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Expression of Human Immunodeficiency Virus Type 1 (HIV-1) *gag*, *pol*, and *env* Proteins from Chimeric HIV-1–Poliovirus Minireplicons

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Recent studies have demonstrated that genomes of poliovirus with deletions in the P1 (capsid) region contain the necessary viral information for RNA replication. To test the effects of the substitution of foreign genes on RNA replication and protein expression, chimeric human immunodeficiency virus type 1 (HIV-1)–poliovirus genomes were constructed in which regions of the *gag*, *pol*, or *env* gene of HIV-1 were substituted for regions of the P1 gene in the infectious cDNA clone of type 1 Mahoney poliovirus. The HIV-1 genes were inserted between nucleotides 1174 and 2956 of the poliovirus cDNA so that the translational reading frame was maintained between the HIV-1 genes and the remaining poliovirus genes. The chimeric genomes were positioned downstream from a T7 RNA polymerase promoter and transcribed in vitro by using T7 RNA polymerase, and the RNA was transfected into HeLa cells. A Northern (RNA blot) analysis of the RNA from transfected cells demonstrated the appropriate-size RNA, corresponding to the full-length chimeric genomes, which increased over time. Immunoprecipitation with antibodies specific for poliovirus RNA polymerase or sera from AIDS patients demonstrated the expression of the poliovirus RNA polymerase and HIV-1 proteins as fusions with the poliovirus P1 protein. The expression of the HIV-1–poliovirus P1 fusion protein was dependent upon an intact RNA polymerase gene, indicating that RNA replication was required for efficient expression. A pulse-chase analysis of the protein expression from the chimeric genomes demonstrated the initial rapid proteolytic processing of the polyprotein from the chimeric genomes to give HIV-1–poliovirus P1 fusion protein in transfected cells; the HIV-1 *gag*–P1 and HIV-1 *pol*–P1 fusion proteins exhibited a greater intracellular stability than the HIV-1 *env*–P1 fusion protein. Finally, superinfection with wild-type poliovirus of HeLa cells which had been transfected with the chimeric genomes did not significantly affect the expression of chimeric fusion protein. The results are discussed in the context of poliovirus RNA replication and demonstrate the feasibility of using poliovirus genomes (minireplicons) as novel vectors for expression of foreign proteins.

The genomic RNA of poliovirus is approximately 7,500 nucleotides in length and functions both as mRNA and as a template for virus replication (11). Expression of the poliovirus genome occurs via the translation of a single protein (polyprotein) which is subsequently processed by virus-encoded proteases (2A and 3C) to give the mature structural (capsid) and nonstructural proteins (10, 11). Poliovirus replication is catalyzed by the virus-encoded RNA-dependent RNA polymerase ($3D^{pol}$), which copies the genomic RNA to give a complementary RNA molecule, which then serves as the template for further RNA production (11; reviewed in reference 13).

A fundamental goal in the study of the replication process of poliovirus is to define the essential viral proteins and delineate the structural features of the poliovirus RNA required for replication. Previous studies have clearly established that the entire poliovirus genome is not required for RNA replication (8, 9). Naturally occurring defective interfering particles (DIs) of poliovirus have been found to consist of subgenomic RNAs which have the capacity for replication (3, 12). The common feature of the poliovirus DI genome is a partial deletion of the capsid (P1) region that still maintains the translational reading frame of the polyprotein (12). In recent years, the availability of infectious cDNA clones of the poliovirus genome has facilitated further studies to define the regions required for RNA replication (22). Specifically, the deletion of 1,782 nucleotides of P1, corresponding to nucleotides 1174 to 2956, resulted in an RNA which can replicate upon transfection into tissue culture cells (i.e., minireplicons [8, 9]).

The fact that regions of the poliovirus genome could be deleted suggested that these minireplicons might be used for studies on protein expression and RNA replication. Furthermore, recent studies have also suggested that if these minireplicons can be encapsidated, a future use would be to express useful compounds in the alimentary canal (8). To further explore this possibility, our experiments were designed to determine the effect of adding foreign, nonpoliovirus RNA on the replication and protein expression of these minireplicons. For these studies, we have inserted DNA corresponding to regions of the *gag*, *pol*, and *env* genes of human immunodeficiency virus type 1 (HIV-1) into the poliovirus cDNA between nucleotides 1174 and 2956 of the infectious poliovirus cDNA so that the translational reading frame is conserved between the HIV-1 and poliovirus genes. We report that the chimeric HIV-1–poliovirus RNA genomes replicate and express the appropriate HIV-1–P1 poliovirus fusion proteins upon transfection into tissue culture cells. The expression of the foreign genes from the chimeric HIV-1–poliovirus genomes is dependent upon RNA replication and is not altered by superinfection with wild-type poliovirus. From the results of these studies, we conclude that poliovirus replication proteins have the potential to replicate foreign RNA when placed in the context of the viral genome, which can be exploited for expression of chimeric proteins.

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MATERIALS AND METHODS

Materials. All chemicals unless otherwise noted were purchased from Sigma Chemical Co. Restriction enzymes and DNA modification enzymes were purchased from Boehringer Mannheim. Synthetic DNA linkers were purchased from New England BioLabs. The *Taq* DNA polymerase and reagents for the polymerase chain reaction were bought from Perkin-Elmer Cetus Company. Sequenase was purchased from U.S. Biochemicals. RNasin was obtained from Promega Biotec Co. Tissue culture media and reagents were purchased from BRL-GIBCO. The T7 RNA polymerase was prepared in this laboratory by the method of Grodberg and Dunn (7). Synthetic DNA oligomers were synthesized at the Cancer Center Oligonucleotide Core Facility at this university. Antipeptide antibodies specific for the HIV-1 reverse transcriptase were made by Southern Biotech Associates, Birmingham, Ala.

Tissue culture cells and viruses. HeLa cells were grown in complete medium; Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum and 5% fetal calf serum. The stock of the poliovirus type 1 Mahoney used in this study was derived from transfection of the infectious cDNA.

