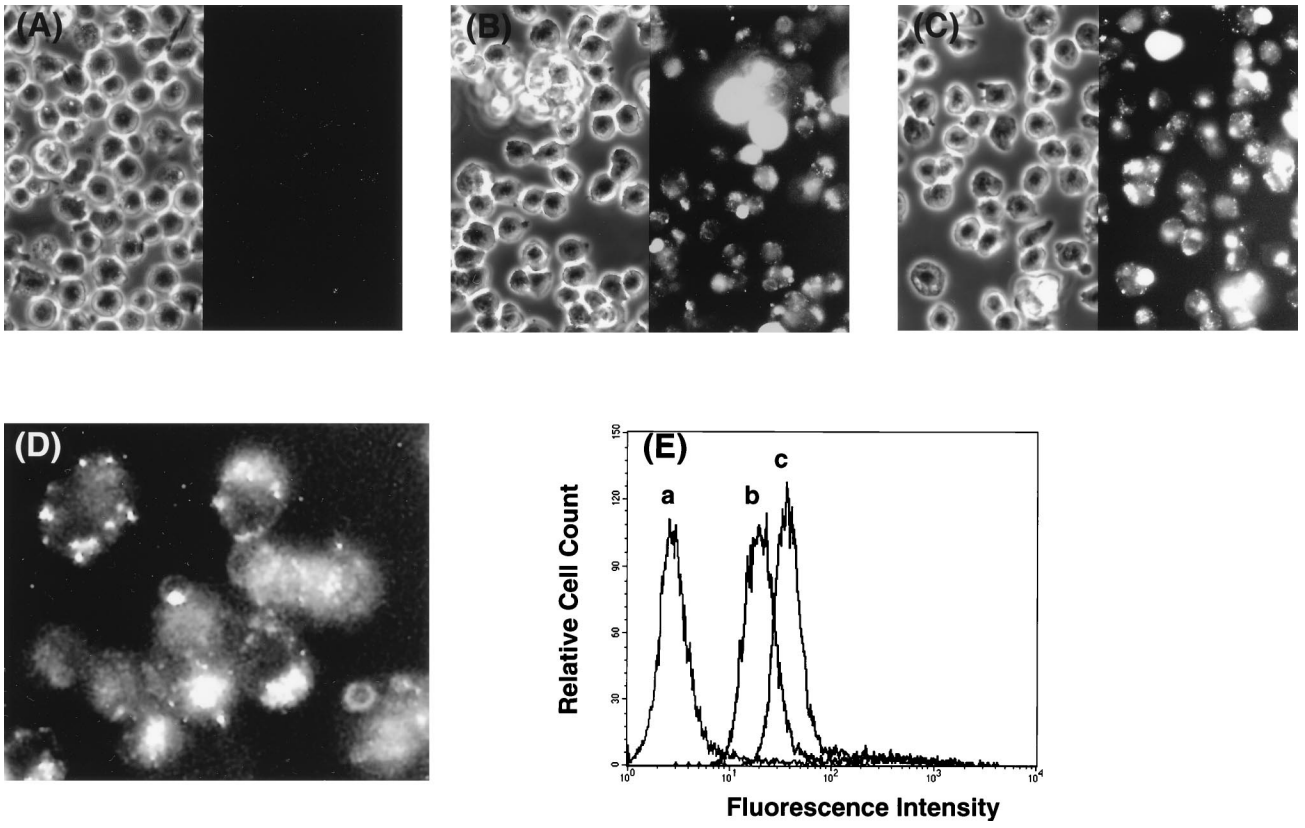


FIG. 2. Cellular uptake of PNA. (A to C) Chronically HIV-1-infected $H9_{LAI}$ cells were incubated with fluorescein-tagged PNA at various concentrations overnight, fixed with 3.7% formaldehyde for 20 min, washed and resuspended in PBS, and examined by fluorescence microscopy. Shown are the cells incubated with 0 (A), 30 (B), and 60 (C) μM fluorescein-tagged PNA_{PR2}. Note that the cells treated with fluorescein-tagged PNA_{PR2} were resuspended in a slightly larger volume of PBS than was the untreated control so that each cell could be clearly visualized. The panel on the right-hand side is the fluorescence image, and that on the left is the phase-contrast image of the same field. The discernible fluorescent signal was seen in the majority of cells at 30 μM PNA or greater. (D) Shown at a higher magnification are the cells incubated with 30 μM PNA_{PR2}. Note that the fluorescent signal is virtually confined to the cytoplasm. (E) $H9_{LAI}$ cells incubated with fluorescein-tagged PNA were also evaluated by fluorescence-activated cell sorter analysis. Curves a, b, and c represent $H9_{LAI}$ cells incubated with 0, 30, and 60 μM fluorescein-tagged PNA_{PR2}, respectively. Similar results were obtained with fluorescein-tagged PNA_{PR4} and PNA_{RT2}.

was specific to PNA_{PR2}-targeted sequence intervention and may have resulted from an accumulation of p24 Gag protein within the cytoplasm and/or a down-modulation of certain host proteins that have yet to be identified, rather than from general toxicity of the PNA molecules.

Next, the processes of virion morphogenesis and virion release from the cell surface were examined by electron microscopy. Treatment with PNA_{PR2} appeared to hinder the steps of virion assembly, unlike protease inhibitors, which halt the process of virion morphological maturation. For 100 cells surveyed within one 60-nm thin section, the estimated average number of HIV-1 virions formed and released from the cells was reduced to less than two per sectioned cell (62 cells with no virion and 38 cells with less than five virions per cell) in PNA_{PR2}-treated $H9_{LAI}$ cells compared to 10 to 15 virions per sectioned cell in untreated $H9_{LAI}$ cells, while there were no apparent changes in virion morphology (Fig. 4).

PNA_{PR2} blocks production of $Pr160^{Gag\text{-}Pol}$ polyprotein and induces excessive intracellular cleavage of $Pr55^{Gag}$. To determine the mechanism(s) of virion assembly inhibition by PNA_{PR2}, $H9_{LAI}$ cells cultured in the presence of 30 to 100 μM PNA_{PR2} were evaluated for viral protein expression and virion production. Consistent with the preliminary results, the level of p24 antigen production and the amount of pelletable virions were significantly reduced in the supernatant of PNA_{PR2}-treated cells compared to the untreated control (Fig. 5A). The amounts of intracellular HIV-1 unspliced and singly spliced RNA transcripts were comparable between PNA_{PR2}-treated and untreated cells (Fig. 5A), indicating that PNA_{PR2} did not interfere with the transcription of the HIV-1 genome. Strikingly, Western blot analysis with anti-$p24^{Gag}$ antibody demonstrated that PNA_{PR2}-treated $H9_{LAI}$ cells contained predominantly p24 Gag protein with Gag precursor $Pr55^{Gag}$ markedly reduced and $Pr160^{Gag\text{-}Pol}$ virtually undetectable within the cells (Fig. 5A).

In order to further examine the expression and subsequent proteolytic processing of Gag protein in PNA_{PR2}-treated cells, $H9_{LAI}$ cells were cultured with an HIV-1 protease inhibitor, indinavir, in addition to PNA_{PR2}. The combination of indinavir and PNA_{PR2} resulted in a reduction of intracellular p24 and p17 Gag proteins and the reappearance of $Pr55^{Gag}$ protein, but not $Pr160^{Gag\text{-}Pol}$ (Fig. 5B). Because the PNA_{PR2}-targeting site was near the 3′ end of the $p6^{Gag}$-encoding region, the proteolytic processing of $Pr55^{Gag}$, also a precursor of $p6^{Gag}$ protein, was examined by Western blot analysis with anti-$p6^{Gag}$ antibody. The peptide ELISA demonstrated that the anti-$p6^{Gag}$ antibody was most reactive to the peptide 480–500, which corresponds to the C-terminal domain of $p6^{Gag}$ protein, with the ODs linearly increasing from 0.303 ± 0.095 to 1.468 ± 0.052 (mean ± SD of triplicate values) in detecting 9.8 to 625 ng of the peptide per well, followed by the peptide 451–470, with ODs ranging from 0.212 ± 0.064 to 0.595 ± 0.067. The affin-

