JOURNAL OF VIROLOGY, Mar. 1996, p. 1941–1952 Vol. 70, No. 3
0022-538X/96/$04.00+0

Capsid Coding Sequence Is Required for Efficient Replication of Human Rhinovirus 14 RNA

KEVIN L. McKNIGHT[1] AND STANLEY M. LEMON[1,2]*

Departments of Microbiology and Immunology[1] and Medicine,[2] The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7030

Received 24 August 1995/Accepted 1 December 1995

Mechanisms by which the plus-sense RNA genomes of picornaviruses are replicated remain poorly defined, but existing models do not suggest a role for sequences encoding the capsid proteins. However, candidate RNA replicons (ΔP1βgal and ΔP1Luc), representing the sequence of human rhinovirus 14 virus (HRV-14) with reporter protein sequences (β-galactosidase or luciferase, respectively) replacing most of the P1 capsid-coding region, failed to replicate in transfected H1-HeLa cells despite efficient primary cleavage of the polyprotein. To determine which P1 sequences might be required for RNA replication, HRV-14 mutants in which segments of the P1 region were removed in frame from the genome were constructed. Mutants with deletions involving the 5′-proximal 1,489 nucleotides of the P1 region replicated efficiently, while those with deletions involving the 3′ 1,079 nucleotides did not. Reintroduction of the 3′ P1 sequence into the nonreplicating ΔP1Luc construct resulted in a new candidate replicon, ΔP1Luc/VP3, which replicated well and expressed luciferase efficiently. Capsid proteins provided in *trans* by helper virus failed to rescue the nonreplicating ΔP1Luc genome but were able to package the larger-than-genome-length ΔP1Luc/VP3 replicon. Thus, a 3′-distal P1 capsid-coding sequence has a previously unrecognized *cis*-active function related to replication of HRV-14 RNA.

The picornaviruses comprise a large and diverse group of positive-strand RNA viruses which have a common genetic organization and replication strategy and which are pathogenic in a variety of animal species. In each of these viruses, the dominant feature of the organization of the genome is the presence of a single large open reading frame (ORF), which encodes a multifunctional viral polyprotein. The viral capsid proteins are encoded by a segment of RNA (P1 segment) located at or near the 5′ end of the ORF, upstream of the P2 and P3 segments, which encodes a variety of nonstructural proteins involved in replication of the virus (46). In two closely related picornavirus genera which are important causes of disease in humans, enteroviruses (which include the polioviruses) and rhinoviruses, the P1 segment is located at the extreme 5′ end of the ORF, and primary cleavage of the viral polyprotein occurs at the carboxyl terminus of the expressed P1 protein. This structural protein intermediate is subsequently further proteolytically processed into several capsid precursor proteins: VP0 (representing VP4 plus VP2), VP3, and VP1 (see Fig. 1). While nontranslated RNA sequences located upstream and downstream of the ORF contain *cis*-active elements which are critical for replication, and while translation of the P2/P3 coding region appears to be *cis* active with respect to viral replication (50), the available evidence suggests that the P1 capsid-coding sequence is not required for viral RNA amplification.

The absence of a role for P1 sequences in the replication of poliovirus is supported by the natural occurrence of defective interfering particles with large deletions in this region of the genome (11, 12, 26). In addition, several studies demonstrate that artificially constructed poliovirus genomes with deletions in the P1 region retain all the genetic information necessary for RNA replication (13, 20, 24). Other, more recent studies indicate that poliovirus P1 sequences can be replaced by RNA encoding heterologous proteins without compromising viral RNA replication (3, 9, 36). Such subgenomic P1-deleted poliovirus genomes have been referred to as replicons or mini-replicons, and those expressing heterologous proteins may be valuable for the study of various aspects of virus multiplication or as general-purpose gene expression vectors (3, 9, 36, 40). Among the poliovirus replicons which have been described, there are several examples of replicating RNAs which contain very large deletions in the P1 region. Some of these deletions are much larger than those present in naturally occurring defective interfering poliovirus particles (23, 26, 41), which usually retain VP4 and VP1 coding sequences near the 5′ and 3′ limits of the P1 segment (10, 20, 26). Despite this, these artificial replicons still retain efficient RNA replication functions (3, 13, 24, 41).

