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A Poliovirus Minireplicon Containing an Inactive 2A Proteinase Is Expressed in Vaccinia Virus-Infected Cells

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It has been difficult to evaluate the role of individual viral proteins in poliovirus replication because a suitable complementation system has not yet been developed. To approach this problem, we constructed a chimeric human immunodeficiency virus type 2 (HIV-2)-*gag*-poliovirus minireplicon in which regions of the *gag* gene of HIV-2 were inserted in the poliovirus genome between nucleotides 1174 and 2470. Transfection of this chimeric RNA into HeLa cells results in the replication of the minireplicon and expression of an HIV-2-*gag*-P1 fusion protein which can be immunoprecipitated with antibodies to HIV-2-*gag*. Expression of the HIV-2-*gag*-P1 fusion protein was dependent on replication of the chimeric RNA genome. Although the chimeric HIV-2-*gag*-poliovirus RNA genome replicated in poliovirus-infected cells, transfection of the chimeric HIV-2-*gag*-poliovirus genome into vaccinia virus-infected cells resulted in increased replication as measured by analysis of chimeric RNA. The increase in replication correlated with an increase in the expression of the HIV-2-*gag*-P1 fusion protein in vaccinia virus-infected cells. To characterize this system, we constructed a mutation in the 2A gene to change a cysteine at amino acid 109 to a serine. Expression of the HIV-2-*gag*-P1 fusion protein was not detected when the HIV-2-*gag*-poliovirus genome containing the 2A mutation was transfected into HeLa cells, demonstrating the mutation was lethal for replication. When the chimeric genome was transfected into poliovirus-infected cells, no RNA replication or expression of the HIV-2-*gag*-P1 fusion protein was observed. In contrast, transfection of this genome into vaccinia virus-infected cells resulted in replication of the chimeric RNA and expression of two proteins with larger molecular masses than the HIV-2-*gag*-P1 proteins, possibly representing HIV-2-*gag*-P1-2A and HIV-2-*gag*-P1-2ABC fusion proteins. The transfection of the chimeric HIV-2-*gag*-poliovirus genome containing the 2A mutation into poliovirus-vaccinia virus coinfected cells resulted in the expression and partial processing of the two larger HIV-2-*gag*-P1 fusion proteins to give the correct molecular mass for the HIV-2-*gag*-P1 fusion protein. The 2A mutation was reconstructed back into the full-length infectious cDNA of poliovirus. Transfection of this cDNA into vaccinia virus-infected cells followed by immunoprecipitation with anticapsid antibodies demonstrated the presence of two proteins with molecular masses larger than P1, possibly P1-2A and P1-2ABC fusion proteins. The results of this study suggest the proteinase activity of $2A^{pro}$ is not required for RNA replication in vaccinia virus-infected cells and demonstrate the use of poliovirus minireplicons as a means to study poliovirus RNA replication.

The single-stranded RNA genome of poliovirus is approximately 7,500 nucleotides in length and functions both as an mRNA as well as a template RNA for virus replication (16). The primary translation product of the poliovirus genome is a single long polyprotein which is subsequently processed by virus-encoded proteases, $2A^{pro}$ and $3C^{pro}$ (16). The polyprotein encompassing the entire poliovirus genome is divided into structural (capsid) proteins, designated as P1, and the nonstructural proteins, designated as P2 and P3 (17, 33). The initial cleavage in the polyprotein between the P1 and P2 regions is catalyzed by $2A^{pro}$ in a cotranslational reaction (25, 29, 38), while the other subsequent proteolytic cleavages are catalyzed by $3C^{pro}$ or its precursor 3CD (14, 25, 41). In addition to the proteolytic cleavage activity, $2A^{pro}$ indirectly induces cleavage of the 220-kDa component of the eukaryotic translational initiation factor, eIF-4F, resulting in a shutoff of host cell protein synthesis (10, 18, 21, 38). Previous studies have identified single mutations in the 2A gene which result in inactivation of both catalytic activities of the $2A^{pro}$ (13, 42, 43).

In recent years, new approaches toward the study of poliovirus replication have been utilized which rely on manipulation of the infectious clone cDNA (32, 36). Important concepts regarding poliovirus replication have emerged from these studies. Based on studies which used molecular genetics to construct numerous mutations in the poliovirus infectious cDNA, it has been suggested that the P2 and P3 regions of the poliovirus genome probably encode *cis*- and *trans*-acting proteins for virus replication. Although the vast majority of these mutant genomes did not result in any infectious virus upon transfection, a few mutations resulted in viruses with a temperature-sensitive phenotype that were rescued at the nonpermissive temperature by coinfection with wild-type virus or with a second mutant virus (4, 7).

A drawback of those previous studies has been the necessity to generate an infectious virus for the complementation experiments. Thus, it has not been possible to effectively analyze poliovirus genomes containing lethal mutations for their capacity to be complemented by poliovirus replication proteins provided in *trans*. To approach this problem, we constructed a chimeric poliovirus genome in which the human immunodeficiency virus type 2 (HIV-2)-*gag* gene was substituted for the majority of the poliovirus P1 gene. Previous studies from this laboratory, as well as others, have demonstrated that deletions in the P1 capsid region still result in RNA with the capacity for replication when transfected into cells (8, 9, 12, 15). Transfection of the chimeric

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HIV-2-*gag*-poliovirus RNAs into HeLa cells or poliovirus-infected HeLa cells resulted in efficient expression of the HIV-2-*gag*-P1 fusion protein which was dependent on replication of the transfected RNA. Expression and replication of the chimeric genome were increased by transfection into cells previously infected with wild-type vaccinia virus. A chimeric genome with an inactive 2A proteinase did not replicate in uninfected or poliovirus-infected cells, but was replication competent when transfected into vaccinia virus-infected cells. The results of these studies are discussed with respect to the role of 2A in replication and the use of this system to study the *cis* and *trans* nature of poliovirus proteins in replication.

MATERIALS AND METHODS

Materials. All chemicals, unless otherwise noted, were purchased from Sigma Chemical Co. Restriction enzymes and DNA modification enzymes 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 Biotech Co. Tissue culture media and reagents were purchased from GIBCO-BRL. The T7 RNA polymerase was prepared in this laboratory by the method of Grodberg and Dunn (11). Synthetic DNA oligomers were synthesized at the UAB Cancer Center Oligonucleotide Core Facility.