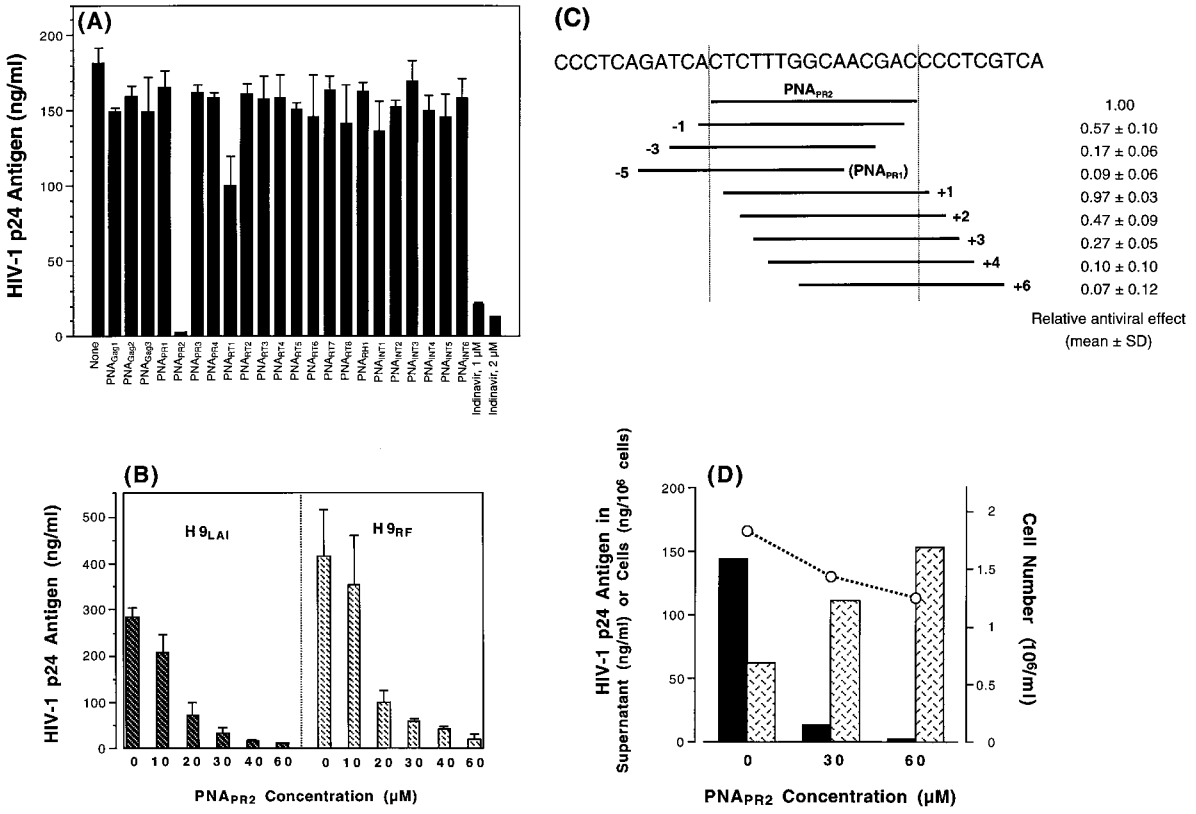


FIG. 3. Effects of PNA oligomers on HIV-1 production in chronically HIV-1-infected cells. (A) Levels of p24 antigen in the supernatants of $H9_{LAI}$ cells cultured in the absence or presence of various PNA oligomers used at a 100 μM concentration. All compounds were tested in triplicate, and the values shown are means with SDs. The p24 antigen level in a supernatant of $H9_{LAI}$ treated with an HIV-1 protease inhibitor, indinavir, is shown as a reference. (B) Levels of p24 antigen in the supernatants of $H9_{LAI}$ and $H9_{RF}$ cells cultured in the absence or presence of various concentrations of PNA_{PR2}. Each dose was tested in triplicate. The values shown are means with SDs. (C) The degrees of inhibition of p24 antigen production in $H9_{LAI}$ cells cultured with 100 μM PNA_{PR2} or other PNA oligomers targeting the sequences proximate to the PNA_{PR2}-annealing site were compared. Each PNA oligomer was tested in triplicate. Each bar represents the target sequence shifted upstream (−) or downstream (+) by one to six nucleotides away from the PNA_{PR2}-annealing site. The values shown are relative antiviral effects (means ± SDs). (D) Amounts of p24 antigen in the supernatant (solid bars) and cell lysate (crosshatched bars) of $H9_{LAI}$ cells cultured in the absence or presence of 30 and 60 μM PNA_{PR2}. The cell numbers are indicated by circles. The experimental results shown are representative of three separate experiments.

ities to the other three peptides were negligible. Western blot analysis with the anti-$p6^{Gag}$ antibody could detect $p6^{Gag}$ protein in the HIV-1 virions (data not shown and reference 106) as well as its precursor ($Pr55^{Gag}$) in the cell lysate of untreated $H9_{LAI}$ cells (Fig. 5B). This $p6^{Gag}$ antibody also demonstrated the $p6^{Gag}$-containing intermediate (p15) in the lysate of untreated $H9_{RF}$ cells (data not shown). Notably, the cell lysate of $H9_{LAI}$ treated with 60 μM PNA_{PR2} contained virtually no $Pr55^{Gag}$ protein which could be detected by the anti-$p6^{Gag}$ antibody, even with the addition of a protease inhibitor, indinavir, while the anti-p24 antibody readily recognized $Pr55^{Gag}$ in the same lysate (Fig. 5B), indicating that the $Pr55^{Gag}$ expressed in PNA_{PR2}-treated cells had insufficient binding determinants for the anti-$p6^{Gag}$ antibody. We then examined the effects of PNA_{PR2} on the expression of $Pr55^{Gag}$ protein in HLtat cells (32, 127) transfected with the Gag expression plasmid p55M1-10, in which $Pr55^{Gag}$ was not expected to be cleaved by the viral protease, as p55M1-10 contained solely the *gag* reading frame (126). While Western blot analysis with anti-$p24^{Gag}$ antibody demonstrated $Pr55^{Gag}$ protein in both untreated and PNA_{PR2}-treated cells, the band intensity of anti-$p6^{Gag}$ antibody-reactive $Pr55^{Gag}$ was significantly diminished in PNA_{PR2}-treated HLtat cells (Fig. 5C).

These data suggested that PNA_{PR2} treatment could induce the truncation of $Pr55^{Gag}$ toward the C terminus of $p6^{Gag}$, presumably by disrupting a translation of the *gag* reading frame at its site of binding to the target mRNA in HIV-1-infected cells. It is also possible that PNA_{PR2} effectively blocked the translation of the *gag-pol* reading frame, resulting in generation of truncated $Pr160^{Gag\text{-}Pol}$, namely, $Pr55^{Gag}$ with $p6^{Pol}$ which could not be detected by the anti-$p6^{Gag}$ antibody. Thus, the intracellular Gag precursor protein in PNA_{PR2}-treated

TABLE 2. HIV-1 virion production from PHA-PBMC infected with clinical MDR isolates and cultured in the absence or presence of 60 μM PNA_{PR2}

MDR isolate no.	Result of MAGI-CCR5 assay[a]	
	No PNA_{PR2}	60 μM PNA_{PR2}
1	467.7 ± 138.6	12.7 ± 4.2 (97.3)
2	486.7 ± 112.0	4.7 ± 5.5 (99.0)
3	210.0 ± 16.10	1.3 ± 0.6 (99.4)
4	589.7 ± 32.10	7.7 ± 4.2 (98.7)
5	214.0 ± 142.6	5.7 ± 1.5 (97.4)
6	219.7 ± 25.20	3.7 ± 1.5 (98.3)
7	208.3 ± 96.70	2.0 ± 1.0 (99.0)

[a] Results are shown as the mean numbers ± SDs of blue cells per well. Values in parentheses are the mean percent reductions.

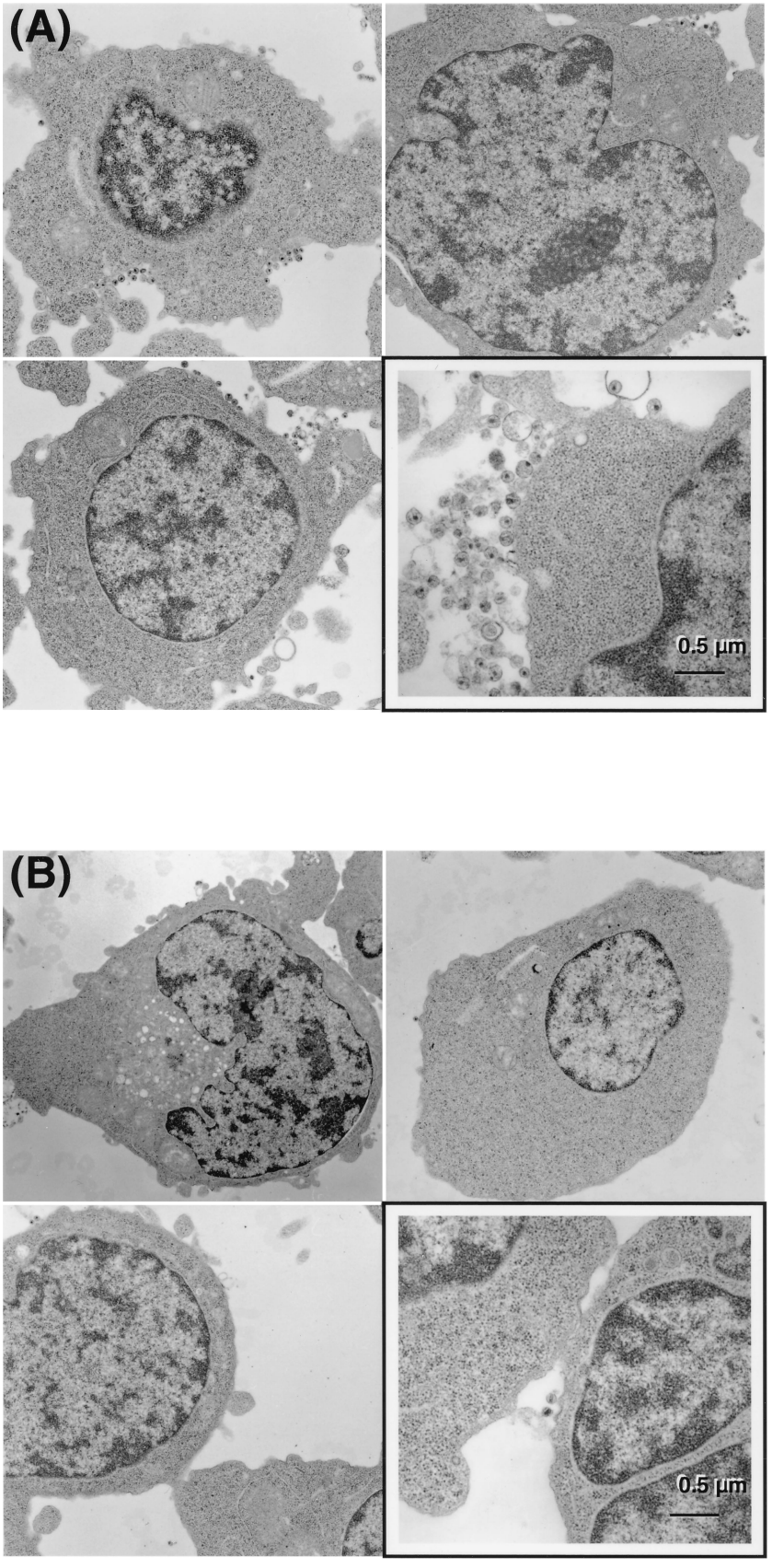


FIG. 4. Electron microscopy of $H9_{LAI}$ cells cultured in the absence (A) or presence (B) of 60 μM PNA_{PR2}. Shown are representatives of 100 cells surveyed in each sample (magnification, ×13,500). Virions are shown at a higher magnification (×54,000) in each inset on the right-hand side at the bottom.