The successful development of poliovirus replicons expressing heterologous proteins led us to consider the possibility that encapsidated rhinovirus replicons have unique potential for the delivery and expression of foreign genes in human airway cells. The rhinoviruses include approximately 100 serologically distinct pathogens which primarily infect human epithelial airway cells and which are important causative agents of the common cold (15, 19). The RNA genomes and nonstructural proteins of these viruses share striking similarities with those of poliovirus, as does the structure of the virus capsid (8, 35, 45). However, among several rhinovirus serotypes, some important differences do exist, including the shorter length of the rhinovirus 5′ nontranslated RNA (5′NTR) (38), sequence requirements for substrate recognition by the rhinovirus proteases (8, 49), and utilization of specific cellular receptors (14, 22).

To our knowledge, there are no prior reports describing the construction of rhinovirus replicons or even descriptions of naturally occurring P1-deleted defective interfering human rhinovirus (HRV) genomes. However, several infectious HRV cDNA clones have been constructed and are available for such studies (17, 28, 32). Here, we describe the successful construction of a subgenomic HRV-14 replicon, ΔP1Luc/VP3, which expresses high levels of a foreign reporter protein, firefly lu-

* Corresponding author. Phone: (919) 966-2536. Fax: (919) 966-6714.

ciferase, in cultured cells. The design of this construct involved the direct fusion of the luciferase coding sequence to the internal ribosomal entry site present in the 5′NTR of HRV-14, resulting in the removal of 1.5 kb of P1 sequence. However, during the course of these experiments, we found that replication of HRV-14 RNA was dependent on the retention of sequence near the 3′ end of the P1 segment (VP3-VP1 coding region). This finding demonstrates an important, previously unrecognized difference in the replication strategies of enteroviruses and rhinoviruses and suggests a novel role for P1 RNA sequences in the replication of rhinoviruses.

MATERIALS AND METHODS

Cells. H1-HeLa cells were obtained from the American Type Culture Collection and maintained in 1× minimal essential medium (MEM) supplemented with Earle's salts (Gibco/BRL, Life Technologies Inc., Grand Island, N.Y.), L-glutamine, and 10% fetal calf serum.

Virus. HRV-14 was recovered following transfection of H1-HeLa cells with RNA transcribed from the infectious cDNA clone, pWR3.26, a generous gift from Roland Rueckert, University of Wisconsin at Madison (28). For preparation of virus stocks, monolayers of H1-HeLa cells in a 100-mm-diameter dish were infected with approximately 10^7 PFU of virus. After removal of the inocula, 5 ml of complete medium was added and the monolayer was incubated at 34°C for 24 h, at which time virus was harvested from cytoplasmic extracts as previously described (28, 48). The titer of the resulting virus stock was $>10^8$ PFU/ml. For plaque assay of HRV-14, H1-HeLa cell monolayers in 60-mm dishes were inoculated with 200 μl of virus diluted in 1× phosphate-buffered saline (PBS) with 0.1% bovine serum albumin and overlaid 30 min later with MEM containing 5% fetal calf serum 1× tryptose-phosphate (Gibco/BRL), and 0.9% agarose. After incubation at 34°C for 48 to 72 h, the cell monolayers were stained with crystal violet or neutral red.

Antibodies. A polyclonal rabbit antiserum to HRV-14 was a kind gift from Anne Mosser, University of Wisconsin at Madison. In a microtiter neutralization assay, a 1:500 dilution of this antiserum protected 5×10^4 HeLa cells against infection with 5×10^4 PFU of HRV-14. Polyclonal rabbit antibodies to β-galactosidase (5Prime-3Prime, Inc., Boulder, Colo.) and luciferase (East Acres Biologicals, Southbridge, Mass.) were obtained from commercial sources.