Construction of poliovirus expression plasmids. All DNA manipulations were performed according to standard procedures (15). Restriction enzymes and DNA modification enzymes were used according to the manufacturer's instructions. The poliovirus plasmid pEV104 used for these studies contains the complete infectious poliovirus cDNA flanked by *Eco*RI restriction sites (27). For our studies, this plasmid was modified by partial *Eco*RI digestion, followed by a fill-in reaction with Klenow enzyme. Synthetic *Sal*I DNA linkers were added, and the DNA was transformed into competent *Escherichia coli* DH5α. The transformants were screened by restriction digest for single *Eco*RI and *Sal*I restriction sites. The plasmid pKCIC contained the poliovirus cDNA with *Eco*RI and *Sal*I restriction sites positioned at the 5′ and 3′ ends, respectively.

The plasmid pKCIC was further modified to delete 19 of the 21 guanine residues at the 5′ end of the cDNA clone, which were generated in the cloning of the poliovirus genome (27). This was accomplished by oligonucleotide site-directed mutagenesis in which a synthetic DNA sequence corresponding to a promoter for the bacteriophage T7 RNA polymerase was added (8a). The resulting plasmid, pT7IC, contains the T7 promoter positioned two guanine residues 5′ to the start of the poliovirus cDNA.

Two separate modifications of pT7IC were made to construct the chimeric genomes. For one, pT7IC was digested at the unique *Sna*BI restriction site at nucleotide position 2956 in the poliovirus genome. Synthetic DNA oligomers corresponding to an *Xho*I restriction site were ligated into this site; the resulting plasmid, pT7IC-*Sna*BI, contains a unique *Xho*I restriction site at position 2956 of the poliovirus genome. A second modified pT7IC was generated by digesting at the unique *Nhe*I restriction site, nucleotide 2470 of the poliovirus genome. The 4-bp extensions were filled in with Klenow enzyme, and synthetic DNA oligomers corresponding to an *Xho*I restriction site were ligated into the clone to generate the final plasmid, pT7IC-NheI.

Construction of chimeric HIV-poliovirus genomes. (i) Chimeric HIV-1 *gag*–poliovirus genomes. The construction of chimeric HIV-1 *gag*–poliovirus genomes was accomplished by the polymerase chain reaction (PCR) to amplify the *gag* gene sequences (25). For this procedure, a *Sac*I-*Sal*I DNA fragment of the HIV-1 proviral genome (nucleotides 223 to 5370 [23]) from pBH10 (28) was subcloned into the phagemid vector pUC119. Single-stranded DNA was generated by previously described techniques (33). The single-stranded DNA was used as the template for the PCR reactions with two DNA oligomers: 1, 5′-CACAGCAGTCAGGTTAAC CAAAA-3′, and 2, 5′-TAGGGGCCCTCGAGTTTCTG-3′. These oligomers correspond to nucleotide positions 703 to 725 and 1539 to 1558 in the HIV-1 proviral genome, flanking the p24 protein of the HIV-1 *gag* gene (approximately 800 bp). The oligonucleotides were designed so that a unique *Hpa*I restriction site (underlined in 1) and an *Xho*I restriction site (underlined in 2) were created at the 5′ and 3′ ends, respectively, of the amplified DNA.

For the PCR reactions, approximately 30 ng of single-stranded template DNA derived from pUC119 containing the *Sac*I-*Sal*I fragment and 300 ng of each primer were used in a final volume of 100 μl in 10 mM Tris-HCl–50 mM NaCl–1.5 mM $MgCl_2$–1 mM each deoxynucleoside triphosphate–2 U of AmpliTaq DNA polymerase. The thermocycler conditions were set at 94°C (1 min), 37°C (3 min), and 72°C (3 min) for a total of 30 amplification cycles. One-tenth of the product was analyzed on 0.8% agarose gels to ensure the amplification of an 800-bp DNA fragment. The remaining DNA was extracted with phenol-chloroform (1:1), followed by ethanol precipitation at −70°C. After centrifugation to pellet the DNA, the sample was digested with *Hpa*I and *Xho*I, followed by a second phenol-chloroform extraction and precipitation with ethanol. Approximately 1 to 5 μg of DNA was recovered from this procedure and ligated directly into pT7IC-SnaBI and pT7IC-NheI which had been previously digested with *Nru*I (nucleotide 1174 in the poliovirus genome) and *Xho*I. Transformation into competent *E. coli* DH5α was performed, and the resulting colonies were screened for the appropriate DNA inserts by restriction enzyme analysis. The desired clone pT7IC-SnaBI-*gag* had the PCR-amplified HIV-1 *gag* gene cloned in the poliovirus genome between nucleotides 1174 and 2956 of the poliovirus genome, while pT7IC-NheI-*gag* had the HIV-1 *gag* gene inserted in the poliovirus genome between nucleotides 1174 and 2470. The plasmid was sequenced at the junctions between the gene fusions of poliovirus and HIV-1 *gag* gene to confirm that the translational reading frame was conserved between the *gag* and poliovirus genes (26).

(ii) Chimeric HIV-1 *pol*–poliovirus genome. A DNA fragment of the HIV-1 *pol* gene, corresponding to nucleotides 2182 to 2203 and 3127 to 3148 (23), was amplified by PCR with the following synthetic DNA primers: 1, 5′-GCCC AA AAG TT AAC CAA TGG CC-3′, and 2, 5′-TGA TAT CTC GAG TGG CCT TG CC-3′. The DNA primers used for PCR amplification were designed to create unique *Hpa*I (underlined in 1) and *Xho*I (underlined in 2) restriction sites for direct subcloning into the poliovirus infectious cDNA clones. A DNA fragment of 967 bp was amplified, and following restriction digestion with *Hpa*I and *Xho*I, the DNA fragment was directly ligated into the pT7IC-NheI plasmid which had been previously digested with *Nru*I and *Xho*I. Following transformation into *E. coli* DH5α, the colonies were screened for the presence of the HIV-1 *pol* gene DNA fragment by restriction digestion. DNA sequencing at the junction regions between the HIV-1 *pol* and poliovirus genes was done to confirm the conservation of the translational reading frame (26). The final clone was designated pT7IC-NheI-*pol*.