Tissue culture cells and viruses. HeLa cells and BSC40 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum and 5% fetal calf serum (complete medium). The stock of the poliovirus type 1 Mahoney used in this study was derived from transfection of the infectious cDNA (36). The coxsackievirus B3 was obtained from American Type Culture Collection, while the encephalomyocarditis virus (EMCV) stock was obtained from A. Palmenberg (University of Wisconsin). The vaccinia virus (strain WR) was obtained from R. Compans, University of Alabama at Birmingham, and grown as previously described (1).

Construction of HIV-2-*gag*-poliovirus chimeric genome. All DNA manipulations were performed by standard procedures (22). A modified poliovirus cDNA was generated by digesting with *Nhe*I at the unique *Nhe*I restriction site at 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-*Nhe*I (8).

For construction of the chimeric HIV-2-*gag*-poliovirus genome, polymerase chain reaction was used to amplify *gag* gene sequences (34). For the polymerase chain reactions, approximately 30 ng of pJSP (19) and 300 ng of each primer were used in a final volume of 100 μl of 10 mM Tris-HCl–50 mM NaCl–1.5 mM $MgCl_2$–1 mM each deoxynucleoside triphosphate–2 U of AmpliTaq DNA polymerase. The oligonucleotide primers used for the amplification were as follows:

1. 5′ GGGAACGGAGTTAACTTCCCC 3′
2. 5′ TGGGTCTCTCGAGGGTGCTGT 3′

The oligonucleotides were designed to create unique *Hpa*I and *Xho*I restriction sites. 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) and then 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 were recovered from this procedure and used for ligation directly into pT7IC-*Nhe*I which had been previously digested with *Nru*I (nucleotide 1174 in the poliovirus genome) and *Xho*I. The ligation mixture was transformed into competent *Escherichia coli* DH5α; the resulting colonies were screened for the appropriate DNA inserts by restriction enzyme analysis. The final clone, designated as pT7IC-Nhe-*gag*, contained the HIV-2-*gag* gene positioned between poliovirus nucleotides 1174 and 2470. The plasmid was sequenced at the junctions between the poliovirus-P1 and HIV-2-*gag* genes to confirm that the translational reading frame was conserved (35).

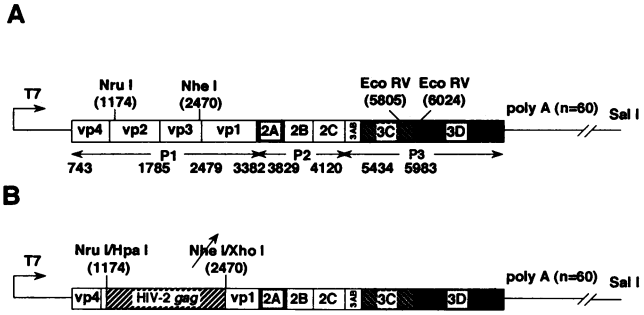

FIG. 1. Chimeric HIV-2-*gag*-poliovirus genome. (A) Wild-type poliovirus genome (type 1 Mahoney) is depicted with the viral proteins. The nucleotide sequence is taken from Kitamura et al. (16). The relevant restriction endonuclease sites are noted. Note that the figure is not drawn to scale. (B) Chimeric HIV-2-*gag*-poliovirus genome. The *gag* gene of HIV-2 was positioned in the poliovirus genome between nucleotides 1174 and 2470. Details of the construction can be found in Materials and Methods.

Construction of the $2A^{pro}$ mutation. A DNA fragment containing nucleotides 2470 to 5805 of the HIV-2-*gag*-poliovirus genome (*Xho*I to *Eco*RV; Fig. 1B), was subcloned into pUC119. The recombinant plasmid was transformed into *E. coli* CJ236, a *dut ung* mutant strain. Single-stranded DNA was prepared by previously described methods (40). For oligonucleotide site-directed mutagenesis, the following DNA oligomer was used: 5′ CAGGGGATAGCGGTGGC 3′ ($2A^{pro}$).

The mutated nucleotides (underlined) will change the codon for cysteine 109 of $2A^{pro}$ to a serine codon. Mutagenesis was performed as previously described (40). Potential mutants were screened by restriction enzyme analysis and DNA sequencing. DNA containing the mutant 2A gene was subcloned into pT7IC-Nhe-*gag* or pT7IC by exchanging the mutant for the wild-type *Bst*EII DNA fragment (nucleotides 3235 to 3925). The region of DNA in pT7IC-Nhe-*gag* or pT7IC containing the mutation was sequenced to confirm the presence of the desired mutation (35).

In vitro transcription reactions. Prior to in vitro transcription, the DNA templates were linearized with the restriction enzyme *Sal*I and then treated with 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 mixture 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. After 60 min, 1/20th of the in vitro-synthesized RNA was analyzed by agarose gel electrophoresis, and the remaining RNA was quantitated by UV absorption at 260/280 nm.

Transfection of in vitro-synthesized RNA. The in vitro-transcribed RNA was transfected into HeLa cells with DEAE-dextran (molecular weight, 500,000) as a facilitator as previously described (8) with the following modifications. Briefly, cells were washed twice with phosphate-buffered saline (PBS) and then incubated at 37°C with approximately 30 μg of the in vitro-synthesized RNA in PBS containing 300 μg of DEAE-dextran per ml. After 2 h, the cells were washed once with DMEM and then incubated in complete medium.

Picornavirus and vaccinia virus infections. In some instances, the cells were first infected with wild-type I poliovirus (20 PFU per cell) or vaccinia virus (WR strain) (5 PFU per cell) or coinfected with each virus 1 h prior to RNA transfection. The infected and transfected cell cultures were then incubated at 37°C for specified times prior to metabolic labeling.

Metabolic labeling of transfected and infected cells and immunoprecipitations. For metabolic labeling, 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 150 μCi/ml final concentration. The cells were washed once with PBS and lysed in radioimmunoprecipitation assay (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 HIV-2-*gag* protein was incubated with the extracts for 16 h at 4°C with constant rocking. The immune complexes were collected after 1 h of incubation with protein A-Sepharose (10 μl of a 1:1 [wt/vol] mixture in RIPA buffer) at room temperature with constant rocking. 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, 5% β-mercaptoethanol, 0.1% bromophenol blue). The proteins were separated on SDS-10% polyacrylamide gels, fluorographed, and exposed to X-ray film (Fuji-RX).