Construction of HRV-14 replicons and P1 segment deletion mutants. Candidate replicons were constructed from the HRV-14 infectious cDNA, pWR3.26 (28), as follows. Nucleotide positions are based on the HRV-14 sequence provided by Lee et al. (28). To facilitate replicon construction, a shuttle vector (pSPHRV5) was prepared by excising the small *Sal*I-*Stu*I fragment (which contains nucleotides [nt] 1 to 851 of HRV-14) from pWR3.26 and inserting this fragment into the *Sal*I and *Pvu*II sites in pSP64 (Promega Corp., Madison, Wis.). The bacterial β-galactosidase and firefly luciferase coding sequences were amplified by PCR from the plasmids pSVβgal and pGem-luc (Promega), respectively, with PCR primers with sequences containing restriction endonuclease sites permitting in-frame fusions with the HRV-14 genome. The PCR primers for β-galactosidase were 5′-GC*TCTAGAtgatcat*gGTCGTTTTACACGTCG-3′ and 5′-CCG*CCCGGGcctagg*tcctaaaccataggatttaatgtcaccttTTGACACCAGACCAA-3′ (where the lowercase bases represent HRV-14 sequences and the italic bases represent unique restriction sites), while the PCR primers for amplification of the luciferase sequence were 5′-GCCCGATCCAA*tgatca*tgGAAGACGCC-3′ and 5′-CAGTTACATTTTA*cctagg*tcctaaaccataggatttaatgtcaccttTCAATTTGGACTTTCCG-3′. PCR-amplified products containing β-galactosidase sequences were digested with *Xba*I and *Sma*I and cloned into unique sites in the vector pSP64 to create the shuttle vector pSPβ-gal. The complete β-galactosidase coding sequence was excised from this plasmid by digestion with *Bcl*I (partial) and *Sma*I and cloned into unique sites of pSPHRV5, creating pSPHRVβ-gal which contains the β-galactosidase coding sequence fused to the HRV-14 internal ribosome entry site. PCR-amplified products containing luciferase were digested with *Bcl*I and *Avr*II and similarly cloned into the unique sites pSPHRVβ-gal, replacing the β-galactosidase coding sequence with the luciferase sequences and creating the plasmid pSPHRVLuc. Large segments within the PCR-amplified reporter gene sequences were subsequently replaced with plasmid DNA (non-PCR amplified) sequences by using unique sites near the ends of each amplified sequence (*Cla*I to *Nde*I for β-galactosidase, and *Xba*I to *Cla*I for luciferase). Any remaining PCR-amplified sequences were confirmed by direct sequencing of plasmid DNA. Final construction of full-length candidate replicons was accomplished by removing the small *Sal*I-*Avr*II fragments from each of the resulting shuttle plasmids. The *Sal*I-*Avr*II β-galactosidase fragment was inserted at unique *Sal*I and *Avr*II sites within pWR3.26 to create pΔP1βgal. The *Sal*I-*Avr*II luciferase fragment was inserted into pΔP1βgal, replacing the 5′NTR–β-galactosidase sequence with 5′NTR-luciferase sequences to create pΔP1Luc. In both of the final constructs, the amino terminus of the reporter sequence was fused in frame to the authentic HRV-14 initiator codon while the carboxyl terminus of the reporter sequence was fused in frame to the carboxyl terminal 7 amino acid residues of VP1, 30 nt upstream of the unique *Avr*II site at nt 3206 of the $2A^{pro}$ coding sequence (see Fig. 1).

Variants of these candidate replicons containing a lethal mutation in the $3D^{pol}$ coding sequence were constructed by linearizing the pWR3.26 plasmid with *Sph*I, removing the resulting 3′ protruding ends with T4 DNA polymerase (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.), and religating the blunt-ended molecules. This created plasmid pWR3.26(pol$^-$), which contains a frameshift in the HRV-14 polyprotein such that a stop codon terminates translation 26 amino acids upstream of the conserved YGDD motif in $3D^{pol}$. The small *Sal*I-*Avr*II fragments containing the reporter gene sequences described above were cloned into pWR3.26(pol$^-$) to create ΔP1βgal(pol$^-$) and ΔP1Luc(pol$^-$).

A third candidate replicon, pΔP1Luc/VP3, which contains sequences coding for part of VP3 and all of VP1, was constructed from pΔP1Luc as follows. A PCR product representing the 3′ end of the luciferase coding sequence was amplified with the primers 5′-GGATACCGGGAAAACGCTGGG-3′ and 5′-CCCACCG TA*CCTAGGccatgg*ACCTTGAAACAAAGCTCCTCCTCCCAATTTGGACTT TCCG-3′. In this case, the downstream PCR primer resulted in a fusion of the luciferase coding sequence with a downstream synthetic $3CD^{pro}$ cleavage site, followed by an *Nco*I site allowing an in-frame fusion within the VP3 sequence of HRV-14. The PCR product was digested with *Ppu*MI (a site 471 nt upstream of the 3′ end of the luciferase coding region) and *Avr*II and inserted into pSPHRV-Luc to create pSPHRVLuc/VP3. Next, the *Sal*I-*Nco*I fragment was excised from this plasmid and cloned into unique sites in pSPcap (see below). Finally, the small *Cla*I-*Avr*II fragment of the resulting clone was inserted into the pΔP1Luc replicon to create pΔP1Luc/VP3 (see Fig. 7A).