(iii) Construction of chimeric HIV-1 *env*–poliovirus genome. A subclone of the HIV-1 *env* gene in the pGEM-3 plasmid,

which contains the entire coding region of the HIV-1 *env* from nucleotides 5748 to 8478 (23), was used as starting material (obtained from R. Owens and R. W. Compans, University of Alabama at Birmingham [19]). The plasmid was digested with *Ava*I, which cuts at nucleotide 7972 in the *env* gene, the 4-bp extensions were filled in with avian myeloblastosis virus reverse transcriptase, and synthetic *Xho*I DNA linkers (8-mers) were ligated. The resulting plasmid, pENV-17XhoI, was digested with restriction enzyme *Stu*I, which cuts at nucleotide 6411. Synthetic *Sma*I DNA linkers (8-mers) were ligated into this plasmid, and a *Sma*I-*Xho*I DNA fragment, corresponding to nucleotides 6411 and 7975 of the HIV-1 *env* gene, was isolated and ligated directly into pT7IC-SnaBI which had been previously digested with *Nru*I and *Xho*I. The plasmid pT7IC-SnaBI-*env* was characterized by restriction digestion and DNA sequencing to ensure the conservation of the translational reading frame between the *env* and poliovirus genes (26).

In vitro transcription reactions. Prior to in vitro transcription, the DNA templates were linearized with the restriction enzyme *Sal*I, followed by successive phenol-chloroform (1:1) and chloroform-isoamyl alcohol (24:1) extractions prior to ethanol precipitation. The in vitro transcription reactions used 3 to 5 μg of linearized DNA template in a 100-μl reaction with the following components: 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM each GTP, UTP, ATP, and CTP, 40 U of RNasin, and approximately 5 μg of purified T7 RNA polymerase per reaction mix. After 60 min, 1/20 of the in vitro-synthesized RNA was analyzed by agarose gel electrophoresis, and the remaining RNA was quantitated by the UV A_{260}.

Transfection of in vitro-synthesized RNA. The in vitro-transcribed RNA was transfected into HeLa cells with DEAE-dextran (M_r 500,000) as a facilitator as previously described except for the following modifications (32). Briefly, cells were washed twice with phosphate-buffered saline (PBS) and then incubated with approximately 10 μg of the in vitro-synthesized RNA with DEAE-dextran at 300 μg/ml in PBS at 37°C. After 2 h, the cells were washed once with DMEM and incubated in complete medium.

Metabolic labeling of transfected cells and immunoprecipitations. The transfected cells were metabolically labeled at 7 h posttransfection. The cells were washed once with DMEM without methionine and incubated in this medium for 45 min, followed by an additional 45 min with DMEM without methionine plus [^{35}S]methionine-cysteine (Translabel; ICN) at 0.3 mCi/ml final concentration. The cells were washed once with PBS and lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 mM NaCl, 25 mM Tris-HCl [pH 7.5]). The cellular DNA and debris were removed by centrifugation at 13,000 × *g* for 10 min at 4°C. A rabbit polyclonal antibody specific for the poliovirus RNA polymerase was used for immunoprecipitation of poliovirus RNA polymerase 3D^{pol} (24). Sera from five AIDS patients were used for immunoprecipitation of HIV-1 *gag*- or *env*-related proteins. A rabbit antipeptide antibody specific for the HIV-1 reverse transcriptase was used for immunoprecipitation of *pol* gene products. The antibodies were incubated with the extracts for 24 h at 4°C with constant rocking. The immune complexes were collected by a 1-h incubation with protein A-Sepharose (20 μl of a 1:1 [wt/vol] mixture in RIPA) at room temperature with constant shaking. The beads were pelleted by centrifugation and washed three times with RIPA buffer. The bound proteins were eluted from the beads by boiling for 5 min in gel sample buffer (50 mM Tris [pH 6.8], 5% SDS, 10% β-mercaptoethanol, 0.1% bromophenol blue). The proteins were separated on 10% SDS–polyacrylamide gels, fluorographed, and exposed to X-ray film (Kodak X-AR) with an intensifying screen.

Nucleic acid hybridization. Total cellular RNAs were prepared from cells at specified times following transfection (2). The cells were washed once in PBS prior to lysis in a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), and 0.5% sarcosyl in diethylpyrocarbonate (DEP)-treated water. Following lysis, the extract was adjusted to 0.2 M sodium acetate, and sequential extractions were performed with water-saturated phenol and chloroform-isoamyl alcohol (24:1). The aqueous phase was mixed with 0.8 volume of isopropanol and placed at −20°C for 1 h, followed by centrifugation at 10,000 × *g* for 20 min. The pellet was dissolved in DEP-treated water, adjusted to 0.2 M sodium acetate, and reprecipitated with 2.5 volumes of ethanol at −70°C for 1 h. Following centrifugation at 10,000 × *g* for 20 min, the pelleted material was dried and resuspended in DEP-treated water. The quantity of nucleic acid was determined by the UV A_{260}.

The Northern (RNA blot) hybridizations were performed by standard procedures (15). Briefly, approximately 20 μg of total cellular RNA was solubilized in RNA gel loading solution (50% formamide, 0.02 M 3-[*N*-morpholino]propanesulfonic acid [pH 7], 5 mM sodium acetate, 1 mM EDTA, 6.3% formaldehyde with 13% glycerol). The RNA was incubated at 65°C for 15 min, chilled on ice, and loaded into a 1% agarose–6.3% formaldehyde gel. Following electrophoresis, the nucleic acids were transferred to a nitrocellulose membrane by capillary blotting, followed by prehybridization under standard conditions (6× SSC [1× SSC is 0.15 M NaCl, 17 mM sodium acetate, pH 7.0], 10 mM EDTA, 5× Denhardt's solution [1× Denhardt's reagent is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin], 0.5% SDS, and 100 μg of salmon sperm DNA per ml) for 2 to 4 h at 42°C. The prehybridization solution was replaced with 6× SSC–0.5% SDS–50% formamide–5× Denhardt's solution–10 mM EDTA–100 μg of salmon sperm DNA per ml containing approximately 10^6 cpm of the riboprobe per ml; hybridization was done at 52°C for 16 h. The blot was washed two times with 2× SSC–0.5% SDS at room temperature for 5 min and then once with 0.1× SSC–0.5% SDS at 65°C for 2 h, dried, and autoradiographed at −70°C.

For detection of poliovirus-specific plus-strand RNA, a 503-base riboprobe was generated which is complementary to nucleotides 671 to 1174 of the poliovirus genome. The poliovirus cDNA from nucleotides 1 to 1174 was subcloned into the pGEM-4 plasmid. The resulting plasmid was linearized prior to in vitro transcription with *Bam*HI, which cuts at positions 220 and 670 in the poliovirus genome. The in vitro transcription was performed under standard conditions (50 mM Tris-HCl [pH 8], 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each ATP, GTP, and CTP, 4 μCi of [α-^{32}P]UTP) with 5 μg of T7 RNA polymerase.