RNA isolation and nucleic acid hybridization. Total cellular RNA was prepared from cells by a guanidinium isothiocyanate procedure as previously described (8). At specific times posttransfection, cells were washed in cold PBS and lysed in solution of 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), and 0.5% Sarkosyl. A 0.1 volume of 2 M sodium acetate (pH 4.0) was then added, followed by sequential addition of water-saturated phenol and chloroform-isoamylalcohol (24:1). A 0.125 volume of 3 M sodium acetate (pH 5.0) and an equal volume of isopropanol were added to the extracted aqueous phase and placed at −20°C for 1 h. After centrifugation at 10,000 × *g* for 20 min, the pelleted RNA was dried and resuspended in diethyl pyrocarbonate-treated water (DEPC-H_2O). The RNA was reprecipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold ethanol and placed at −70°C for 1 h or overnight at −20°C. Following centrifugation at 10,000 × *g* for 20 min, the pelleted RNA was suspended in DEPC-H_2O.

The RNA (approximately 30 μg) in DEPC-H_2O was treated with gel loading buffer (50% formamide, 6.3% formaldehyde, 0.02 M 3-[*N*-morpholine]propanesulfonic acid [pH 7], 5 mM sodium acetate, 1 mM EDTA, 13% glycerol) at 65°C for 15 min, chilled on ice, and loaded on a 1% agarose–6.3% formaldehyde gel. After 2 h of electrophoresis, the nucleic acids were transferred to nitrocellulose by capillary blotting. Following transfer, the blot was air dried and baked for 2 h at 80°C in vacuo. The blot was then prehybridized for 2 h at 42°C in prehybridization solution consisting of 50% formamide, 5× Denhardt's solution, 5× SSC, 0.1% SDS, 50 μg of salmon sperm DNA per ml (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin; 1× SSC is 0.15 M NaCl plus 17 mM sodium acetate [pH 7.0]). The blot was then hybridized overnight at 50°C in fresh prehybridization solution plus 8% dextran sulfate to which 10^6 cpm of the RNA riboprobe specific for the plus strand of HIV-2-*gag* was added. To make this riboprobe, a DNA fragment from nucleotides 422 (*Kpn*I) to 2986 (*Pst*I) of HIV-2 ST proviral DNA (19) was subcloned into pTZ19U (U.S. Biochemical). The cloned DNA was linearized with *Sst*I in the polycloning site of pTZ19U prior to in vitro transcription. The procedure for generating radiolabeled riboprobes from in vitro transcription was as previously described (8). Following overnight incubation, the blot was washed once in 1× SSC–0.1% SDS for 2 min at room temperature, followed by 0.1× SSC–0.1% SDS for 90 min at 65°C. To reduce nonspecific background, we treated the blot with RNase A (1 μg/ml in 2× SSC) for 15 min at room temperature (22°C). The blot was rinsed for 30 min at 50°C with 0.1× SSC–0.1% SDS, air dried, and autoradiographed at −70°C.

RESULTS

Construction of chimeric HIV-2-*gag*–poliovirus genome. For these studies, a modification of the poliovirus cDNA was constructed to generate an *Xho*I site at nucleotide 2470 (an *Nhe*I site) to facilitate construction of the chimeric genome described in this report (Fig. 1A). The *gag* gene of HIV-2 was used for construction of the chimeric genomes because of availability of the complete nucleic acid sequence, necessary plasmids, and immunological reagents needed for detection of the HIV-2-*gag* gene product. A region of the HIV-2-*gag* gene, corresponding to nucleotides 935 to 1936 (19), was amplified by polymerase chain reaction with DNA primers that created unique *Hpa*I and *Xho*I restriction sites 5′ and 3′, respectively. The amplified DNA fragments were cloned into pT7IC-*Nhe*I previously digested with *Nru*I and *Xho*I. The resulting plasmid was named pT7IC-Nhe-*gag*. The DNA sequence at the 5′ and 3′ ends of the inserted *gag* gene (nucleotides 1174 and 2470) was determined to confirm the desired clone (Fig. 1B).

Expression of HIV-2-*gag*–P1 fusion protein in cells transfected with RNA derived from pT7IC-Nhe-*gag*. Previous reports from this laboratory have described expression of HIV-1-related proteins with poliovirus minireplicons (8). A similar strategy was used to analyze expression of the HIV-2-*gag*-related protein from transfection of chimeric RNA genomes derived from in vitro transcription of pT7IC-Nhe-*gag*. Expression of the HIV-2-*gag*–P1 fusion protein was detected in transfected cells after 4 h and at 6 h (Fig. 2A). At later time points, the expression actually decreased, possibly because of cell death (data not shown). To determine the effect of poliovirus infection on expression of HIV-2-*gag*–P1 protein, HeLa cells were first infected with poliovirus for 2 h, followed by transfection with RNA

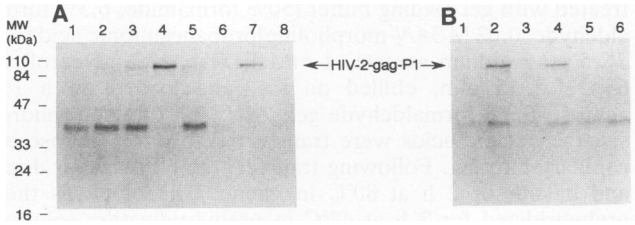

FIG. 2. Expression of HIV-2-*gag*-P1 fusion protein from transfected chimeric RNA genome. RNA derived from in vitro transcription of pT7IC-Nhe-*gag* was used to transfect HeLa cells or HeLa cells previously infected with poliovirus. After specified times, the cells were metabolically labeled, and expression of the HIV-2-*gag*-P1 fusion protein was analyzed by immunoprecipitation with anti-HIV-2-*gag* antibodies. (A) Kinetics of HIV-2-*gag*-P1 expression in HeLa cells and poliovirus-infected HeLa cells. The samples in lanes 1 to 4 are from transfected HeLa cells, whereas lanes 5 to 8 are from transfected HeLa cells infected with poliovirus 2 h prior to transfection. The order of the samples is as follows: lane 1, mock transfected; lanes 2 and 5, 1 h posttransfection; lanes 3 and 6, 4 h posttransfection; lanes 4 and 7, 6 h posttransfection; lane 8, poliovirus-infected HeLa cells metabolically labeled 6 h posttransfection. (B) Replication of the chimeric genome is required for expression of the HIV-2-*gag*-P1 fusion protein. To determine whether replication of the RNA was required for protein expression, a truncated chimeric RNA genome was generated by in vitro transcription from an *Eco*RV-linearized template (Fig. 1). The RNA was then transfected into cells, and expression of the chimeric HIV-2-*gag*-P1 fusion protein was determined by immunoprecipitation. The order of the samples is as follows: lane 1, mock transfected; lane 2, transfected with RNA from pT7IC-Nhe-*gag* (full length); lane 3, transfected with RNA from the same genome linearized with *Eco*RV; lane 4, transfected with RNA from pT7IC-Nhe-*gag* into poliovirus-infected HeLa cells; lane 5, transfected with *Eco*RV RNA from pT7IC-Nhe-*gag* into poliovirus-infected HeLa cells; lane 6, poliovirus-infected HeLa cells. MW, molecular weight.