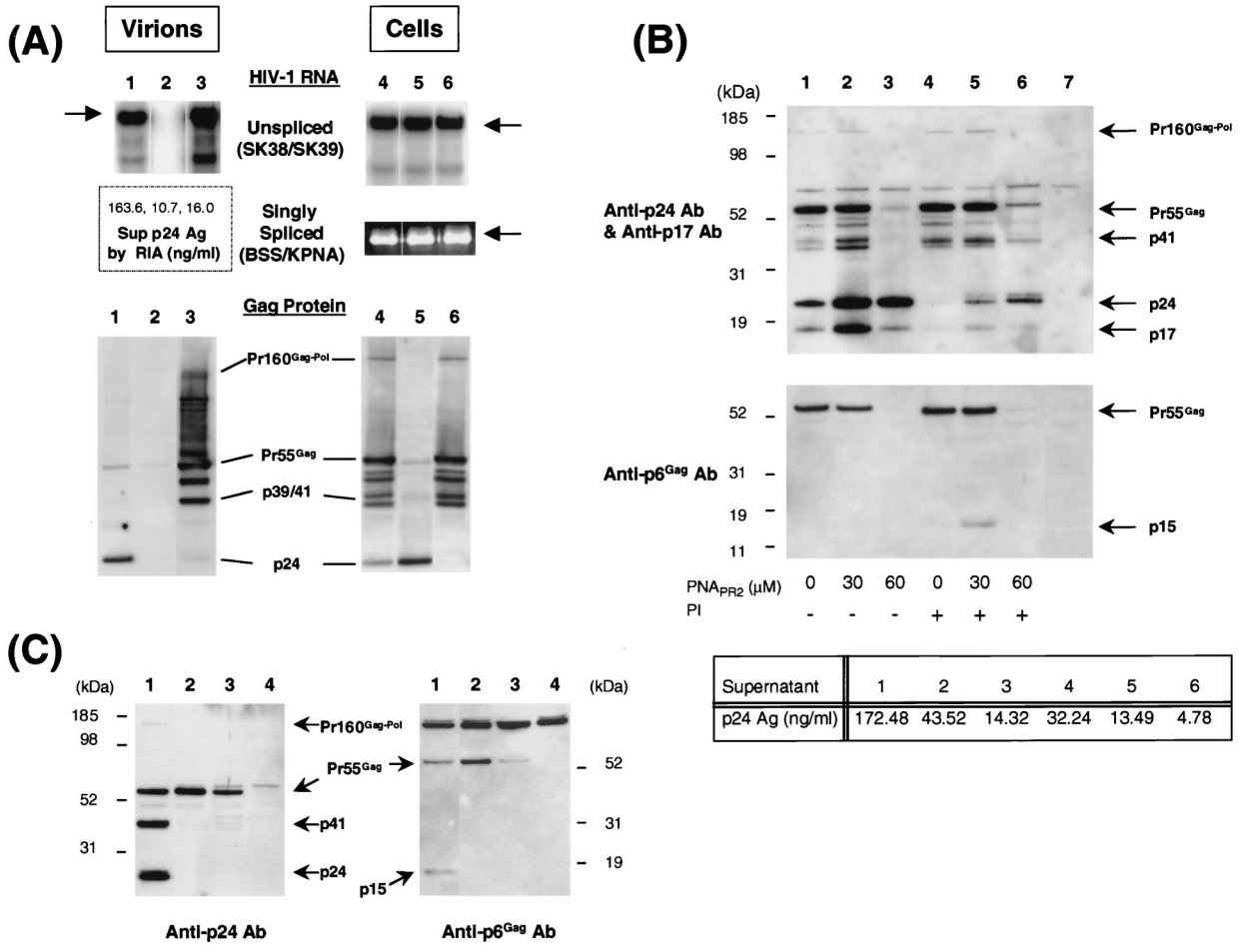
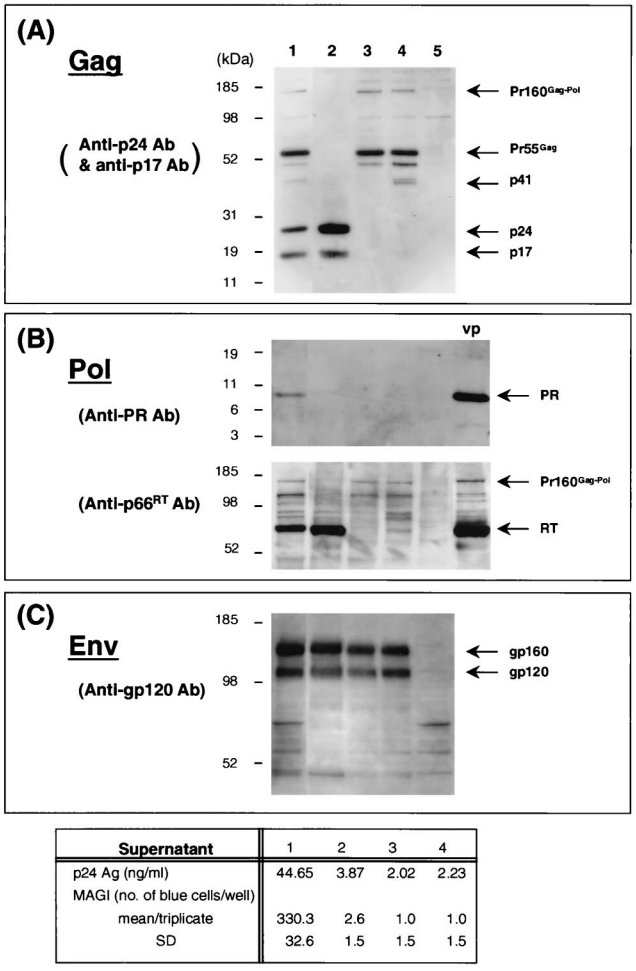


FIG. 5. Effects of PNA_{PR2} on Gag-Pol expression and virion production in HIV-1-infected cells (A and B) and on Gag protein expression in HLtat cells transfected with the Gag expression plasmid p55M1-10 (C). (A) The amounts of viral RNA in the culture supernatant (pelleted virion derived) (lanes 1 to 3) and in the cells (lanes 4 to 6) were evaluated by RT-PCR (top). Arrows indicate the pertinent PCR products for each primer pair. The virion-derived Gag protein in the culture supernatant (lanes 1 to 3) and intracellular Gag protein processing (lanes 4 to 6) were evaluated by Western blot analysis using anti-$p24^{Gag}$ antibody (bottom). Shown are the positions of $Pr160^{Gag\text{-}Pol}$, $Pr55^{Gag}$, p41, and p24 Gag proteins. Lanes of each gel represent $H9_{LAI}$ cells cultured alone (lanes 1 and 4) or incubated with 100 μM PNA_{PR2} (lanes 2 and 5) and 2 μM indinavir (lanes 3 and 6). (B) Western blot analyses with anti-$p24^{Gag}$ antibody and anti-$p17^{Gag}$ antibody (top) and with anti-$p6^{Gag}$ antibody (bottom) of cell lysates processed from $H9_{LAI}$ cells cultured alone (lane 1) or in the presence of 30 μM PNA_{PR2} (lane 2), 60 μM PNA_{PR2} (lane 3), 2 μM indinavir alone (lane 4), 30 μM PNA_{PR2} plus 2 μM indinavir (lane 5), or 60 μM PNA_{PR2} plus 2 μM indinavir (lane 6). Cell lysate of uninfected H9 cells is shown in lane 7. PI − or + represents the absence or presence of a protease inhibitor, indinavir, respectively. The level of p24 antigen in each culture supernatant is indicated at the bottom. (C) Western blot analyses with anti-$p24^{Gag}$ antibody (left) and anti-$p6^{Gag}$ antibody (right) of cell lysates processed from HLtat cells transfected with pHXB2-based plasmid pSUM9 (136) (lanes 1) or with p55M1-10 and cultured alone (lanes 2) or in the presence of 100 μM PNA_{RR2} (lanes 3). The cell lysate of mock-transfected HLtat cells is included as a reference (lanes 4). Sup, supernatant; Ag, antigen; Ab, antibody.

cells appeared to be predominantly $Pr55^{Gag}$ lacking the full-size $p6^{Gag}$ protein, which was excessively cleaved to the final products by a certain amount of viral protease.

PNA_{PR2} reduces synthesis of viral protease as well as triggering its premature activation. We then asked whether preexisting viral protease at the beginning of $H9_{LAI}$ cell culture subsequently influenced the proteolytic processing of the viral protein. To this end, nonpermissive COS-7 cells were transfected with an infectious HIV-1 molecular clone, pNL4-3, immediately followed by incubation with 100 μM PNA_{PR2} in tissue culture. Virion production was significantly suppressed in PNA_{PR2}-treated COS-7 cells as demonstrated by the reduced p24 antigen level and the decreased number of infectious virion particles in the culture supernatant determined by the MAGI assay (65). The intracellular Gag protein was predominantly p24 and p17 with significantly decreased amounts of $Pr55^{Gag}$ and virtually undetectable $Pr160^{Gag\text{-}Pol}$ as observed in chronically HIV-1-infected H9 cells (Fig. 6A). Consistent with the decreased quantity of full-size $Pr160^{Gag\text{-}Pol}$, the amount of intracellular viral protease was reduced in PNA_{PR2}-treated cells compared to untreated control (Fig. 6B). It should be noted that the antiprotease antibody that we employed in the current study detected a monomeric form of viral protease, not a homodimer, an active form of viral protease (112, 146). The latter is known to continually undergo autodegradation as it is being activated (123, 135). Thus, the amount of intracellular monomeric viral protease may not necessarily reflect the total amount of active viral protease within the cytoplasm but may simply correlate with the amount of precursor polyprotein. These results strongly suggested that the disrupted translation of *gag-pol* mRNA induced by PNA_{PR2} subsequently led to a premature activation of viral protease, albeit synthesized in probably a much smaller quantity than untreated control, resulting in an excessive intracel-



Supernatant	1	2	3	4
p24 Ag (ng/ml)	44.65	3.87	2.02	2.23
MAGI (no. of blue cells/well)				
mean/triplicate	330.3	2.6	1.0	1.0
SD	32.6	1.5	1.5	1.5

FIG. 6. Effects of PNA_{PR2} on *gag*, *pol*, and *env* gene expression and virion production in COS-7 cells transfected with an infectious HIV-1 molecular clone, pNL4-3. Shown are results of Western blot analyses with anti-$p24^{Gag}$ antibody and anti-$p17^{Gag}$ antibody (A), antiprotease antibody and anti-$p66^{RT}$ antibody (B), and anti-gp120 antibody (C) of pNL4-3-transfected COS-7 cells cultured with no drug (lanes 1), 100 μM PNA_{PR2} (lanes 2), 1 μM saquinavir (also a protease inhibitor) (lanes 3), or 3 μM indinavir (lanes 4). The cell lysate of mock-transfected COS-7 cells is shown in lanes 5. Viral lysate from pelleted virion particles (vp) is included in the Western blot analyses for the *pol* gene products as a reference. The levels of p24 antigen in supernatant and the infectious titers determined by MAGI assay are shown at the bottom. Ab, antibody; Ag, antigen.

lular cleavage of the Gag protein and an impairment of HIV-1 virion morphogenesis.

Interestingly, the amount of another *pol* gene product, RT, appeared to be disproportionately high compared to the low-level expression of its precursor, $Pr160^{Gag\text{-}Pol}$ polyprotein, in PNA_{PR2}-treated cells. Western blot analysis using a monoclonal antibody against $p66^{RT}$, which also recognizes the precursor (p160) and several intermediates, but not $p51^{RT}$ (NIH AIDS Research and Reference Reagent Program Catalog, 1998), clearly demonstrated $p66^{RT}$ in both untreated and PNA_{PR2}-treated cells (Fig. 6B). However, the cell lysate of PNA_{PR2}-treated COS-7 cells contained fewer precursor intermediates, smaller than 100 kDa in size (Fig. 6B). These data suggested that, in PNA_{PR2}-treated cells, the translation of *gag-pol* mRNA may have been reinitiated at the appropriate AUG codon located downstream of the PNA_{PR2}-targeting site by the mechanism described previously as ribosomal leaky scanning (71, 74, 75). A consensus sequence for the efficient initiation of translation by eukaryotic ribosomes has been reported as xx-PuxxAUGG with a purine (Pu) in the −3 position (three nucleotides upstream of the AUG codon) having a dominant effect (70, 72, 73). The potential AUG codons downstream of the PNA_{PR2}-annealing site were identified at various positions, including 2358 to 2360 (GAAAUGA), 2388 to 2390 (AAAAUGA), 2595 to 2597 (GGAAUGG), and 2670 to 2672 (GAGAUGG) of the HIV-1 HXB2 genome, preserving the amino acid sequence of the remaining Pol. If the translation is internally initiated at the sites listed above, resulting Pol proteins will be approximately 103.7, 102.5, 95.2, and 92.3 kDa, respectively. These Pol proteins may be cleaved by viral protease expressed in *trans* or by host proteases (34, 87). A similar pattern of $p66^{RT}$ expression was also observed in chronically infected H9 cells, and the addition of a viral protease inhibitor, indinavir, to PNA_{PR2} did not restore detection of the full-size precursor p160 (data not shown). Expression of HIV-1 Env was not affected by PNA_{PR2} in either COS-7 cells transfected with pNL4-3 (Fig. 6C) or chronically infected H9 cells (data not shown).