RESULTS

Construction of chimeric HIV-1–poliovirus genomes. The plasmid used in these studies, pT7IC, contained the promoter sequence for the T7 RNA polymerase positioned 5′ to the start of the complete poliovirus cDNA (Fig. 1A). For in vitro transcriptions, the plasmid was linearized with *Sal*I; the in vitro-synthesized RNA then corresponded to the full-length poliovirus genome with only 2 guanine residues at the 5′ end and 89 nucleotides following the 60-nucleotide

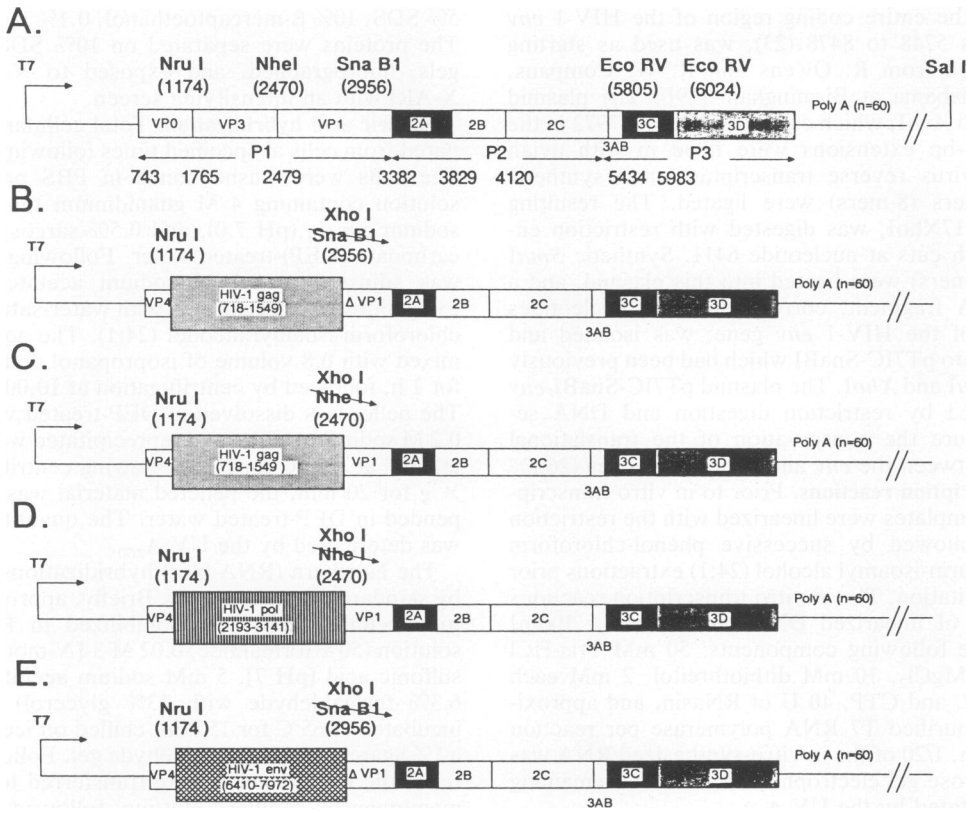

FIG. 1. Chimeric HIV-1–poliovirus genomes. (A) Plasmid containing the complete poliovirus infectious cDNA clone. The nucleotides at which proteolytic cleavage occurs to give the mature polypeptides are shown with the relevant restriction enzyme sites. The numbering of the nucleotides is according to the published poliovirus type 1 Mahoney sequence (10). For in vitro transcriptions, the plasmid, which contains a promoter for T7 RNA polymerase, is digested with either *Sal*I (for full-length transcripts) or *Eco*RV (nucleotide 5805; truncated). Two modified plasmids were constructed for these studies: pT7IC-SnaBI contains an *Xho*I restriction site at position 2956, while pT7IC-NheI contains an *Xho*I restriction site at nucleotide 2470. All of the gene fusions described in this report are within the P1 region of poliovirus and contain an intact coding region for VP4 and either a truncated (Δ) or complete coding sequence for VP1. (B) Construction of pT7IC-SnaBI-*gag*. An 832-base fragment of the HIV-1 *gag* gene (nucleotides 718 to 1549 [23]) was amplified by PCR and subcloned directly into the poliovirus cDNA genome between nucleotides 1174 and 2956 (pT7IC-SnaBI). (C) Construction of pT7IC-NheI-*gag*. The 832-base *gag* fragment was amplified by PCR and subcloned into poliovirus cDNA plasmid pT7IC-NheI. (D) Construction of T7IC-NheI-*pol*. A 949-base fragment of the HIV-1 *pol* gene (nucleotides 2193 to 3141 [23]) was amplified by PCR and cloned into poliovirus cDNA pT7IC-NheI. (E) Construction of T7IC-SnaBI-*env*. A 1,563-base fragment of the HIV-1 *env* gene (nucleotides 6410 to 7972 [23]), prepared and modified as described in Materials and Methods, was subcloned into poliovirus cDNA pT7IC-SnaBI.

poly(A) tract at the 3′ end. In preliminary experiments, the in vitro-transcribed RNA from pT7IC exhibited infectivity of approximately 10^5 PFU/μg of RNA upon transfection into HeLa cells (1b).

Two separate modifications of the poliovirus cDNA were made for the construction of the HIV-1–poliovirus chimeric genomes. In the first, the *Sna*BI restriction site, at nucleotide 2956 of the poliovirus genome, was converted to an *Xho*I restriction site by using synthetic DNA linkers. Previous studies have demonstrated that deletion of nucleotides 1174 (an *Nru*I site) to 2956 (a *Sna*BI restriction site) resulted in a defective poliovirus genome which contained all of the necessary genetic information for RNA replication (9). A second, separate modification of the poliovirus cDNA was constructed to generate an *Xho*I site at nucleotide 2470 (an *Nhe*I site). Previous studies demonstrated that the genomes of the majority of the naturally occurring poliovirus DIs contain deletions between nucleotides 1226 and 2705 of the poliovirus genome (12). Both clones, designated pT7IC-SnaBI and pT7IC-NheI, respectively, were used to construct the chimeric genomes described in this report.