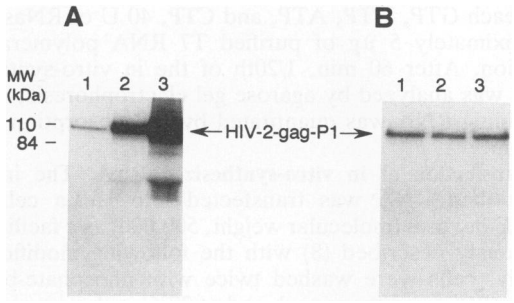

FIG. 3. Kinetics of expression of HIV-2-*gag*-P1 fusion protein in vaccinia virus-infected cells. (A) RNA derived from in vitro transcription of pT7IC-Nhe-*gag* was transfected into HeLa cells previously infected 2 h earlier with wild-type vaccinia virus. At specified times posttransfection, the cells were metabolically labeled, and expression of HIV-2-*gag*-P1 fusion protein was analyzed by immunoprecipitation. The order of the samples is as follows: lane 1, 1 h posttransfection; lane 2, 4 h posttransfection; lane 3, 6 h posttransfection. No immunoreactive material was detected from vaccinia virus-infected cells (see Fig. 4D). The molecular mass markers and HIV-2-*gag*-P1 fusion protein are noted. (B) To determine whether replication is required for expression, vaccinia virus-infected HeLa cells were transfected with RNA derived from pT7IC-Nhe-*gag* containing a deletion from nucleotides 5805 to 6024, and expression of the HIV-2-*gag*-P1 fusion protein was analyzed at the following times posttransfection: lane 1, 1 h posttransfection; lane 2, 4 h posttransfection; lane 3, 6 h posttransfection.

derived from in vitro transcription of pT7IC-Nhe-*gag*. Expression of the HIV-2-*gag*-P1 fusion protein was detected at 4 h posttransfection and reached a level comparable to that expressed from cells transfected with the chimeric RNA genome alone (Fig. 2A). The slight decrease in the levels of the expression of the HIV-2-*gag*-P1 fusion protein at the 6-h time point in poliovirus-infected cells in comparison with the 6-h time point in uninfected cells might be attributable to cell death as a result of poliovirus infection. Alternatively, it is also possible that the chimeric RNA genome and poliovirus genome compete for limiting host cell factors required for protein expression and genome replication.

We next determined whether replication of the chimeric HIV-2-*gag*-poliovirus RNA genome was required for expression of the HIV-2-*gag*-P1 protein. For these experiments, we tested full-length chimeric RNA genomes and genomes which had been linearized at the *Eco*RV restriction endonuclease site (nucleotide 5805) (Fig. 1). The RNAs were transfected into uninfected HeLa cells, or HeLa cells previously infected with poliovirus, and expression of the HIV-2-*gag*-P1 protein was detected by immunoprecipitation (Fig. 2B). No protein expression was detected upon transfection of the truncated RNA genomes into either uninfected cells or poliovirus-infected cells. A second protein with a molecular mass of approximately 40 kDa was detected by this anti-HIV-2-*gag* serum. This protein (possibly actin) was detected in normal cells and probably represents a nonspecific interaction with the antiserum or protein A-Sepharose beads.

Effect of vaccinia virus infection on replication and expression of HIV-2-*gag*-poliovirus chimeric genome. In preliminary experiments, we observed that expression of the HIV-2-*gag*-P1 protein was dramatically increased when the chimeric RNA genome was transfected into cells previously infected with vaccinia virus. No enhancement of protein expression was observed in cells which were infected with vaccinia virus after transfection (data not shown). To further characterize this result, we analyzed the kinetics of HIV-2-*gag*-P1 expression in the vaccinia virus-infected cells transfected with the chimeric RNA genome (Fig. 3A). A low level of expression of the HIV-2-*gag*-P1 fusion protein was detected at 1 h posttransfection. The expression increased substantially at later times posttransfection for up to 6 h. At later times, though, the levels of the HIV-2-*gag*-P1 fusion protein detected were actually lower, possibly because of cell death as a result of vaccinia virus infection (data not shown).

To further characterize this result, we determined whether replication of the chimeric HIV-2-*gag*-poliovirus genome was required for expression. For these studies, we constructed a chimeric HIV-2-*gag*-poliovirus genome containing a deletion from nucleotides 5805 to 6024 (ΔRV), which interrupts the coding region for $3C^{pro}$ and $3D^{pol}$ and thus prevents the replication of this chimeric genome (Fig. 1B). Transfection of this chimeric HIV-2-*gag*-poliovirus RNA genome into vaccinia virus-infected cells also resulted in expression of HIV-2-*gag*-P1 (Fig. 3B). In contrast to the wild-type genome, though, the expression of the HIV-2-*gag*-P1 fusion protein from the chimeric genome containing the deletion between nucleotides 5805 and 6024 (ΔRV) did not increase over the incubation time.

To further substantiate this result, we compared the replication of the chimeric RNA in uninfected, poliovirus-infected, and vaccinia virus-infected HeLa cells (Fig. 4). For these studies, we utilized a riboprobe specific for the plus