DISCUSSION

The introduction of protease inhibitors (55–57, 63, 77, 121) to conventional RT inhibitor-based antiretroviral therapy (23, 46, 95–97, 149, 150) has induced unprecedented degrees of viral load reduction and a recovery of $CD4^+$ T-lymphocyte counts even in patients with advanced HIV-1 disease (20, 22, 43, 44, 91, 125). Substantial declines in AIDS incidence and death observed in the United States and other industrialized countries for the past 2 years have also been attributed to this improved treatment regimen (1, 15), often referred to as highly active antiretroviral therapy. While RT inhibitors, which block de novo HIV-1 infection of uninfected cells, have no control over production of virions from HIV-1-infected cells, protease inhibitors actively exert their effect in infected cells, driving them to release noninfectious immature virions and thereby preventing the spread of HIV-1. Dramatic effects of highly active antiretroviral therapy on individual cases of HIV-1 infection as well as on the global AIDS epidemic, although they may possibly be short-lived, underscore the importance of targeting the existing HIV-1 reservoir (HIV-1-infected cells) to better control HIV-1 disease, rather than simply providing protective shields for uninfected cells.

In the current study, we attempted to identify the viral gene sequences that were not only critical for the virion production but also accessible to gene-intervening molecules in chronically HIV-1-infected, provirus-laden cells. We showed that PNA_{PR2}, which is complementary to a region of the viral *gag-pol* mRNA sequence near the 3′ end of the transframe domain, encoding protease residues 4 to 8 and the C-terminal Vpr binding motif of $p6^{Gag}$ protein, can effectively interrupt a translation of *gag-pol* mRNA. The disrupted translation of *gag-pol* mRNA at the PNA_{PR2}-annealing site gave rise to $Pr55^{Gag}$ as a predominant Gag precursor, which lacked a full-length $p6^{Gag}$ protein due to a C-terminal truncation of $p6^{Gag}$ and/or production of $Pr55^{Gag}$ containing $p6^{Pol}$ protein. The arrest of *gag-pol* mRNA translation by PNA_{PR2} was not complete, thereby permitting a synthesis of a small quantity of full-size $Pr160^{Gag\text{-}Pol}$ polyprotein, which was presumably processed to viral protease.

Despite the small amount of viral protease synthesized in PNA_{PR2}-treated cells, Gag precursors were almost entirely cleaved to the final products of p24 and p17 within the cytoplasm. These seemingly counterintuitive findings prompted us to examine the intracellular expression profiles of viral protease. The Western blot analysis of cell lysate with antiprotease

antibody demonstrated a significantly decreased amount of monomeric viral protease in PNA_{PR2}-treated COS-7 cells transfected with an HIV-1 molecular clone compared to untreated control, whereas an active form of homodimeric viral protease (112, 146) could not be visualized in either PNA_{PR2}-treated or untreated cells, probably because the homodimer had undergone autolysis as it was being activated (123, 135). It has previously been reported that the level of viral protease activity required to properly process the precursor proteins may be reduced by 4- to 50-fold, below which a limited amount of proteolytic processing can still be demonstrated (122). Therefore, it would not have been surprising to see some degree of Gag precursor processing in PNA_{PR2}-treated cells even if the actual quantity of active viral protease had been significantly decreased. However, the extent of intracellular Gag precursor cleavage observed in PNA_{PR2}-treated cells notably exceeded that in the untreated control, resulting in a significant accumulation of p24 and p17 within the cells. These data strongly suggested that the rate or timing of protease activation was markedly accelerated within the cytoplasm of PNA_{PR2}-treated cells.

The impact of PNA_{PR2}-mediated translation arrest of *gag-pol* mRNA seems to be severalfold. Not only did it impede the synthesis of full-size $Pr160^{Gag\text{-}Pol}$, leading to decreased production of viral protease, but it also appeared to trigger the premature activation of viral protease, resulting in excessive and untimely intracellular cleavage of Gag proteins and significantly reduced virion production. In HIV-1, approximately 5 to 10% of *gag-pol* mRNA translational events are mediated by −1 ribosomal frameshifting to produce $Pr160^{Gag\text{-}Pol}$ precursor polyprotein (52, 143), which is eventually incorporated into viral particles to provide viral protease, RT, and integrase. This delicately balanced synthesis of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ and their intermolecular association are believed to play a critical role in coordinating sequential events of virion assembly and release. The final stages of virion morphogenesis begin with the interaction of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ polyprotein (80, 81, 108, 133), which then attach to the plasma membrane of host cells (13, 41, 116). Viral protease embedded in $Pr160^{Gag\text{-}Pol}$ remains inactive until Gag and Gag-Pol precursors reach the plasma membrane, where precursor protein processing and virion assembly are initiated upon activation of the viral protease, followed by the budding and completion of virion maturation (58). Although viral protease-mediated precursor cleavage may take place within the cytoplasm (13, 41, 59), the membrane-associated precursor processing and assembly events must occur in a regulated sequence in order for the infectious virions to be released (58).

Excessive intracellular processing of Gag precursor proteins without concomitant extracellular production of mature virions as induced by PNA_{PR2} has also been observed in a number of other conditions, including (i) sole expression of $Pr160^{Gag\text{-}Pol}$ encoded in a single reading frame and thus lacking $p6^{Gag}$ (60, 109), (ii) $p6^{Gag}$-deletion or -truncation HIV-1 molecular clones (40, 49, 152), and (iii) HIV-1 molecular clones containing $p6^{Gag}$ with mutated Vpr-binding motifs (49, 152). Notably, inactivation of the viral protease induced by mutational changes could restore the production of virions, albeit immature virions, in the $p6^{Gag}$-deletion HIV-1 provirus-containing cells (49). These data suggest that the majority of viral protease may be prematurely released from the precursors and activated in the absence of fully functional $p6^{Gag}$. How $p6^{Gag}$ protein exerts such a regulatory effect on viral protease has yet to be determined. It is possible that $p6^{Gag}$ may actively participate in the process of intermolecular association of $Pr55^{Gag}$ and $Pr160^{Gag}$, in addition to the major homology domain of capsid protein (48, 134), such that the release of viral protease occurs in a coordinated fashion at the plasma membrane. Whether the binding of Vpr to $p6^{Gag}$ plays any role in regulating the processes of Gag and Gag-Pol precursor association and subsequent protease activation is unclear, since some studies have shown that a deletion of Vpr had no effect on viral infectivity or replication (3, 37).

In the current study, we could not determine whether the $Pr55^{Gag}$ synthesized in the PNA_{PR2}-treated cells was the product of the *gag* reading frame and thus contained C-terminally truncated $p6^{Gag}$ or was predominantly the product of the *gag-pol* reading frame and had $p6^{Pol}$ protein. It is conceivable that the antisense PNA oligomer targeted downstream of the stem-loop structure within the *gag-pol* overlap region may have enhanced the ribosomal frameshifting, as has been demonstrated with the use of antisense oligonucleotides (138), resulting in increased synthesis of *gag-pol* gene products, most of which were nonetheless truncated toward the N terminus of the viral protease in PNA_{PR2}-treated cells. Regardless, the lack of fully functional $p6^{Gag}$ protein was evidently as important a factor as the reduced synthesis of viral protease, both resulting from the PNA_{PR2}-mediated interference with the translation of *gag-pol* mRNA, in blocking virion production from HIV-1-infected cells. In addition to these posttranscriptional events, it is also possible that the reverse transcription may be inhibited by the PNA oligomers tightly bound to the viral RNA target (69), thus preventing the integration of viral DNA in uninfected cells. This may potentially augment the overall antiviral effect of PNA_{PR2} in the setting of de novo HIV-1 infection.

One of the most challenging aspects of the development of antisense or antigene strategies is, in general, to identify the genetic targets the expression of which is crucial to the pathological process of disease. Targeting various regulatory genes of the HIV-1 genome has not always been effective against diverse HIV-1 strains (2, 64, 66, 83–85, 92, 141). The structural *gag* gene has also been targeted by antisense PsODN at a *gag* translation initiation site or various regions of capsid-encoding domain (4, 5, 66, 86, 141, 148). Such an anti-*gag* antisense strategy, however, exhibited only a modest, at most, antiviral effect in tissue culture. These previous studies have clearly demonstrated that a complete disruption of *gag* gene expression is difficult to achieve and that the moderate suppression of Gag protein production may not be substantially detrimental to HIV-1. Furthermore, even if the anti-*gag* antisense could block the translation of full-length Gag protein, eukaryotic ribosomes would most likely scan the downstream *gag* or *gag-pol* mRNA and identify the optimal initiation codon by leaky scanning as discussed above. The resulting truncated Gag and Gag-Pol may still be sufficient for virion particle assembly and release (11), or compensatory downstream mutations may arise to negate deletional effects and restore replication competency, as has been demonstrated for similar deletion mutants (82). The viral gene sequence identified by the current study appeared to be sufficiently accessible to the PNA oligomers within the living cells and presented a narrow window of opportunity to invade HIV-1 structural genes, as shifting the target by a few nucleotides upstream or downstream resulted in a significant loss of antiviral activity. This sequence, therefore, can be considered a vulnerable spot of the HIV-1 genome. Such genetically vulnerable spots may also be identified in other viruses that adopt a frameshift for the translation of critical proteins (12, 30, 53, 54, 89, 98, 100) and possibly exploited to develop pathogen-specific antiviral genetic intervention.

Development of optimal genome-blocking molecules with an appropriate delivery system is another critical step toward a successful antigene-antisense intervention. Antisense PsODN targeting the same sequence as PNA_{PR2} ($PsODN_{PR2}$) did not show a substantial antiviral effect in chronically infected $H9_{LAI}$ or

$H9_{RF}$ cells in our laboratory (data not shown). The antiviral effects of antisense PsODN are exhibited through several mechanisms, including sequence-specific suppression of transcription or translation and a sequence-independent inhibition of viral adsorption (83, 85, 148). Moreover, the translational suppression induced by antisense PsODN is predominantly mediated by degradation of target RNA by RNase H (9, 14, 139), unlike PNA, which blocks ribosomal elongation in an RNase H-independent manner (45). Thus, PsODN may not be the best possible agent to efficiently block genes in cells that are already infected with HIV-1, and this may explain the discrepancy between the antiviral effects of PNA_{PR2} and $PsODN_{PR2}$ observed in our laboratory. Although PNA exhibits seemingly superior properties as a DNA-RNA-blocking tool compared with other oligonucleotide analogs, the current unmodified form of PNA cannot immediately be utilized as a therapeutic agent. Because of the poor cellular uptake of PNA, relatively higher concentrations of PNA molecules in tissue culture were required to sufficiently block the expression of the target sequence in our study. A conjugation of certain transporter molecules to the PNA may facilitate its cellular uptake (113, 114) and may prove useful if the gene-blocking ability of such a modified PNA construct is maintained. Innovative and optimal modifications to the existing PNA molecules or development of novel gene-intervening agents will clearly advance the efforts to develop potent antiviral therapeutics targeting viral genes.