The *gag*, *pol*, and *env* genes of HIV-1 were used for the construction of the chimeric genomes because of the availability of the complete nucleic acid sequence, the plasmids, and the immunological reagents needed for the detection of HIV-1 gene products. The chimeric HIV-1 *gag*–poliovirus genomes were constructed so that nucleotides 718 to 1549 of the *gag* gene of HIV-1 were amplified by PCR and subcloned into pT7IC-SnaBI or pT7IC-NheI (Fig. 1B and C). A similar strategy was used for the construction of chimeric HIV-1–poliovirus genomes containing regions of the *pol* gene corresponding to the coding region for reverse transcriptase. The final plasmid, pT7IC-NheI-*pol*, has the HIV-1 *pol* gene subcloned into the poliovirus cDNA to conserve the translational reading frame (Fig. 1D). A fourth chimeric HIV-poliovirus genome was constructed in which the HIV *env* gene from nucleotides 6410 to 7972, encoding partial genes for gp120 and gp41 of HIV-1, was subcloned into pT7IC-SnaBI to give the final plasmid, pT7IC-SnaBI-*env* (Fig. 1E).

Replication of chimeric HIV-1–poliovirus genomes. To determine whether the chimeric HIV-poliovirus RNAs replicate, the RNAs derived from in vitro transcription were

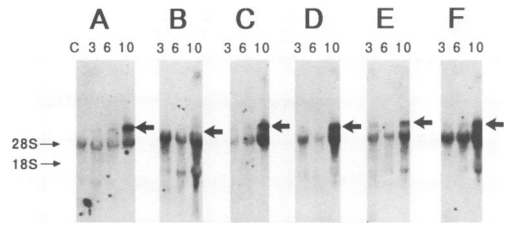

FIG. 2. Northern blot analysis of RNA from HeLa cells transfected with in vitro-transcribed RNA. At 3, 6, and 10 h posttransfection of HeLa cells, total cellular RNA was prepared, fractionated in a 1% agarose–6.3% formaldehyde gel, blotted onto a nitrocellulose membrane, and probed with a radioactive riboprobe complementary to the poliovirus virion RNA (nucleotides 670 through 1172). (A) Lane C, RNA derived from mock-transfected cells; lanes 3, 6, and 10 (representing time of transfection in hours), RNA from cells transfected with RNA derived from in vitro transcription of pT7IC. (B) Lanes 3, 6, and 10, RNA from cells transfected with RNA derived from in vitro transcription of pT7IC linearized with *Eco*RV for 3, 6, or 10 h. (C) Lanes 3, 6, and 10, RNA derived from cells transfected with RNA derived from in vitro transcription of pT7IC-SnaBI-*gag* for 3, 6, or 10 h. (D) Lanes 3, 6, and 10, RNA from cells transfected with RNA derived from the in vitro transcription of pT7IC-NheI-*gag* for 3, 6, or 10 h. (E) Lanes 3, 6, and 10, RNA from cells transfected with RNA derived from in vitro transcription of pT7IC-NheI-*pol* for 3, 6, or 10 h. (F) Lanes 3, 6, and 10, RNA isolated from cells transfected with RNA derived from in vitro transcription of pT7IC-SnaBI-*env* for 3, 6, or 10 h. Arrows indicate the migration of the expected size of each full-length transfected RNA molecule relative to that of the 28S and 18S cellular rRNAs.

transfected into HeLa cells. At the indicated times, equivalent amounts of RNA were analyzed by Northern blot with a riboprobe complementary to nucleotides 671 to 1174 of the poliovirus genome (Fig. 2). Minor background hybridization was detected from RNA isolated from the mock-transfected cells corresponding to 28S RNA (Fig. 2A, lane C); previous studies have also noted background hybridization between 28S RNA and poliovirus RNA (16). The hybridization signal for RNA derived from in vitro transcription of pT7IC at a position consistent with the known size for full-length poliovirus RNA (7.5 kb) was evident at increasing times posttransfection (Fig. 2A, lanes 3, 6, and 10).

To test whether the increase in the hybridization signal correlates with the replication of the transfected RNA, transcripts derived from in vitro transcription of pT7IC linearized with *Eco*RV, which does not encode a functional RNA polymerase gene, were transfected into tissue culture cells. No increase in the hybridization signal corresponding to the predicted 5.8-kb RNA was evident (Fig. 2B, lane 10). From these results, we conclude that replication of the transfected RNA is necessary for the observed increase in the hybridization. Each of the RNAs derived from the in vitro transcription of the HIV-1–poliovirus chimeric genomes replicated, as evidenced by the increase in the hybridization signal posttransfection (Fig. 2C, D, E, and F). The size of the amplified RNA, approximately 6.5 to 7.2 kb, is that expected for the entire chimeric genomes, suggesting that the full-length RNA of the chimeric genomes had replicated rather than deleted genomes consisting of a smaller subspecies. The differences in the hybridization signal between the chimeric genomes were not apparent upon repeat transfections. Finally, no hybridization signal was detected when the chimeric RNA transcripts were generated from plasmids linearized with *Eco*RV (data not shown).

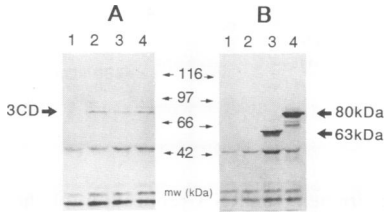

FIG. 3. Analysis of the poliovirus $3D^{pol}$ and HIV-1 *gag* fusion protein expression in transfected cells. HeLa cells were transfected with RNA derived from the in vitro transcription of the designated plasmids, metabolically labeled with [^{35}S]methionine-cysteine, followed by immunoprecipitation with anti-$3D^{pol}$ antibodies (A) or sera from AIDS patients (B) and analysis on SDS-polyacrylamide gels: Lane 1, mock-transfected cells; lane 2, RNA from pT7IC; lane 3, RNA from pT7IC-SnaBI-*gag*; lane 4, RNA derived from pT7IC-NheI-*gag*. The positions of the molecular mass standards, the poliovirus 3CD protein, and the 63-kDa and 80-kDa HIV-1 *gag*–P1 fusion proteins are marked.