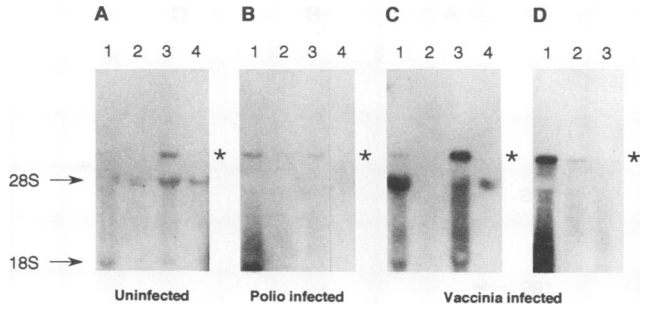

FIG. 4. Analysis of chimeric RNA from transfected cells. HeLa cells were transfected with in vitro-transcribed RNA, and total cellular RNA was extracted from cells at 0, 6, and 9 h posttransfection. RNA was separated in a formaldehyde-agarose gel and transferred onto a nitrocellulose membrane. The blot was probed with ^{32}P-labeled HIV-2-*gag*-specific minus-strand RNA transcript. (A) Transfection of HeLa cells with RNA derived from in vitro transcription of pT7IC-Nhe-*gag*. The order of the samples is as follows: lane 1, RNA from cells transfected and immediately processed; lane 2, RNA from cells 6 h posttransfection; lane 3, RNA from cells 9 h posttransfection; lane 4, RNA from mock-transfected cells. (B) Cells were infected with poliovirus prior to transfection with RNA derived from pT7IC-Nhe-*gag*. The order of the samples is as follows: lane 1, RNA from cells transfected and immediately processed; lane 2, RNA from cells 6 h posttransfection; lane 3, RNA from cells 9 h posttransfection; lane 4, RNA from poliovirus-infected cells at 9 h. (C) Cells were infected with vaccinia virus prior to transfection with RNA transcribed from pT7IC-Nhe-*gag*. The order of the samples is as follows: lane 1, RNA from cells transfected and immediately processed; lane 2, RNA from cells 6 h posttransfection; lane 3, RNA from cells 9 h posttransfection; lane 4, RNA from vaccinia virus-infected cells at 9 h. (D) Cells were infected with vaccinia virus and then transfected with RNA derived from pT7IC-Nhe-*gag*-ΔRV. The order of the samples is as follows: lane 1, RNA from cells transfected and immediately processed; lane 2, RNA from cells 6 h posttransfection; lane 3, RNA from cells 9 h posttransfection. The migration of 28S and 18S RNA was determined by ethidium bromide staining of the agarose gels and UV florescence prior to blotting. The amounts of 28S and 18S RNA in each lane were approximately equal prior to transfer; complete transfer was confirmed by restaining the gel after transfer. The chimeric RNA genome is denoted by an asterisk (*).

strand of the HIV-2-*gag* sequence in the chimeric RNA genome. At early times posttransfection, we detected low amounts of the chimeric RNA genome in uninfected, poliovirus-infected, and vaccinia virus-infected cells. The levels of chimeric RNA detected under the different conditions at this time posttransfection were similar and probably represent the initial transfected RNA. At later times posttransfection (6 h), we did not detect chimeric RNA in uninfected, poliovirus-infected, or vaccinia virus-infected cells. This result is consistent with previous studies which demonstrated that the majority of the transfected RNA was degraded (8). At 9 h posttransfection, we detected increases in the levels of the chimeric RNA genome in both uninfected and poliovirus-infected cells when compared with the 6-h time point. Increased levels of the chimeric RNA genome were also detected in vaccinia virus-infected cells at 9 h, reaching a final level greater than that in the uninfected or poliovirus-infected cells. Thus, the levels of chimeric RNA in vaccinia virus-infected cells versus the levels in uninfected or poliovirus-infected cells correlated with the increase in the expression of the HIV-2-*gag*-P1 fusion protein. The levels of chimeric RNA in vaccinia virus-infected cells transfected with chimeric RNA containing the ΔRV deletion also paralleled the expression of the HIV-2-*gag*-P1 fusion protein from the RNA. At 1 h posttransfection, we detected greater levels of the chimeric RNA containing the ΔRV deletion than the wild-type RNA; this result is consistent with the levels of HIV-2-*gag*-P1 fusion protein detected at this time posttransfection (Fig. 3B). Since the chimeric RNA containing the ΔRV deletion could not replicate, the levels of RNA did not increase over the incubation time; in fact, at 9 h posttransfection, the levels of the chimeric transfected RNA containing the ΔRV deletion were below the sensitivity for detection with a Northern (RNA) blot. This result is consistent with the fact that the expression of the HIV-2-*gag*-P1 fusion protein from the chimeric RNA genome containing the ΔRV deletion, in contrast to the wild-type chimeric genome, did not increase at later times posttransfection (Fig. 3B).

In summary, these results demonstrate the increased expression and replication of the chimeric RNA genomes in vaccinia virus-infected cells compared with that in uninfected or poliovirus-infected cells.

Complementation of poliovirus genome with a mutation in 2A^{pro}. In previous studies, a mutant 2A^{pro} in which a cysteine at amino acid 109 was changed to a serine was found to have little or no proteolytic activity and did not induce the degradation of p220 (13, 42, 43). A poliovirus cDNA containing this mutation was noninfectious (13). Since previous studies have demonstrated that replication of poliovirus genomes with certain mutations in 2A were complemented in *trans*, we tested whether a chimeric HIV-2-*gag*-poliovirus genome containing a cysteine-to-serine change at amino acid 109 in 2A^{pro} (designated as pT7IC-Nhe-*gag*-Cys 109-Ser 2A^{pro}) could be complemented by 2A^{pro} provided in *trans*. As might be expected, transfection of RNA derived from transcription of pT7IC-Nhe-*gag*-Cys 109-Ser 2A^{pro} into HeLa cells did not result in the expression of chimeric HIV-2-*gag*-P1 protein (Fig. 5A). We also did not detect any expression if this RNA was transfected into poliovirus-infected cells (Fig. 5B). Thus, 2A^{pro} provided by the wild-type poliovirus did not complement the defective chimeric HIV-2-*gag*-poliovirus genome. Surprisingly, though, the expression of two larger HIV-2-*gag*-P1 fusion proteins was evident upon transfection of this RNA into vaccinia virus-infected cells (Fig. 5C). On the basis of recent studies describing the poliovirus protein-processing cascade (20), we believe the larger HIV-2-*gag*-P1 proteins correspond to HIV-2-*gag*-P1-2A and HIV-2-*gag*-P1-2ABC fusion proteins. The expression of the two larger HIV-2-*gag*-P1 fusion proteins increased over the incubation time, suggesting that the HIV-2-*gag*-poliovirus genome containing the 2A^{pro} mutation replicated in vaccinia virus-infected cells (Fig. 5D). To confirm this, we performed a Northern blot of RNA extracted from uninfected, poliovirus-infected and vaccinia virus-infected cells transfected with RNA derived from transcription of pT7IC-Nhe-*gag*-Cys 109-Ser 2A^{pro} (Fig. 6). At 0, 6, and 9 h posttransfection, no chimeric RNA was detected in uninfected or poliovirus-infected cells, while a clear increase in chimeric RNA was apparent in vaccinia virus-infected cells. Taken together, these results demonstrate that vaccinia virus infection of cells provides the appropriate cellular environment for the expression and replication of the chimeric RNA genome containing the mutation in 2A^{pro}.