ACKNOWLEDGMENTS

We thank Robert Wittes, John Erickson, and Yoshitatsu Sei for critical reviews of the manuscript; Louis E. Henderson for kindly providing anti-$p6^{Gag}$ and antiprotease antisera and George Pavlakis for kindly providing p55M1-10; Douglas Ferris, Eiichi Kodama, and Mutsuko Kumagai for helpful discussions; Elena Afonina for help with fluorescence microscopy; and Kathleen Noer for fluorescence-activated cell sorting analysis.

This work was supported, in part, by federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-56000.

REFERENCES

1. **Aalen, O. O., V. T. Farewell, D. De Angelis, N. E. Day, and O. N. Gill.** 1999. New therapy explains the fall in AIDS incidence with a substantial rise in number of persons on treatment expected. AIDS **13:**103–108.
2. **Agrawal, S., T. Ikeuchi, D. Sun, P. S. Sarin, A. Konopka, J. Maizel, and P. C. Zamecnik.** 1989. Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues. Proc. Natl. Acad. Sci. USA **86:** 7790–7794.
3. **Aldrovandi, G. M., and J. A. Zack.** 1996. Replication and pathogenicity of human immunodeficiency virus type 1 accessory gene mutants in SCID-hu mice. J. Virol. **70:**1505–1511.
4. **Anazodo, M. I., E. Duta, A. D. Friesen, and J. A. Wright.** 1996. Relative levels of inhibition of p24 gene expression by different 20-mer antisense oligonucleotide sequences targeting nucleotides + 1129 to + 1268 of the HIV-1 gag genome: an analysis of mechanism. Biochem. Biophys. Res. Commun. **229:**305–309.
5. **Anazodo, M. I., M. A. Wainberg, A. D. Friesen, and J. A. Wright.** 1995. 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. J. Virol. **69:** 1794–1801.
6. **Aoki-Sei, S., R. Yarchoan, S. Kageyama, D. Hoekzema, J. M. Pluda, K. M. Wyvill, S. Broder, and H. Mitsuya.** 1992. Plasma HIV-1 viremia in HIV-1 infected individuals assessed by polymerase chain reaction. AIDS Res. Hum. Retrovir. **8:**1263–1270.
7. **Artico, M., R. Di Santo, R. Costi, E. Novellino, G. Greco, S. Massa, E. Tramontano, M. E. Marongiu, A. De Montis, and P. La Colla.** 1998. Geometrically and conformationally restrained cinnamoyl compounds as inhibitors of HIV-1 integrase: synthesis, biological evaluation, and molecular modeling. J. Med. Chem. **41:**3948–3960.
8. **Barrie, K. A., E. E. Perez, S. L. Lamers, W. G. Farmerie, B. M. Dunn, J. W. Sleasman, and M. M. Goodenow.** 1996. Natural variation in HIV-1 protease, Gag p7 and p6, and protease cleavage sites within gag/pol polyproteins: amino acid substitutions in the absence of protease inhibitors in mothers and children infected by human immunodeficiency virus type 1. Virology **219:**407–416.
9. **Boiziau, C., N. T. Thuong, and J. J. Toulme.** 1992. Mechanisms of the inhibition of reverse transcription by antisense oligonucleotides. Proc. Natl. Acad. Sci. USA **89:**768–772.
10. **Bonham, M. A., S. Brown, A. L. Boyd, P. H. Brown, D. A. Bruckenstein, J. C. Hanvey, S. A. Thomson, A. Pipe, F. Hassman, J. E. Bisi, et al.** 1995. An assessment of the antisense properties of RNase H-competent and steric-blocking oligomers. Nucleic Acids Res. **23:**1197–1203.
11. **Borsetti, A., A. Ohagen, and H. G. Gottlinger.** 1998. The C-terminal half of the human immunodeficiency virus type 1 Gag precursor is sufficient for efficient particle assembly. J. Virol. **72:**9313–9317.
12. **Brierley, I., M. E. Boursnell, M. M. Binns, B. Bilimoria, V. C. Blok, T. D. Brown, and S. C. Inglis.** 1987. An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV. EMBO J. **6:** 3779–3785.
13. **Bryant, M., and L. Ratner.** 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc. Natl. Acad. Sci. USA **87:**523–527.
14. **Cazenave, C., C. A. Stein, N. Loreau, N. T. Thuong, L. M. Neckers, C. Subasinghe, C. Helene, J. S. Cohen, and J. J. Toulme.** 1989. Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides. Nucleic Acids Res. **17:**4255–4273.
15. **Centers for Disease Control and Prevention.** 1998. HIV/AIDS surveillance report, midyear ed. vol. 10. Centers for Disease Control and Prevention, Public Health Service, Department of Health and Human Services, Atlanta, Ga.
16. **Chackerian, B., E. M. Long, P. A. Luciw, and J. Overbaugh.** 1997. Human immunodeficiency virus type 1 coreceptors participate in postentry stages in the virus replication cycle and function in simian immunodeficiency virus infection. J. Virol. **71:**3932–3939.
17. **Checroune, F., X. J. Yao, H. G. Gottlinger, D. Bergeron, and E. A. Cohen.** 1995. Incorporation of Vpr into human immunodeficiency virus type 1: role of conserved regions within the P6 domain of Pr55gag. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **10:**1–7.
18. **Chun, T. W., D. Engel, M. M. Berrey, T. Shea, L. Corey, and A. S. Fauci.** 1998. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc. Natl. Acad. Sci. USA **95:**8869–8873.
19. **Chun, T. W., L. Stuyver, S. B. Mizell, L. A. Ehler, J. A. Mican, M. Baseler, A. L. Lloyd, M. A. Nowak, and A. S. Fauci.** 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc. Natl. Acad. Sci. USA **94:**13193–13197.
20. **Collier, A. C., R. W. Coombs, D. A. Schoenfeld, R. L. Bassett, J. Timpone, A. Baruch, M. Jones, K. Facey, C. Whitacre, V. J. McAuliffe, H. M. Friedman, T. C. Merigan, R. C. Reichman, C. Hooper, and L. Corey.** 1996. Treatment of human immunodeficiency virus infection with saquinavir, zidovudine, and zalcitabine. AIDS Clinical Trials Group. N. Engl. J. Med. **334:**1011–1017.
21. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, et al.** 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:**569–571.
22. **Danner, S. A., A. Carr, J. M. Leonard, L. M. Lehman, F. Gudiol, J. Gonzales, A. Raventos, R. Rubio, E. Bouza, V. Pintado, et al.** 1995. A short-term study of the safety, pharmacokinetics, and efficacy of ritonavir, an inhibitor of HIV-1 protease. European-Australian Collaborative Ritonavir Study Group. N. Engl. J. Med. **333:**1528–1533.
23. **De Clercq, E.** 1992. HIV inhibitors targeted at the reverse transcriptase. AIDS Res. Hum. Retrovir. **8:**119–134.
24. **Demidov, V. V., V. N. Potaman, M. D. Frank-Kamenetskii, M. Egholm, O. Buchard, S. H. Sonnichsen, and P. E. Nielsen.** 1994. Stability of peptide nucleic acids in human serum and cellular extracts. Biochem. Pharmacol. **48:**1310–1313.
25. **Donzella, G. A., D. Schols, S. W. Lin, J. A. Este, K. A. Nagashima, P. J. Maddon, G. P. Allaway, T. P. Sakmar, G. Henson, E. De Clercq, and J. P. Moore.** 1998. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat. Med. **4:**72–77.
26. **Doranz, B. J., K. Grovit-Ferbas, M. P. Sharron, S. H. Mao, M. B. Goetz, E. S. Daar, R. W. Doms, and W. A. O'Brien.** 1997. A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. J. Exp. Med. **186:**1395–1400.
27. **Egholm, M., O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden, and P. E. Nielsen.** 1993. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature **365:**566–568.
28. **Egholm, M., O. Buchardt, P. E. Nielsen, and R. H. Berg.** 1992. Peptide nucleic acids (PNA). Oligonucleotide analogues with an achiral peptide backbone. J. Am. Chem. Soc. **114:**1895–1897.
29. **Eich, E., H. Pertz, M. Kaloga, J. Schulz, M. R. Fesen, A. Mazumder, and Y.**