Protein expression from chimeric HIV-poliovirus genomes. The expression of HIV-1 and poliovirus proteins from the transfected chimeric genomes was next determined. Under our experimental conditions, approximately 10 to 20% of the cells are transfected. In preliminary experiments, we were unable to detect the expression of viral proteins by the analysis of total labeled cell extracts. Subsequently, cells transfected with RNA derived from pT7IC, pT7IC-SnaBI-*gag*, and pT7IC-NheI-*gag* were analyzed for the expression of poliovirus RNA polymerase with anti-$3D^{pol}$ antibodies (Fig. 3A). Under the conditions used for metabolic labeling, a precursor of the mature $3D^{pol}$, consisting of the viral protease $3C^{pro}$ and $3D^{pol}$, designated 3CD, was immunoprecipitated from the cells transfected with RNA derived from pT7IC, pT7IC-SnaBI-*gag*, and pT7IC-NheI-*gag*.

The expression of the HIV-1 *gag*-related protein from the cells transfected with the RNA derived from the chimeric genomes pT7IC-SnaBI-*gag* and pT7IC-NheI-*gag* was determined by immunoprecipitation with pooled sera from five AIDS patients (Fig. 3B). Preliminary studies were performed to ensure that all of the relevant HIV-1 proteins were immunoprecipitated from extracts of radiolabeled SupT1 cells infected with the IIIB strain of HIV-1 (data not shown). The HIV-1-related proteins, representing an HIV-1 *gag*–P1 poliovirus fusion protein, were 80 kDa from cells transfected with RNA derived from pT7IC-NheI-*gag* and 63 kDa from cells transfected with RNA derived from pT7IC-SnaBI-*gag*, which is consistent with the notion that proteolytic processing of the poliovirus polyprotein occurred at the tyrosine-glycine amino acid pair separating the VP1 and 2A genes (Fig. 1). Indeed, anti-VP1 antibody immunoprecipitated a protein of 80 or 63 kDa from cells transfected with RNA derived from pT7IC-NheI-*gag* and pT7IC-SnaBI-*gag*, respectively (data not shown). No immunoreactive material was detected from the mock-transfected cells or from cells transfected with RNA derived from in vitro transcription of pT7IC.

To test whether RNA replication is required for protein expression, the RNAs from the in vitro transcription of pT7IC-NheI-*gag* linearized at the *Sal*I restriction site (full-length RNA) or the *Eco*RV restriction site (nucleotide 5805), which deletes the $3C^{pro}$ and $3D^{pol}$ gene, were transfected into HeLa cells, followed by immunoprecipitation with pooled sera from AIDS patients (Fig. 4). The HIV-1 *gag*–P1 fusion protein was only immunoprecipitated from cells trans-

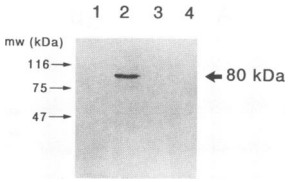

FIG. 4. Requirement for RNA replication for the expression of HIV-1 *gag*–P1 fusion protein. RNA derived from the designated plasmids was transfected into HeLa cells, and expression of the HIV-1–P1 fusion protein was determined by immunoprecipitation with sera from AIDS patients and analysis by SDS-polyacrylamide gel electrophoresis. Lane 1, mock-transfected cells; lane 2, RNA from pT7IC-NheI-*gag*; lane 3, RNA derived from transfection of pT7IC-NheI-*gag* linearized with *Eco*RV; lane 4, RNA from pT7IC. The positions of the molecular mass standards and 80-kDa HIV-1 *gag*–P1 fusion protein are marked.

fected with the full-length chimeric HIV-1–poliovirus genomes (lane 2). No immunoreactive material was detected from cells transfected with the RNA derived from pT7IC-NheI-*gag* linearized with *Eco*RV or from pT7IC. Thus, the replication of the chimeric genome is required for efficient expression of the HIV-1–P1 fusion proteins.

To test for the expression of HIV-1 *env* and *pol* proteins from the respective chimeric genomes, the RNAs from pT7IC-SnaBI-*env* and pT7IC-NheI-*pol* were transfected into HeLa cells. Immunoprecipitation of cell lysates with anti-$3D^{pol}$ antibody revealed the presence of 3CD proteins from cells transfected with RNA derived from the wild-type or mutant genome (data not shown). The expression of HIV-1-related protein was determined by using a polyclonal anti-peptide antibody to HIV-1 reverse transcriptase (for *pol*) or serum from AIDS patients (for *env*). Extracts from cells transfected with RNA transcribed from pT7IC-NheI-*pol* and immunoprecipitated with anti-reverse transcriptase antibodies revealed the presence of a specific 85-kDa protein (Fig. 5A). No immunoreactive proteins were detected from HeLa cells transfected with no RNA or with RNA from in vitro-transcribed pT7IC. A protein with an approximate molecular mass of 90 kDa was immunoprecipitated from cells transfected with pT7IC-SnaBI-*env* (Fig. 5B), with no immunoreactive proteins detected from mock-transfected cells or cells transfected with RNA derived from pT7IC. Again, the molecular masses of the HIV-1 *pol* and *env* proteins expressed from the respective chimeric genomes were consistent with those predicted if proteolytic processing had occurred at the VP1-2A junction.

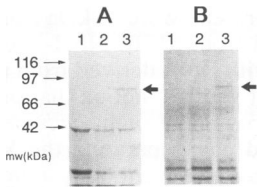

FIG. 5. Analysis of HIV-1 *pol*- and *env*-related protein expression from chimeric genomes. In vitro-transcribed RNA from the designated plasmids was transfected into HeLa cells, and protein expression was determined by immunoprecipitation with rabbit anti-reverse transcriptase (for *pol*, panel A) or sera from AIDS patients (for *env*, panel B) followed by SDS-polyacrylamide gel electrophoresis. (A) Analysis of HIV-1 *pol*-related protein expression. Lane 1, mock-transfected cells; lane 2, RNA derived from pT7IC; lane 3, RNA derived from pT7IC-NheI-*pol*. The positions of molecular mass standards and the HIV-1 *pol*–P1 fusion protein are marked. (B) Analysis of expression of HIV-1 *env*-related protein expression. Lane 1, mock-transfected cells; lane 2, RNA derived from pT7IC; lane 3, RNA derived from pT7IC-SnaBI-*env*. The positions of the molecular mass standards and the HIV-1 *env*–P1 fusion protein (large arrows) are marked.