To further characterize the HIV-2-*gag*-P1 fusion protein expressed from the chimeric genome containing the mutation in 2A, we wanted to determine whether 2A^{pro} produced by poliovirus would act in *trans* and proteolytically process

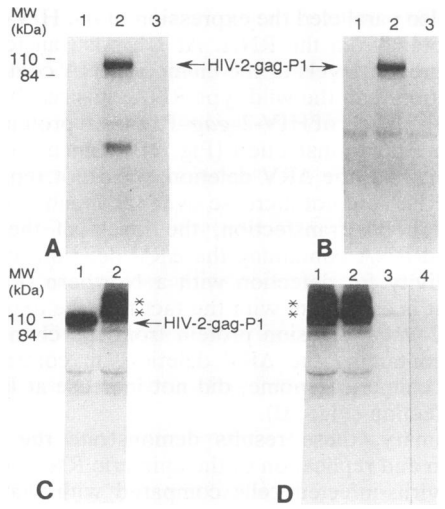

FIG. 5. Expression of HIV-2-*gag*–P1 from an HIV-2-*gag*–poliovirus minireplicon containing a mutation in $2A^{pro}$ in vaccinia virus-infected cells. A cysteine-to-serine change at amino acid 109 of $2A^{pro}$ was made by oligonucleotide site-directed mutagenesis. The mutation was subcloned back into the HIV-2-*gag*–poliovirus minireplicon and designated as pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$. Following in vitro transcription, RNA was transfected into HeLa cells, poliovirus-infected HeLa cells, or vaccinia virus-infected HeLa cells. Expression of HIV-2-*gag*–P1 was analyzed by immunoprecipitation with anti-HIV-2-*gag*–antibodies. (A) Expression in uninfected cells of HIV-2-*gag*–P1 from RNA derived from transcription of pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$. The order of the samples is as follows: lane 1, mock transfected; lane 2, cells transfected with RNA derived from transcription of pT7IC-Nhe-*gag* (wild-type genome); lane 3, cells transfected with RNA derived from transcription of pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$. (B) Expression of HIV-2-*gag*–P1 from transfected RNA transcribed from pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$ in poliovirus-infected cells. The order of the samples is the same as that for panel A. (C) Expression of HIV-2-*gag*–P1 fusion protein in vaccinia virus-infected HeLa cells. Infected cells were transfected with RNA derived from transcription of pT7IC-Nhe-*gag* (lane 1) or pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$ (lane 2). Four hours posttransfection, the cells were metabolically labeled, and expression of HIV-2-*gag*–P1 fusion protein was analyzed by immunoprecipitation. The migration of molecular mass markers, HIV-2-*gag*–P1, and two larger proteins (*) are noted. (D) Kinetics of expression of HIV-2-*gag*–P1 fusion protein in vaccinia virus-infected cells transfected with RNA derived from transcription of pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$. HeLa cells were infected with vaccinia virus for 2 h and then transfected with RNA derived from pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$; they were then metabolically labeled at the designated times. The order of the samples is as follows: lane 1, 6 h posttransfection; lane 2, 4 h posttransfection; lane 3, 1 h posttransfection; lane 4, mock-transfected vaccinia virus-infected HeLa cells at 6 h postinfection. Migrations of the larger HIV-2-*gag*–P1 precursor are noted (*).

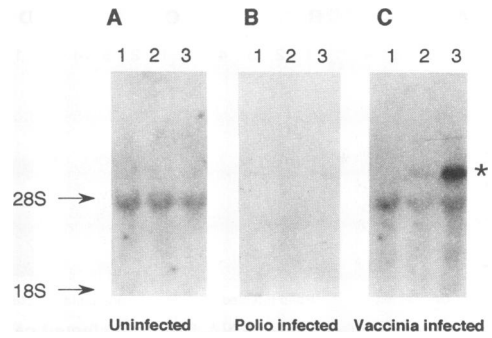

FIG. 6. Analysis of the replication of transfected RNA from in vitro-transcribed pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$. Total cellular RNA was extracted from cells at 0, 6, and 9 h posttransfection. RNA was fractionated in formaldehyde-agarose gel, blotted, and then analyzed with an HIV-*gag*-specific RNA probe. (A) Cells were transfected with RNA from in vitro-transcribed pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$. The order of the samples is as follows: lane 1, RNA from cells transfected and immediately processed; lane 2, RNA from cells 6 h posttransfection; lane 3, RNA from cells 9 h posttransfection. (B) Cells were first infected with poliovirus and then transfected with RNA derived from in vitro transcription of pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$. The order of the samples is as follows: lane 1, RNA from cells transfected and immediately processed; lane 2, RNA from cells 6 h posttransfection; lane 3, RNA from cells 9 h posttransfection. (C) Cells were first infected with vaccinia virus followed by transfection with RNA derived from in vitro transcription of pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$. The order of the samples is as follows: lane 1, RNA from cells transfected and immediately processed; lane 2, RNA from cells 6 h posttransfection; lane 3, RNA from cells 9 h posttransfection. The migration of 28S and 18S RNA was determined by ethidium bromide staining of the agarose gels and UV florescence prior to blotting. The amounts of 28S and 18S RNA in each lane were similar prior to transfer; complete transfer was confirmed by restaining the gel after transfer. The chimeric RNA genome is noted by an asterisk (*).

the fusion protein. To do this, we transfected the chimeric HIV-2-*gag*–poliovirus genome into cells previously coinfected with wild-type poliovirus and vaccinia virus (Fig. 7). As controls, we also transfected the chimeric HIV-2-*gag*–poliovirus genomes into vaccinia virus-EMCV (which does not encode a 2A protein [26, 29]) or vaccinia virus-coxsackievirus B3 coinfected cells to determine whether complementation would be poliovirus specific. Using anti-HIV-2-*gag* antibodies, we detected the two proteins larger than the HIV-2-*gag*–P1 fusion protein in all the picornavirus-vaccinia virus-coinfected cultures. However, we clearly detected an HIV-2-*gag*–P1 protein in the poliovirus-vaccinia virus-coinfected cells which had a molecular mass consistent with that for authentic HIV-2-*gag*–P1 fusion protein. Some of the processed HIV-2-*gag*–P1 fusion protein in the EMCV-vaccinia virus- and coxsackievirus B3-vaccinia virus-infected cells appeared to be processed, although the levels were much less than that from the poliovirus-vaccinia virus-infected cells. Whether this represents actual processing of the HIV-2-*gag*–P1–2A junction by the EMCV or coxsackievirus B3 proteases in this system is unknown and will require further studies. Taken together, though, these results demonstrate that the larger HIV-2-*gag*–P1 fusion proteins can undergo a limited proteolytic processing by $2A^{pro}$ provided in *trans* from wild-type poliovirus.