Pommier. 1996. (−)-Arctigenin as a lead structure for inhibitors of human immunodeficiency virus type-1 integrase. J. Med. Chem. **39:**86–95.
30. **Falk, H., N. Mador, R. Udi, A. Panet, and A. Honigman.** 1993. Two *cis*-acting signals control ribosomal frameshift between human T-cell leukemia virus type II *gag* and *pro* genes. J. Virol. **67:**6273–6277.
31. **Farnet, C. M., B. Wang, M. Hansen, J. R. Lipford, L. Zalkow, W. E. Robinson, Jr., J. Siegel, and F. Bushman.** 1998. Human immunodeficiency virus type 1 cDNA integration: new aromatic hydroxylated inhibitors and studies of the inhibition mechanism. Antimicrob. Agents Chemother. **42:** 2245–2253.
32. **Felber, B. K., C. M. Drysdale, and G. N. Pavlakis.** 1990. Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. J. Virol. **64:**3734–3741.
33. **Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **278:**1295–1300.
34. **Flexner, C., S. S. Broyles, P. Earl, S. Chakrabarti, and B. Moss.** 1988. Characterization of human immunodeficiency virus gag/pol gene products expressed by recombinant vaccinia viruses. Virology **166:**339–349.
35. **Freed, E. O., J. M. Orenstein, A. J. Buckler-White, and M. A. Martin.** 1994. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. J. Virol. **68:**5311–5320.
36. **Furtado, M. R., D. S. Callaway, J. P. Phair, K. J. Kunstman, J. L. Stanton, C. A. Macken, A. S. Perelson, and S. M. Wolinsky.** 1999. Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy. N. Engl. J. Med. **340:**1614–1622.
37. **Gibbs, J. S., D. A. Regier, and R. C. Desrosiers.** 1994. Construction and in vitro properties of HIV-1 mutants with deletions in "nonessential" genes. AIDS Res. Hum. Retrovir. **10:**343–350.
38. **Gonda, M. A., F. Wong-Staal, R. C. Gallo, J. E. Clements, O. Narayan, and R. V. Gilden.** 1985. Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus. Science **227:**173–177.
39. **Gong, W., O. M. Howard, J. A. Turpin, M. C. Grimm, H. Ueda, P. W. Gray, C. J. Raport, J. J. Oppenheim, and J. M. Wang.** 1998. Monocyte chemotactic protein-2 activates CCR5 and blocks CD4/CCR5-mediated HIV-1 entry/replication. J. Biol. Chem. **273:**4289–4292.
40. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA **88:**3195–3199.
41. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:** 5781–5785.
42. **Gray, G. D., S. Basu, and E. Wickstrom.** 1997. Transformed and immortalized cellular uptake of oligodeoxynucleoside phosphorothioates, 3′-alkylamino oligodeoxynucleotides, 2′-O-methyl oligoribonucleotides, oligodeoxynucleoside methylphosphonates, and peptide nucleic acids. Biochem. Pharmacol. **53:**1465–1476.
43. **Gulick, R. M., J. W. Mellors, D. Havlir, J. J. Eron, C. Gonzalez, D. McMahon, D. D. Richman, F. T. Valentine, L. Jonas, A. Meibohm, E. A. Emini, and J. A. Chodakewitz.** 1997. Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N. Engl. J. Med. **337:**734–739.
44. **Hammer, S. M., K. E. Squires, M. D. Hughes, J. M. Grimes, L. M. Demeter, J. S. Currier, J. J. Eron, Jr., J. E. Feinberg, H. H. Balfour, Jr., L. R. Deyton, J. A. Chodakewitz, and M. A. Fischl.** 1997. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. N. Engl. J. Med. **337:**725–733.
45. **Hanvey, J. C., N. J. Peffer, J. E. Bisi, S. A. Thomson, R. Cadilla, J. A. Josey, D. J. Ricca, C. F. Hassman, M. A. Bonham, K. G. Au, et al.** 1992. Antisense and antigene properties of peptide nucleic acids. Science **258:**1481–1485.
46. **Hargrave, K. D., J. R. Proudfoot, K. G. Grozinger, E. Cullen, S. R. Kapadia, U. R. Patel, V. U. Fuchs, S. C. Mauldin, J. Vitous, M. L. Behnke, et al.** 1991. Novel non-nucleoside inhibitors of HIV-1 reverse transcriptase. 1. Tricyclic pyridobenzo- and dipyridodiazepinones. J. Med. Chem. **34:**2231–2241.
47. **Ho, D. D., T. Toyoshima, H. Mo, D. J. Kempf, D. Norbeck, C. M. Chen, N. E. Wideburg, S. K. Burt, J. W. Erickson, and M. K. Singh.** 1994. Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C_2-symmetric protease inhibitor. J. Virol. **68:**2016–2020.
48. **Huang, M., and M. A. Martin.** 1997. Incorporation of $Pr160^{gag\text{-}pol}$ into virus particles requires the presence of both the major homology region and adjacent C-terminal capsid sequences within the Gag-Pol polyprotein. J. Virol. **71:**4472–4478.
49. **Huang, M., J. M. Orenstein, M. A. Martin, and E. O. Freed.** 1995. p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J. Virol. **69:** 6810–6818.
50. **Husson, R. N., T. Shirasaka, K. M. Butler, P. A. Pizzo, and H. Mitsuya.** 1993. High-level resistance to zidovudine but not to zalcitabine or didanosine in human immunodeficiency virus from children receiving antiretroviral therapy. J. Pediatr. **123:**9–16.
51. **Igloi, G. L.** 1998. Variability in the stability of DNA-peptide nucleic acid (PNA) single-base mismatched duplexes: real-time hybridization during affinity electrophoresis in PNA-containing gels. Proc. Natl. Acad. Sci. USA **95:**8562–8567.
52. **Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus.** 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. Nature **331:**280–283.
53. **Jacks, T., K. Townsley, H. E. Varmus, and J. Majors.** 1987. Two efficient ribosomal frameshifting events are required for synthesis of mouse mammary tumor virus gag-related polyproteins. Proc. Natl. Acad. Sci. USA **84:** 4298–4302.
54. **Jacks, T., and H. E. Varmus.** 1985. Expression of the Rous sarcoma virus pol gene by ribosomal frameshifting. Science **230:**1237–1242.
55. **Johnson, V. A., D. P. Merrill, T. C. Chou, and M. S. Hirsch.** 1992. Human immunodeficiency virus type 1 (HIV-1) inhibitory interactions between protease inhibitor Ro 31-8959 and zidovudine, 2′,3′-dideoxycytidine, or recombinant interferon-alpha A against zidovudine-sensitive or -resistant HIV-1 in vitro. J. Infect. Dis. **166:**1143–1146.
56. **Kageyama, S., T. Mimoto, Y. Murakawa, M. Nomizu, H. Ford, Jr., T. Shirasaka, S. Gulnik, J. Erickson, K. Takada, H. Hayashi, S. Broder, Y. Kiso, and H. Mitsuya.** 1993. In vitro anti-human immunodeficiency virus (HIV) activities of transition state mimetic HIV protease inhibitors containing allophenylnorstatine. Antimicrob. Agents Chemother. **37:**810–817.
57. **Kageyama, S., J. N. Weinstein, T. Shirasaka, D. J. Kempf, D. W. Norbeck, J. J. Plattner, J. Erickson, and H. Mitsuya.** 1992. In vitro inhibition of human immunodeficiency virus (HIV) type 1 replication by C_2 symmetry-based HIV protease inhibitors as single agents or in combinations. Antimicrob. Agents Chemother. **36:**926–933.
58. **Kaplan, A. H., M. Manchester, and R. Swanstrom.** 1994. The activity of the protease of human immunodeficiency virus type 1 is initiated at the membrane of infected cells before the release of viral proteins and is required for release to occur with maximum efficiency. J. Virol. **68:**6782–6786.
59. **Kaplan, A. H., and R. Swanstrom.** 1991. Human immunodeficiency virus type 1 Gag proteins are processed in two cellular compartments. Proc. Natl. Acad. Sci. USA **88:**4528–4532.
60. **Karacostas, V., E. J. Wolffe, K. Nagashima, M. A. Gonda, and B. Moss.** 1993. Overexpression of the HIV-1 gag-pol polyprotein results in intracellular activation of HIV-1 protease and inhibition of assembly and budding of virus-like particles. Virology **193:**661–671.
61. **Kavlick, M. F., T. Shirasaka, E. Kojima, J. M. Pluda, F. Hui, Jr., R. Yarchoan, and H. Mitsuya.** 1995. Genotypic and phenotypic characterization of HIV-1 isolated from patients receiving (−)-2′,3′-dideoxy-3′-thiacytidine. Antivir. Res. **28:**133–146.
62. **Kavlick, M. F., K. Wyvill, R. Yarchoan, and H. Mitsuya.** 1998. Emergence of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 variants, viral sequence variation, and disease progression in patients receiving antiretroviral chemotherapy. J. Infect. Dis. **177:**1506–1513.
63. **Kempf, D. J., K. C. Marsh, J. F. Denissen, E. McDonald, S. Vasavanonda, C. A. Flentge, B. E. Green, L. Fino, C. H. Park, X. P. Kong, et al.** 1995. ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. Proc. Natl. Acad. Sci. USA **92:** 2484–2488.
64. **Kim, S. G., T. Hatta, S. Tsukahara, H. Nakashima, N. Yamamoto, Y. Shoji, K. Takai, and H. Takaku.** 1995. Antiviral effect of phosphorothioate oligodeoxyribonucleotides complementary to human immunodeficiency virus. Bioorg. Med. Chem. **3:**49–54.
65. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J. Virol. **66:**2232–2239.
66. **Kinchington, D., S. Galpin, J. W. Jaroszewski, K. Ghosh, C. Subasinghe, and J. S. Cohen.** 1992. A comparison of gag, pol and rev antisense oligodeoxynucleotides as inhibitors of HIV-1. Antivir. Res. **17:**53–62.
67. **Kojima, E., T. Shirasaka, B. D. Anderson, S. Chokekijchai, S. M. Steinberg, S. Broder, R. Yarchoan, and H. Mitsuya.** 1995. Human immunodeficiency virus type 1 (HIV-1) viremia changes and development of drug-related mutations in patients with symptomatic HIV-1 infection receiving alternating or simultaneous zidovudine and didanosine therapy. J. Infect. Dis. **171:** 1152–1158.
68. **Kondo, E., and H. G. Gottlinger.** 1996. A conserved LXXLF sequence is the major determinant in $p6^{gag}$ required for the incorporation of human immunodeficiency virus type 1 Vpr. J. Virol. **70:**159–164.
69. **Koppelhus, U., V. Zachar, P. E. Nielsen, X. Liu, J. Eugen-Olsen, and P. Ebbesen.** 1997. Efficient in vitro inhibition of HIV-1 gag reverse transcription by peptide nucleic acid (PNA) at minimal ratios of PNA/RNA. Nucleic Acids Res. **25:**2167–2173.
70. **Kozak, M.** 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. **12:**857–872.
71. **Kozak, M.** 1980. Evaluation of the "scanning model" for initiation of protein synthesis in eucaryotes. Cell **22:**7–8.

72. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44:** 283–292.
73. **Kozak, M.** 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. **9:**5233–5262.
74. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.
75. **Kozak, M.** 1984. Selection of initiation sites by eucaryotic ribosomes: effect of inserting AUG triplets upstream from the coding sequence for preproinsulin. Nucleic Acids Res. **12:**3873–3893.
76. **Kozal, M. J., N. Shah, N. Shen, R. Yang, R. Fucini, T. C. Merigan, D. D. Richman, D. Morris, E. Hubbell, M. Chee, and T. R. Gingeras.** 1996. Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. Nat. Med. **2:**753–759.
77. **Lam, P. Y., P. K. Jadhav, C. J. Eyermann, C. N. Hodge, Y. Ru, L. T. Bacheler, J. L. Meek, M. J. Otto, M. M. Rayner, Y. N. Wong, et al.** 1994. Rational design of potent, bioavailable, nonpeptide cyclic ureas as HIV protease inhibitors. Science **263:**380–384.
78. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731–1734.
79. **Lech, W. J., G. Wang, Y. L. Yang, Y. Chee, K. Dorman, D. McCrae, L. C. Lazzeroni, J. W. Erickson, J. S. Sinsheimer, and A. H. Kaplan.** 1996. In vivo sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects. J. Virol. **70:**2038–2043.
80. **Lee, Y. M., C. J. Tian, and X. F. Yu.** 1998. A bipartite membrane-binding signal in the human immunodeficiency virus type 1 matrix protein is required for the proteolytic processing of Gag precursors in a cell type-dependent manner. J. Virol. **72:**9061–9068.
81. **Lee, Y. M., and X. F. Yu.** 1998. Identification and characterization of virus assembly intermediate complexes in HIV-1-infected CD4+ T cells. Virology **243:**78–93.
82. **Liang, C., L. Rong, M. Laughrea, L. Kleiman, and M. A. Wainberg.** 1998. Compensatory point mutations in the human immunodeficiency virus type 1 Gag region that are distal from deletion mutations in the dimerization initiation site can restore viral replication. J. Virol. **72:**6629–6636.
83. **Lisziewicz, J., D. Sun, M. Klotman, S. Agrawal, P. Zamecnik, and R. Gallo.** 1992. Specific inhibition of human immunodeficiency virus type 1 replication by antisense oligonucleotides: an in vitro model for treatment. Proc. Natl. Acad. Sci. USA **89:**11209–11213.
84. **Lisziewicz, J., D. Sun, A. Lisziewicz, and R. C. Gallo.** 1995. Antitat gene therapy: a candidate for late-stage AIDS patients. Gene Ther. **2:**218–222.
85. **Lisziewicz, J., D. Sun, V. Metelev, P. Zamecnik, R. C. Gallo, and S. Agrawal.** 1993. Long-term treatment of human immunodeficiency virus-infected cells with antisense oligonucleotide phosphorothioates. Proc. Natl. Acad. Sci. USA **90:**3860–3864.
86. **Lisziewicz, J., D. Sun, F. F. Weichold, A. R. Thierry, P. Lusso, J. Tang, R. C. Gallo, and S. Agrawal.** 1994. Antisense oligodeoxynucleotide phosphorothioate complementary to Gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells. Proc. Natl. Acad. Sci. USA **91:**7942–7946.
87. **Lowe, D. M., A. Aitken, C. Bradley, G. K. Darby, B. A. Larder, K. L. Powell, D. J. Purifoy, M. Tisdale, and D. K. Stammers.** 1988. HIV-1 reverse transcriptase: crystallization and analysis of domain structure by limited proteolysis. Biochemistry **27:**8884–8889.
88. **Lu, Y.-L., R. P. Bennett, J. W. Wills, R. Gorelick, and L. Ratner.** 1995. A leucine triplet repeat sequence $(LXX)_4$ in $p6^{gag}$ is important for Vpr incorporation into human immunodeficiency virus type 1 particles. J. Virol. **69:** 6873–6879.
89. **Mador, N., A. Panet, and A. Honigman.** 1989. Translation of *gag*, *pro*, and *pol* gene products of human T-cell leukemia virus type 2. J. Virol. **63:** 2400–2404.
90. **Markowitz, M., H. Mo, D. J. Kempf, D. W. Norbeck, T. N. Bhat, J. W. Erickson, and D. D. Ho.** 1995. Selection and analysis of human immunodeficiency virus type 1 variants with increased resistance to ABT-538, a novel protease inhibitor. J. Virol. **69:**701–706.
91. **Markowitz, M., M. Saag, W. G. Powderly, A. M. Hurley, A. Hsu, J. M. Valdes, D. Henry, F. Sattler, A. La Marca, J. M. Leonard, et al.** 1995. A preliminary study of ritonavir, an inhibitor of HIV-1 protease, to treat HIV-1 infection. N. Engl. J. Med. **333:**1534–1539.
92. **Matsukura, M., G. Zon, K. Shinozuka, M. Robert-Guroff, T. Shimada, C. A. Stein, H. Mitsuya, F. Wong-Staal, J. S. Cohen, and S. Broder.** 1989. Regulation of viral expression of human immunodeficiency virus in vitro by an antisense phosphorothioate oligodeoxynucleotide against rev (art/trs) in chronically infected cells. Proc. Natl. Acad. Sci. USA **86:**4244–4248.
93. **Mazumder, A., N. Neamati, J. O. Ojwang, S. Sunder, R. F. Rando, and Y. Pommier.** 1996. Inhibition of the human immunodeficiency virus type 1 integrase by guanosine quartet structures. Biochemistry **35:**13762–13771.
94. **McDonnell, N. B., R. N. De Guzman, W. G. Rice, J. A. Turpin, and M. F. Summers.** 1997. Zinc ejection as a new rationale for the use of cystamine and related disulfide-containing antiviral agents in the treatment of AIDS. J. Med. Chem. **40:**1969–1976.
95. **Mitsuya, H., and S. Broder.** 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. USA **83:**1911–1915.
96. **Mitsuya, H., R. F. Jarrett, M. Matsukura, F. Di Marzo Veronese, A. L. DeVico, M. G. Sarngadharan, D. G. Johns, M. S. Reitz, and S. Broder.** 1987. Long-term inhibition of human T-lymphotropic virus type III/lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2′,3′-dideoxynucleosides in vitro. Proc. Natl. Acad. Sci. USA **84:**2033–2037.
97. **Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder.** 1985. 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA **82:**7096–7100.
98. **Moore, R., M. Dixon, R. Smith, G. Peters, and C. Dickson.** 1987. Complete nucleotide sequence of a milk-transmitted mouse mammary tumor virus: two frameshift suppression events are required for translation of *gag* and *pol*. J. Virol. **61:**480–490.
99. **Murakami, T., T. Nakajima, Y. Koyanagi, K. Tachibana, N. Fujii, H. Tamamura, N. Yoshida, M. Waki, A. Matsumoto, O. Yoshie, T. Kishimoto, N. Yamamoto, and T. Nagasawa.** 1997. A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. J. Exp. Med. **186:**1389–1393.
100. **Nam, S. H., T. D. Copeland, M. Hatanaka, and S. Oroszlan.** 1993. Characterization of ribosomal frameshifting for expression of *pol* gene products of human T-cell leukemia virus type I. J. Virol. **67:**196–203.
101. **Neamati, N., H. Hong, J. M. Owen, S. Sunder, H. E. Winslow, J. L. Christensen, H. Zhao, T. R. Burke, Jr., G. W. Milne, and Y. Pommier.** 1998. Salicylhydrazine-containing inhibitors of HIV-1 integrase: implication for a selective chelation in the integrase active site. J. Med. Chem. **41:**3202–3209.
102. **Neumann, M., J. Harrison, M. Saltarelli, E. Hadziyannis, V. Erfle, B. K. Felber, and G. N. Pavlakis.** 1994. Splicing variability in HIV type 1 revealed by quantitative RNA polymerase chain reaction. AIDS Res. Hum. Retrovir. **10:**1531–1542.
103. **Nielsen, P. E., M. Egholm, R. H. Berg, and O. Buchardt.** 1991. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science **254:**1497–1500.
104. **Noble, S. A., M. A. Bonham, J. E. Bisi, D. A. Bruckenstein, P. H. Brown, S. C. Brown, R. Cadilla, M. D. Gaul, J. C. Hanvey, C. F. Hassman, J. A. Josey, M. J. Luzzio, P. M. Myers, A. J. Pipe, D. J. Ricca, C. W. Su, C. L. Stevenson, S. A. Thomson, R. W. Wiethe, and L. E. Babiss.** 1995. Impact of biophysical parameters on the biological assessment of peptide nucleic acids, antisense inhibitors of gene expression. Drug Dev. Res. **34:**184–195.
105. **Oberlin, E., A. Amara, F. Bachelerie, C. Bessia, J. L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J. M. Heard, I. Clark-Lewis, D. F. Legler, M. Loetscher, M. Baggiolini, and B. Moser.** 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature **382:**833–835. (Erratum, **384:**288.)
106. **Ott, D. E., L. V. Coren, T. D. Copeland, B. P. Kane, D. G. Johnson, R. C. Sowder II, Y. Yoshinaka, S. Oroszlan, L. O. Arthur, and L. E. Henderson.** 1998. Ubiquitin is covalently attached to the $p6^{Gag}$ proteins of human immunodeficiency virus type 1 and simian immunodeficiency virus and to the $p12^{Gag}$ protein of Moloney murine leukemia virus. J. Virol. **72:**2962–2968.
107. **Otto, M. J., S. Garber, D. L. Winslow, C. D. Reid, P. Aldrich, P. K. Jadhav, C. E. Patterson, C. N. Hodge, and Y. S. Cheng.** 1993. In vitro isolation and identification of human immunodeficiency virus (HIV) variants with reduced sensitivity to C-2 symmetrical inhibitors of HIV type 1 protease. Proc. Natl. Acad. Sci. USA **90:**7543–7547.
108. **Park, J., and C. D. Morrow.** 1992. The nonmyristylated $Pr160^{gag\text{-}pol}$ polyprotein of human immunodeficiency virus type 1 interacts with $Pr55^{gag}$ and is incorporated into viruslike particles. J. Virol. **66:**6304–6313.
109. **Park, J., and C. D. Morrow.** 1991. Overexpression of the *gag-pol* precursor from human immunodeficiency virus type 1 proviral genomes results in efficient proteolytic processing in the absence of virion production. J. Virol. **65:**5111–5117.
110. **Partaledis, J. A., K. Yamaguchi, M. Tisdale, E. E. Blair, C. Falcione, B. Maschera, R. E. Myers, S. Pazhanisamy, O. Futer, A. B. Cullinan, C. M. Stuver, R. A. Byrn, and D. J. Livingston.** 1995. In vitro selection and characterization of human immunodeficiency virus type 1 (HIV-1) isolates with reduced sensitivity to hydroxyethylamino sulfonamide inhibitors of HIV-1 aspartyl protease. J. Virol. **69:**5228–5235.
111. **Patick, A. K., R. Rose, J. Greytok, C. M. Bechtold, M. A. Hermsmeier, P. T. Chen, J. C. Barrish, R. Zahler, R. J. Colonno, and P. F. Lin.** 1995. Characterization of human immunodeficiency virus type 1 variant with reduced sensitivity to an aminodiol protease inhibitor. J. Virol. **69:**2148–2152.
112. **Pearl, L. H., and W. R. Taylor.** 1987. A structural model for the retroviral proteases. Nature **329:**351–354.
113. **Pooga, M., M. Hallbrink, M. Zorko, and U. Langel.** 1998. Cell penetration by transportan. FASEB J. **12:**67–77.
114. **Pooga, M., U. Soomets, M. Hallbrink, A. Valkna, K. Saar, K. Rezaei, U.**

Kahl, J. X. Hao, X. J. Xu, Z. Wiesenfeld-Hallin, T. Hokfelt, T. Bartfai, and U. Langel. 1998. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat. Biotechnol. **16:**857–861.