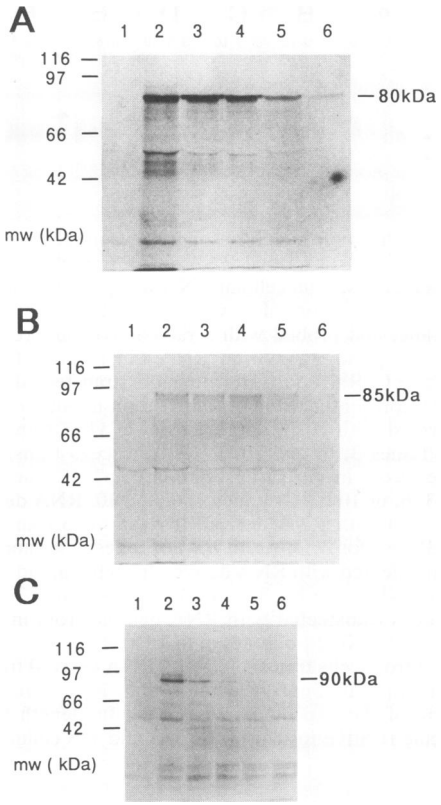

FIG. 6. Pulse-chase analysis of expression of HIV-1 *gag*, *pol*, and *env* fusion proteins with P1 from the respective chimeric genomes. The in vitro-transcribed RNAs from the designated plasmids were transfected into HeLa cells. After 7 h, the cells were metabolically labeled with [^{35}S]methionine-cysteine for 30 min (pulse), followed by the change of complete medium for 0.5, 1, 1.5, and 2 h. After the pulse and each chase period, cell extracts were immunoprecipitated with sera from AIDS patient (A and C) or anti-reverse transcriptase antibodies (B), followed by SDS-gel electrophoresis. (A) HeLa cells transfected with RNA derived from pT7IC-NheI-*gag*; (B) HeLa cells transfected with RNA derived from pT7IC-NheI-*pol*; (C) HeLa cells transfected with RNA derived from pT7-SnaBI-*env*. The order of each gel is the same: lane 1, mock-transfected cells; lane 2, pulse label; lane 3, 0.5-h chase; lane 4, 1-h chase; lane 5, 1.5-h chase; lane 6, 2-h chase. The positions of the molecular mass standards and the individual HIV-1–P1 fusion proteins are marked.

Pulse-chase experiments were performed to characterize the expression and stability of the HIV-1–poliovirus P1 fusion proteins. The RNAs derived from in vitro transcription of pT7IC-NheI-*gag*, pT7IC-NheI-*pol*, and pT7IC-SnaBI-*env* were transfected into HeLa cells; at 7 h posttransfection, the cells were metabolically labeled for 30 min and incubated in medium (chase) for 2 h (Fig. 6). The chimeric HIV-1 *gag*–P1 fusion protein was readily detected during the pulse labeling and was stable in transfected cells for most of

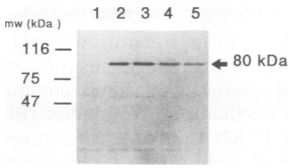

FIG. 7. Effect of poliovirus infection on the expression of HIV-1 *gag*–P1 fusion protein. HeLa cells were transfected with in vitro-transcribed RNA from pT7IC-NheI-*gag*. After 2 h, the cells were infected with poliovirus (type 1 Mahoney) at the specified MOI and metabolically labeled 4 h later. The HIV-1-related proteins were immunoprecipitated with sera from AIDS patients, followed by analysis by SDS-polyacrylamide gel electrophoresis. Lane 1, mock-transfected cells; lane 2, transfected with RNA derived from pT7IC-NheI-*gag*; lane 3, transfected with RNA derived from pT7IC-NheI-*gag* and infected with 5 PFU of poliovirus type 1 Mahoney per cell; lane 4, transfected with RNA derived from pT7IC-NheI-*gag* and infected with 20 PFU of poliovirus per cell; lane 5, transfected with RNA derived from pT7IC-NheI-*gag* and infected with 50 PFU of poliovirus per cell. The positions of the molecular mass standards and the 80-kDa HIV-1 *gag*–P1 fusion protein are marked.

the 2-h chase period (Fig. 6A, lanes 2 to 6). No difference was observed in the overall stability of the HIV-1 *gag*–P1 fusion protein when the RNA was derived from the in vitro transcription of pT7IC-SnaBI-*gag* (data not shown). Similar results were observed with the expression and the relative stability of the HIV-1 *pol*–P1 fusion protein (Fig. 6B, lanes 2 to 6). At later times during the chase period, however, both the *gag*-P1 and *pol*-P1 fusion proteins disappeared, indicating some instability. In contrast, the chimeric HIV-1 *env*–P1 fusion protein was immunoprecipitated after the pulse period but demonstrated greater instability, as evidenced by the loss of immunoreactive material after the 30-min chase period (Fig. 6C, lanes 2 to 6).

Expression of HIV-1–poliovirus chimeric proteins in poliovirus-infected cells. Specific *cis*-acting elements in the 5′ noncoding region of the poliovirus genome have been found to be essential for translation during the host cell shutoff characteristic of poliovirus infections (4, 20, 21, 29). Since the chimeric HIV-1–poliovirus genomes contain intact 5′ noncoding regions, we expect that the expression of the HIV-1–P1 fusion protein would be resistant to inhibition of translation caused by poliovirus infection. To test this, we transfected RNA derived from pT7IC-NheI-*gag* into cells, followed by superinfection with poliovirus at varying multiplicities of infection (MOIs). The expression of HIV-1 *gag*–P1 proteins was analyzed by immunoprecipitation (Fig. 7). The expression of the HIV-1 *gag*–P1 chimeric protein was comparable in uninfected and poliovirus-infected cells at a low MOI, while a slight decrease in expression was observed at the higher MOIs (20 and 50 PFU/cell). In parallel experiments, we determined that under these experimental conditions, a complete shutoff of host cell protein synthesis was evident in the HeLa cells infected with poliovirus at an MOI of greater than 3 (data not shown). We also passaged these extracts (up to eight passages) to determine whether the chimeric RNA was encapsidated as a DI genome. Furthermore, we attempted to encapsidate the chimeric genomes by cotransfection with the poliovirus infectious RNA. After protein (immunoprecipitation) and RNA (Northern blot) analyses of HeLa cells infected by the passaged virus, we found no evidence of encapsidation of the chimeric genomes (data not shown).