The expression of the chimeric HIV-2-*gag*–poliovirus genome encoding the $2A^{pro}$ mutation in vaccinia virus-infected cells suggested that full-length poliovirus genomes containing an identical mutation in the 2A gene might express P1 proteins in vaccinia virus-infected cells. To test this possibility, we reconstructed the 2A mutation into the infectious cDNA clone of poliovirus (designated as pT7IC-Cys 109-Ser $2A^{pro}$). In preliminary experiments, we confirmed that RNA derived from in vitro transcription of pT7IC-Cys 109-Ser $2A^{pro}$ was noninfectious both by plaque assay and by RNA replication (data not shown). The in vitro-transcribed RNA from pT7IC-Cys 109-Ser $2A^{pro}$ was next transfected into cells previously infected with wild-type vaccinia virus (Fig. 8). Using anti-poliovirus capsid antiserum, we detected completely processed poliovirus capsid

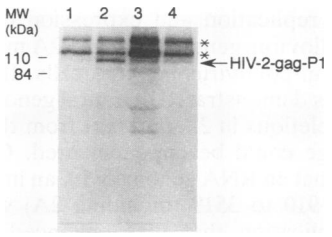

FIG. 7. Proteolytic processing of HIV-2-*gag*–P1 fusion protein by $2A^{pro}$ provided in *trans*. The chimeric HIV-2-*gag*–poliovirus genome containing the $2A^{pro}$ mutation was transfected into cells previously infected with vaccinia virus alone or coinfected with vaccinia virus and poliovirus, vaccinia virus and EMCV, or vaccinia virus and coxsackievirus B3. For this study, cells were infected with vaccinia virus (5 PFU per cell), and then after 1 h of infection, cells were superinfected with the designated picornavirus (20 PFU per cell). After 1 h of incubation, RNA derived from transcription of pT7IC-Nhe-*gag*-Cys 109-Ser $2A^{pro}$ was transfected into the cells. At 4 h posttransfection, the cells were metabolically labeled, and expression of HIV-2-*gag*–P1 fusion protein was analyzed by immunoprecipitation. The order of the samples is as follows: lane 1, vaccinia virus-infected cells; lane 2, vaccinia virus-poliovirus-infected cells; lane 3, vaccinia virus-EMCV-infected cells; lane 4, vaccinia virus-coxsackievirus B3-infected cells. Migrations of HIV-2-*gag*–P1 protein and the two larger precursor proteins (*) are noted. MW, molecular weight.

proteins VP0, VP3, and VP1 from vaccinia virus-infected cells which had been transfected with wild-type poliovirus genome. In contrast, we detected VP0, VP3, and VP1-2A from vaccinia virus-infected cells transfected with the wild-type genome containing the $2A^{pro}$ mutation. Two proteins with higher molecular mass (denoted by the asterisk) probably consisting of P1-2A or P1-2ABC fusions were also immunoprecipitated. To determine whether the defect in $2A^{pro}$ of the poliovirus genome could be complemented by $2A^{pro}$ provided in *trans*, we cotransfected the chimeric HIV-2-*gag*–poliovirus RNA genome and the wild-type genome containing the $2A^{pro}$ mutation into vaccinia virus-infected cells and then immunoprecipitated them with anti-capsid antibodies. We detected proteolytically processed

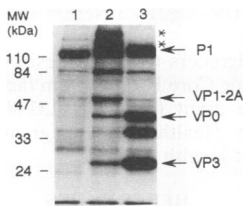

FIG. 8. Expression of poliovirus genomes containing a $2A^{pro}$ mutation. The mutation in the 2A gene was reconstituted back into the poliovirus infectious cDNA and designated as pT7IC-Cys 109-Ser $2A^{pro}$. Following in vitro transcription, the RNA was transfected into vaccinia virus-infected cells. The cells were metabolically labeled, and expression of poliovirus capsid proteins was determined by immunoprecipitation with antipoliovirus antibodies. The order of the lanes is as follows: lane 1, vaccinia virus-infected cells; lane 2, vaccinia virus-infected cells transfected with RNA derived from transcription of pT7IC-Cys 109-Ser $2A^{pro}$; lane 3, vaccinia virus-infected cells transfected with RNA derived from transcription of pT7IC (the wild-type poliovirus genome). The migrations of the poliovirus capsid precursor P1 and processed capsid proteins VP1-2A, VP0, and VP3 are noted. The larger P1-P2 precursor proteins resulting from the loss of $2A^{pro}$ activity are also noted (*). MW, molecular weight.

VP0, VP3, and a small amount of VP1 from the cotransfected cells, indicating that the $2A^{pro}$ provided from the chimeric genome had processed the VP1-2A from the wild-type genome containing the $2A^{pro}$ mutation (data not shown). To determine whether the cotransfection of the chimeric genome with the wild-type genome containing the $2A^{pro}$ mutation would result in infectious virus, we cotransfected cells, and after 24 h, any poliovirus in the supernatant was isolated by ultracentrifugation; the pelleted material was then used to reinfect cells in the presence of vaccinia virus. We metabolically labeled those cells after infection and analyzed the lysates for poliovirus capsid proteins by immunoprecipitation. We were unable to detect any poliovirus using this approach, possibly because of the low levels of complementation by $2A^{pro}$ provided in *trans* (data not shown).

DISCUSSION

In this report, we described the construction and characterization of poliovirus genomes in which the HIV-2-*gag* gene was substituted for nucleotides 1174 to 2470 of the P1 gene. Transfection of this chimeric HIV-2-*gag*–poliovirus genome into cells resulted in expression of an HIV-2-*gag*–P1 fusion protein which was immunoprecipitated by antibodies to HIV-2-*gag*. Expression of the HIV-2-*gag*–P1 fusion protein and replication of the chimeric RNA genome were not affected by preinfection of cells with poliovirus, but were increased in cells that had been previously infected with vaccinia virus. A chimeric HIV-2-*gag*–poliovirus minireplicon with a single amino acid change in the $2A^{pro}$ protein was incapable of replication in uninfected or poliovirus-infected cells but replicated efficiently in vaccinia virus-infected cells. Reconstruction of the 2A mutation into the infectious poliovirus cDNA resulted in a genome which expressed viral protein but was unable to produce infectious virus if cotransfected with a wild-type chimeric HIV-2-*gag*–poliovirus genome.