115. **Puras Lutzke, R. A., N. A. Eppens, P. A. Weber, R. A. Houghten, and R. H. Plasterk.** 1995. Identification of a hexapeptide inhibitor of the human immunodeficiency virus integrase protein by using a combinatorial chemical library. Proc. Natl. Acad. Sci. USA **92:**11456–11460.
116. **Rhee, S. S., and E. Hunter.** 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. J. Virol. **61:** 1045–1053.
117. **Rice, W. G., D. C. Baker, C. A. Schaeffer, L. Graham, M. Bu, S. Terpening, D. Clanton, R. Schultz, J. P. Bader, R. W. Buckheit, Jr., L. Field, P. K. Singh, and J. A. Turpin.** 1997. Inhibition of multiple phases of human immunodeficiency virus type 1 replication by a dithiane compound that attacks the conserved zinc fingers of retroviral nucleocapsid proteins. Antimicrob. Agents Chemother. **41:**419–426.
118. **Rice, W. G., C. A. Schaeffer, B. Harten, F. Villinger, T. L. South, M. F. Summers, L. E. Henderson, J. W. Bess, Jr., L. O. Arthur, J. S. McDougal, et al.** 1993. Inhibition of HIV-1 infectivity by zinc-ejecting aromatic C-nitroso compounds. Nature **361:**473–475.
119. **Rice, W. G., J. G. Supko, L. Malspeis, R. W. Buckheit, Jr., D. Clanton, M. Bu, L. Graham, C. A. Schaeffer, J. A. Turpin, J. Domagala, et al.** 1995. Inhibitors of HIV nucleocapsid protein zinc fingers as candidates for the treatment of AIDS. Science **270:**1194–1197.
120. **Rice, W. G., J. A. Turpin, C. A. Schaeffer, L. Graham, D. Clanton, R. W. Buckheit, Jr., D. Zaharevitz, M. F. Summers, A. Wallqvist, and D. G. Covell.** 1996. Evaluation of selected chemotypes in coupled cellular and molecular target-based screens identifies novel HIV-1 zinc finger inhibitors. J. Med. Chem. **39:**3606–3616.
121. **Roberts, N. A., J. A. Martin, D. Kinchington, A. V. Broadhurst, J. C. Craig, I. B. Duncan, S. A. Galpin, B. K. Handa, J. Kay, A. Krohn, et al.** 1990. Rational design of peptide-based HIV proteinase inhibitors. Science **248:** 358–361.
122. **Rose, J. R., L. M. Babe, and C. S. Craik.** 1995. Defining the level of human immunodeficiency virus type 1 (HIV-1) protease activity required for HIV-1 particle maturation and infectivity. J. Virol. **69:**2751–2758.
123. **Rose, J. R., R. Salto, and C. S. Craik.** 1993. Regulation of autoproteolysis of the HIV-1 and HIV-2 proteases with engineered amino acid substitutions. J. Biol. Chem. **268:**11939–11945.
124. **Saltarelli, M. J., E. Hadziyannis, C. E. Hart, J. V. Harrison, B. K. Felber, T. J. Spira, and G. N. Pavlakis.** 1996. Analysis of human immunodeficiency virus type 1 mRNA splicing patterns during disease progression in peripheral blood mononuclear cells from infected individuals. AIDS Res. Hum. Retrovir. **12:**1443–1456.
125. **Schapiro, J. M., M. A. Winters, F. Stewart, B. Efron, J. Norris, M. J. Kozal, and T. C. Merigan.** 1996. The effect of high-dose saquinavir on viral load and CD4+ T-cell counts in HIV-infected patients. Ann. Intern. Med. **124:** 1039–1050.
126. **Schneider, R., M. Campbell, G. Nasioulas, B. K. Felber, and G. N. Pavlakis.** 1997. Inactivation of the human immunodeficiency virus type 1 inhibitory elements allows Rev-independent expression of Gag and Gag/protease and particle formation. J. Virol. **71:**4892–4903.
127. **Schwartz, S., B. K. Felber, D. M. Benko, E. M. Fenyo, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64:**2519–2529.
128. **Sei, S., H. Akiyoshi, J. Bernard, D. J. Venzon, C. H. Fox, D. J. Schwartzentruber, B. D. Anderson, J. B. Kopp, B. U. Mueller, and P. A. Pizzo.** 1996. Dynamics of virus versus host interaction in children with human immunodeficiency virus type 1 infection. J. Infect. Dis. **173:**1485–1490.
129. **Sei, S., D. E. Kleiner, J. B. Kopp, R. Chandra, P. E. Klotman, R. Yarchoan, P. A. Pizzo, and H. Mitsuya.** 1994. Quantitative analysis of viral burden in tissues from adults and children with symptomatic human immunodeficiency virus type 1 infection assessed by polymerase chain reaction. J. Infect. Dis. **170:**325–333.
130. **Shirasaka, T., M. F. Kavlick, T. Ueno, W. Y. Gao, E. Kojima, M. L. Alcaide, S. Chokekijchai, B. M. Roy, E. Arnold, R. Yarchoan, et al.** 1995. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proc. Natl. Acad. Sci. USA **92:**2398–2402.
131. **Shoji, Y., S. Akhtar, A. Periasamy, B. Herman, and R. L. Juliano.** 1991. Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. Nucleic Acids Res. **19:**5543–5550.
132. **Simmons, G., P. R. Clapham, L. Picard, R. E. Offord, M. M. Rosenkilde, T. W. Schwartz, R. Buser, T. N. C. Wells, and A. E. Proudfoot.** 1997. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. Science **276:**276–279.
133. **Smith, A. J., N. Srinivasakumar, M. L. Hammarskjold, and D. Rekosh.** 1993. Requirements for incorporation of $Pr160^{gag\text{-}pol}$ from human immunodeficiency virus type 1 into virus-like particles. J. Virol. **67:**2266–2275.
134. **Srinivasakumar, N., M. L. Hammarskjold, and D. Rekosh.** 1995. Characterization of deletion mutations in the capsid region of human immunodeficiency virus type 1 that affect particle formation and Gag-Pol precursor incorporation. J. Virol. **69:**6106–6114.
135. **Strickler, J. E., J. Gorniak, B. Dayton, T. Meek, M. Moore, V. Magaard, J. Malinowski, and C. Debouck.** 1989. Characterization and autoprocessing of precursor and mature forms of human immunodeficiency virus type 1 (HIV 1) protease purified from Escherichia coli. Proteins **6:**139–154.
136. **Tanaka, M., R. V. Srinivas, T. Ueno, M. F. Kavlick, F. K. Hui, A. Fridland, J. S. Driscoll, and H. Mitsuya.** 1997. In vitro induction of human immunodeficiency virus type 1 variants resistant to 2′-β-fluoro-2′,3′-dideoxyadenosine. Antimicrob. Agents Chemother. **41:**1313–1318.
137. **Turpin, J. A., Y. Song, J. K. Inman, M. Huang, A. Wallqvist, A. Maynard, D. G. Covell, W. G. Rice, and E. Appella.** 1999. Synthesis and biological properties of novel pyridinioalkanoyl thiolesters (PATE) as anti-HIV-1 agents that target the viral nucleocapsid protein zinc fingers. J. Med. Chem. **42:**67–86.
138. **Vickers, T. A., and D. J. Ecker.** 1992. Enhancement of ribosomal frameshifting by oligonucleotides targeted to the HIV gag-pol region. Nucleic Acids Res. **20:**3945–3953.
139. **Walder, R. Y., and J. A. Walder.** 1988. Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. Proc. Natl. Acad. Sci. USA **85:** 5011–5015.
140. **Wang, S., and B. J. Dolnick.** 1993. Quantitative evaluation of intracellular sense: antisense RNA hybrid duplexes. Nucleic Acids Res. **21:**4383–4391.
141. **Weichold, F. F., J. Lisziewicz, R. A. Zeman, L. S. Nerurkar, S. Agrawal, M. S. Reitz, Jr., and R. C. Gallo.** 1995. Antisense phosphorothioate oligodeoxynucleotides alter HIV type 1 replication in cultured human macrophages and peripheral blood mononuclear cells. AIDS Res. Hum. Retrovir. **11:**863–867.
142. **Willey, R. L., M. A. Martin, and K. W. Peden.** 1994. Increase in soluble CD4 binding to and CD4-induced dissociation of gp120 from virions correlates with infectivity of human immunodeficiency virus type 1. J. Virol. **68:**1029–1039.
143. **Wilson, W., M. Braddock, S. E. Adams, P. D. Rathjen, S. M. Kingsman, and A. J. Kingsman.** 1988. HIV expression strategies: ribosomal frameshifting is directed by a short sequence in both mammalian and yeast systems. Cell **55:** 1159–1169.
144. **Wittung, P., J. Kajanus, K. Edwards, G. Haaima, P. E. Nielsen, B. Norden, and B. G. Malmstrom.** 1995. Phospholipid membrane permeability of peptide nucleic acid. FEBS Lett. **365:**27–29. (Correction and republication, **375:**27–29.)
145. **Wittung, P., P. E. Nielsen, O. Buchardt, M. Egholm, and B. Norden.** 1994. DNA-like double helix formed by peptide nucleic acid. Nature **368:**561–563.
146. **Wlodawer, A., M. Miller, M. Jaskolski, B. K. Sathyanarayana, E. Baldwin, I. T. Weber, L. M. Selk, L. Clawson, J. Schneider, and S. B. Kent.** 1989. Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease. Science **245:**616–621.
147. **Wong, J. K., M. Hezareh, H. F. Günthard, D. V. Havlir, C. C. Ignacio, C. A. Spina, and D. D. Richman.** 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **278:**1291–1295.
148. **Yamaguchi, K., B. Papp, D. Zhang, A. N. Ali, S. Agrawal, and R. A. Byrn.** 1997. The multiple inhibitory mechanisms of GEM 91, a gag antisense phosphorothioate oligonucleotide, for human immunodeficiency virus type 1. AIDS Res. Hum. Retrovir. **13:**545–554.
149. **Yarchoan, R., R. W. Klecker, K. J. Weinhold, P. D. Markham, H. K. Lyerly, D. T. Durack, E. Gelmann, S. N. Lehrman, R. M. Blum, D. W. Barry, et al.** 1986. Administration of 3′-azido-3′-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-related complex. Lancet **i:**575–580.
150. **Yarchoan, R., H. Mitsuya, R. V. Thomas, J. M. Pluda, N. R. Hartman, C. F. Perno, K. S. Marczyk, J. P. Allain, D. G. Johns, and S. Broder.** 1989. In vivo activity against HIV and favorable toxicity profile of 2′,3′-dideoxyinosine. Science **245:**412–415.
151. **Yoshimura, K., R. Kato, K. Yusa, M. F. Kavlick, V. Maroun, A. Nguyen, T. Mimoto, T. Ueno, M. Shintani, J. Falloon, H. Masur, H. Hayashi, J. Erickson, and H. Mitsuya.** 1999. JE-2147: a dipeptide protease inhibitor (PI) that potently inhibits multi-PI-resistant HIV-1. Proc. Natl. Acad. Sci. USA **96:** 8675–8680.
152. **Yu, X. F., L. Dawson, C. J. Tian, C. Flexner, and M. Dettenhofer.** 1998. Mutations of the human immunodeficiency virus type 1 $p6^{Gag}$ domain result in reduced retention of Pol proteins during virus assembly. J. Virol. **72:** 3412–3417.
153. **Zhang, L., B. Ramratnam, K. Tenner-Racz, Y. He, M. Vesanen, S. Lewin, A. Talal, P. Racz, A. S. Perelson, B. T. Korber, M. Markowitz, and D. D. Ho.** 1999. Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. N. Engl. J. Med. **340:**1605–1613.
154. **Zhao, H., N. Neamati, H. Hong, A. Mazumder, S. Wang, S. Sunder, G. W. Milne, Y. Pommier, and T. R. Burke, Jr.** 1997. Coumarin-based inhibitors of HIV integrase. J. Med. Chem. **40:**242–249.
155. **Zhao, H., N. Neamati, S. Sunder, H. Hong, S. Wang, G. W. Milne, Y. Pommier, and T. R. Burke, Jr.** 1997. Hydrazide-containing inhibitors of HIV-1 integrase. J. Med. Chem. **40:**937–941.