DISCUSSION

In this article, we described the construction and characterization of chimeric HIV-1–poliovirus genomes. Segments of HIV-1 proviral DNA containing the *gag*, *pol*, or *env* gene were inserted into the poliovirus cDNA so that the translational reading frame was conserved between the HIV-1 and poliovirus genes. The RNAs derived from the in vitro transcription of these genomes, when transfected into cells, replicated and expressed the appropriate HIV-1 protein as a fusion with the poliovirus P1 protein. Protein expression was dependent upon replication of the chimeric genomes and was resistant to inhibition by superinfection with poliovirus. The results of this study establish that the poliovirus proteins required for replication will copy foreign RNA in the context of the poliovirus genome and demonstrate the use of poliovirus as a vector to express foreign proteins.

Previous studies have described poliovirus minireplicons with deleted regions of the genome dispensable for RNA replication. Kaplan and Racaniello demonstrated that the deletion of 1,781 bp, corresponding to the entire VP3 and parts of the VP2 and VP1 capsid genes, did not affect the ability of the RNA to replicate when transfected into cells (9). Further studies by Hagino-Yamagishi and Nomoto have described poliovirus minireplicons with deletions between nucleotides 1663 and 2478; these genomes not only have the capacity to replicate but will act as DI genomes, as demonstrated by the capacity to be encapsidated after superinfection with wild-type poliovirus (8). Our study represents the first demonstration that foreign genes, namely HIV-1 *gag*, *pol*, and *env*, can be substituted for the deleted poliovirus capsid regions without adversely affecting the replication of the RNA. Few expression systems analogous to that presented in this study based on self-replicating RNA have been described. The RNA virus brome mosaic virus has been used as an expression vector by inserting the gene for chloramphenicol acetyltransferase (CAT) into one of the three RNA components of the virus genome (5). Cotransfection of all three brome mosaic virus RNAs into plant protoplasts resulted in efficient expression of the CAT protein. The cDNAs of subgenomic DIs of the positive-strand RNA virus Sindbis virus have also been used to express foreign genes (14, 34).

The HIV-1–P1 fusion protein immunoprecipitated from cells transfected with RNA derived from the chimeric genome is the result of the translation of the polyprotein and subsequent autocatalytic processing by the 2A proteinase. The cleavage of the poliovirus polyprotein is very rapid and most likely occurs while the polyprotein is still on the ribosome (30, 35). This notion is consistent with the results of our pulse-chase experiments, demonstrating that at the earliest time examined, only the HIV-1–P1 fusion protein was observed rather than the entire poliovirus polyprotein. In addition, no further processing of the HIV-1–P1 fusion protein was detected. If processing of the HIV-1–P1 fusion protein had occurred, it would be at glutamine-glycine amino acids by the $3C^{pro}$ proteinase of poliovirus. However, previous studies have established that in addition to the glutamine-glycine amino acid pair, a specific three-dimensional structure is essential for the efficient processing of the P1 polyprotein (35). It is unlikely that the HIV-1–P1 fusion proteins adopt the correct three-dimensional structure necessary for efficient processing.

The analysis of the expression of the chimeric proteins revealed a difference in the intracellular stability of the chimeric proteins in transfected cells that generally followed

a *gag>pol>>env* pattern. Since the amino termini of the HIV-1–P1 fusion proteins have the entire coding region for VP4, the fusion proteins, like the poliovirus P1 protein, are probably located in the cytoplasm of transfected cells. In HIV-1-infected cells, both the *gag* and *pol* proteins are found in the cytoplasm, while the *env* protein is associated with the intracellular organelles involved in protein secretion (for review, see reference 17). In all probability, the HIV-1 envelope protein has many potential proteolytic cleavage sites which are generally not exposed to the cytoplasmic environment because of organelle compartmentalization and glycosylation. In support of this notion is the fact that the expression of several normally glycosylated proteins in an underglycosylated, defective form resulted in rapid intracellular degradation (18). Interestingly, earlier studies analyzing the protein expression of poliovirus proteins from the DI RNA genomes noted that the truncated P1 protein was rapidly degraded in infected cells (3). This suggests that fusion of the HIV-1 *gag* or *pol* protein to the truncated P1 protein might help to stabilize the fusion proteins. Experiments are under way to further examine this possibility.

The fact that the chimeric poliovirus genomes express HIV-1 proteins demonstrates the use of poliovirus as a vector for protein expression. Trono et al. described gene fusions between the poliovirus 5′ noncoding region and the bacterial gene encoding CAT and demonstrated that this protein was efficiently expressed in the transfected cells after infection with poliovirus (31). It is clear that expression of the chimeric HIV-1–poliovirus genome is also not affected by superinfection with poliovirus. An important question is whether the chimeric HIV-1–poliovirus genomes are encapsidated, like DI genomes, by the coinfecting wild-type poliovirus. Previous studies with defined poliovirus DI genomes have suggested the possibility of using poliovirus as a novel system by which to deliver antigen to the alimentary canal (8). We have analyzed virus passages derived from cells transfected with the HIV-1 chimeric genomes and superinfected with wild-type poliovirus and found no evidence for the encapsidation of the chimeric genomes. In fact, cotransfection of the chimeric genomes with wild-type poliovirus RNA inhibited plaque formation by wild-type poliovirus (1a, 9).

At present, it is not clear why the chimeric genomes are not encapsidated like DI genomes. The genome size of the chimeric RNA is consistent with that reported for DIs of poliovirus, approximately 87 to 96% of the wild-type genome, and the deletions for the RNAs (1174 to 2470) correspond to approximate deletions observed for naturally occurring DIs (12). It is possible that specific structures of the genome RNA might be required for efficient encapsidation. Indeed, a stem-loop structure, with a protruding adenine residue, has been postulated to be a signal for RNA binding to empty viral particles in other RNA viruses (1, 6). It is possible that the introduction of foreign genes into the poliovirus genome would disrupt this secondary RNA structure. Experiments are ongoing to determine whether a similar case might exist for poliovirus.

In summary, we have constructed chimeric HIV-1–poliovirus genomes and have demonstrated the replication and expression of appropriate HIV-1–P1 fusion proteins upon transfection of the genomic RNA into HeLa cells. These chimeric HIV-1–poliovirus genomes will be of interest in understanding different aspects of expression and replication of the poliovirus genome. Furthermore, the results of this study provide the basis for the future use of chimeric poliovirus genomes as novel expression vectors.

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