One of the important results of this study is that transfection of the minireplicons into vaccinia virus-infected cells provides a sensitive assay for the expression and subsequent replication competence of the RNA. Numerous studies have used the infectious cDNA clone of poliovirus to construct and characterize mutations in viral proteins (4, 5, 7, 13). The vast majority of these mutations, though, were lethal as evidenced by the lack of production of infectious virus upon transfection of the mutant genomes. A few mutations, however, resulted in infectious virus with a temperature-sensitive phenotype (4, 7). This experimental approach determined that replication of virus genomes with certain mutations in the P2 and P3 region proteins could be complemented at the nonpermissive temperature by viral proteins provided in *trans* by wild-type poliovirus. In most cases, complementation of the mutant viruses by wild-type poliovirus was measured at the nonpermissive temperature by plaque assay. While these studies provided useful information, this experimental approach generally suffers from a lack of sensitivity because the virus has to undergo multiple rounds of replication to visualize a plaque. This problem has been overcome to some degree by the recent studies of Charini et al. (7), who used a biochemical assay to assess complementation. The use of the chimeric HIV-2-*gag*–poliovirus genome presented in this study provides an additional, novel approach to this problem.

A surprising result of this study was the increased expression of the HIV-2-*gag*–poliovirus genome when transfected

into vaccinia virus-infected cells. We believe this might be due, in part, to a more efficient translation of the HIV-2–*gag*–poliovirus genome in vaccinia virus-infected cells. This idea is based on the result that expression of the HIV-2–*gag*–P1 fusion protein was detected from the transfection of HIV-2–*gag*–poliovirus genomes containing the ΔRV deletion into vaccinia virus-infected cells; the ΔRV deletion results in a genome which does not encode a $3C^{pro}$ or a $3D^{pol}$. No expression of HIV-2–*gag*–P1 protein was detected when the chimeric RNA genome containing the ΔRV deletion was transfected into uninfected or poliovirus-infected cells. This result demonstrated that prior infection of cells with vaccinia virus provided a suitable environment for the translation of the chimeric HIV-2–*gag*–poliovirus genome. Although the mechanism of the enhanced expression of the chimeric HIV-2–*gag*–poliovirus genome is unknown, previous studies have established that vaccinia virus infection results in an inhibition of host cell protein synthesis (24). Specifically, translation of some of the vaccinia virus mRNAs does not absolutely require a cap structure (2). Previous studies have established that poliovirus translation does not require a cap structure and uses an internal ribosome entry site located within the 5′ nontranslated region of the viral genome (30, 31, 37, 39). We do not believe, though, that increased translation due to the presence of the internal ribosome entry site is the sole reason for enhanced expression in vaccinia virus-infected cells. Previous studies have demonstrated that the interferon-induced double-stranded RNA-activated protein kinase (P68) is highly activated in poliovirus-infected cells (6, 27). The natural substrate for this kinase is the translation factor eIF-2α. It is likely that the double-stranded RNAs generated as a result of replication of the chimeric RNA genomes stimulate the activity of the p68 kinase which, in turn, would lead to a reduction in the expression of proteins from the chimeric RNA genomes. Recent studies have demonstrated that vaccinia virus encodes an 88-amino-acid protein which has sequence homology to eIF-2α and has been proposed to interact with the P68 kinase to prevent inactivation of the cellular eIF-2α (3). It is possible that the increased expression of the chimeric genomes in vaccinia virus-infected cells is also a result of the inhibition of the P68 kinase. In support of this, we observed decreased expression of the chimeric genomes in cells preinfected with a vaccinia virus recombinant containing a deletion in the K3L open reading frame, which encodes the eIF-2α homolog (28). However, it is important to note that previous studies have suggested a role for the P68 kinase in poliovirus replication, possibly as a host factor required for replication of the viral genome (23). It is possible then, that the effect of vaccinia virus infection on the activity of the P68 kinase also, in an as yet unknown way, modulates the activity of the kinase in viral replication. Future studies will be needed to resolve this question.

To further characterize expression of the minireplicons in vaccinia virus-infected cells, we constructed a mutation in the 2A gene based on previous studies which demonstrated that substitution of cysteine 109 with serine destroyed the majority, if not all, of the proteolytic activity of $2A^{pro}$ polyprotein self-processing; the mutation completely destroyed the capacity of $2A^{pro}$ for activation of the p220-specific protease (13, 42, 43). Previous studies have also demonstrated that poliovirus genomes containing this mutation were noninfectious (13). Consistent with these results, we did not detect replication or expression of the HIV-2–*gag*–P1 fusion protein in HeLa cells transfected with the chimeric HIV-2–*gag*–poliovirus genomes containing the 2A mutation. The replication and expression of the chimeric HIV-2–*gag*–poliovirus genome with the 2A mutation was not complemented in poliovirus-infected cells. In contrast, two previous studies demonstrated that viral genomes containing mutations or deletions in 2A different from the cysteine 109 to serine change could be complemented. Collis et al. (9) demonstrated that an RNA genome with an in-frame deletion of nucleotides 910 to 3519 (including 2A) was capable of limited self-replication that was enhanced in poliovirus-infected cells. In an earlier study, Bernstein et al. (4) demonstrated that transfection of the poliovirus cDNA containing linker insertion mutations in the 2A gene resulted in a temperature-sensitive virus which could be complemented at the nonpermissive temperature by coinfection with another mutant virus. The reason for the difference between the previous studies by Collis et al. (9) and Bernstein et al. (4) and this study is unknown. One difference between the studies is that the mutant genomes described by Collis et al. (9) and Bernstein et al. (4) had the capacity for self-replication, whereas the viral genome containing the single amino acid mutation in 2A did not replicate. It is possible that early events in poliovirus replication may require *cis*-acting functions of 2A. A *trans* function for the proteolytic activity of 2A was demonstrated in our system. The transfection of the HIV-2–*gag*–poliovirus genome containing the $2A^{pro}$ mutation into cells coinfected with poliovirus and vaccinia virus provided the environment for processing of the HIV-2–*gag*–P1–2A or HIV-2–*gag*–P1–2ABC proteins by $2A^{pro}$ provided in *trans*.

Finally, both the HIV-2–*gag*–poliovirus and the poliovirus genome containing the 2A mutation expressed viral proteins and replicated when transfected into vaccinia virus-infected cells, even though the proteolytic activity of $2A^{pro}$ was severely reduced or lost. These results suggest that the proteinase activity of $2A^{pro}$ is probably not essential for RNA replication or that vaccinia virus provides some factor (as yet undefined) to allow poliovirus replication in the absence of $2A^{pro}$ function. Further studies will be required to identify this factor provided by vaccinia virus.

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