The construction of in-frame deletions within the capsid-coding P1 sequence of pWR3.26 was facilitated by subcloning the entire P1 region (*Sal*I-*Avr*II fragment) in the vector pSP64 to create pSPcap. This plasmid was digested by several different enzymes to create a series of deletion mutants (see Fig. 4A). When necessary, 3′ ends were filled in by treatment with Klenow enzyme, prior to gel purification and religation. The modified capsid sequences were excised from the pSPcap derivatives by digestion with *Sal*I and *Avr*II and reinserted into the pWR3.26 vector. To retain the authentic AUG start codon in the pCapd4 construct, the linker 5′-GATC**ATG**AAGAACCAGTTAAAGATC-3′ was inserted between the *Bcl*I and *Bgl*II sites. The initiator AUG codon (boldface type) flanks the first nucleotide of the Thr-54 codon at nt 788 of the HRV-14 genome.

Plasmid DNA manipulations were done by standard procedures (47). DNA for use in ligations was purified by the GeneClean II procedure (Bio101, Inc., Vista, Calif.). Each construct was sequenced across regions where replacements took place to confirm the validity of the construction. The deletion constructs were sequenced in the region of the deletion to confirm preservation of the reading frame. DNA sequencing was done by the USB Δ*Taq* cycle sequencing system (United States Biochemical Corp., Cleveland, Ohio).

In vitro transcription. For the production of virus from infectious transcripts, pWR3.26 plasmid DNA was linearized with *Mlu*I, which cleaves immediately 3′ of the poly(A) tail of the virus genome (28). For the replicon constructs, DNA templates were linearized with *Sma*I, as the β-galactosidase gene contains numerous *Mlu*I sites. For experiments comparing replication potential of transcripts, other templates were also linearized with *Sma*I to maintain consistency. *Sma*I cleaves the DNA templates 9 nt downstream of the poly(A) sequence. The infectivity of RNA transcripts derived from pWR3.26 linearized with *Sma*I was approximately twofold lower than that of transcripts derived from *Mlu*I-linearized templates (31). RNA transcripts were synthesized at 37°C for 1 h in 100-μl reaction mixtures containing 1 to 2 μg of linearized template DNA, 40 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine-HCl, 100 μg of nuclease-free bovine serum albumin per ml, 1.5 mM each nucleoside triphosphate, 5 μl of RNasin (Promega), and 200 to 500 U of T7 RNA polymerase (Promega or New England Biolabs Inc., Beverly, Mass.). To remove template DNA prior to transfection, 5 U of RQ1-DNase (Promega) was added to each reaction mixture and the mixtures were incubated at 37°C for an additional 30 min. RNA products were quantified by determining the amount of [α-^{32}P]CTP incorporated into acid-insoluble precipitates. Alternatively, the RNA content was determined by spectrophotometric analysis. Aliquots of transcription reaction products were examined by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis in comparison with RNA standards to confirm transcript integrity. These reaction conditions consistently produced 1 to 3 μg of RNA per μl.

RNA transfections. H1-HeLa cells were transfected by electroporation as described by Liljestrom et al. (29). Optimal transfection of infectious RNA transcripts was obtained at a setting of 980 V and 25 μF with a gene pulser (Bio-Rad, Richmond, Calif.) with the pulse controller unit set at maximum resistance. Cells (0.5 ml at a concentration of 10^7 cells per ml) were transfected with 10 to 20 μg of transcript RNA and subsequently plated into two 60-mm dishes. Specific infectivities derived from monolayers transfected with pWR3.26 transcripts were consistently above 10^7 PFU/μg of RNA, with <10% of the cells being killed by the transfection procedure alone. RNA transfection of a reporter RNA transcript containing the 5′NTR of HRV-14 fused to the β-galactosidase coding sequence, followed by in situ staining for β-galactosidase activity (Promega), indicated that approximately 60 to 70% of cells were successfully transfected (31).

Analysis of β-galactosidase and luciferase enzyme activity. Transfected cells were processed for enzyme assays by the addition of 250 μl of lysis buffer

(luciferase assay kit; Promega) per 60-mm dish. The protein content of the cell lysates was determined by the Bradford method (protein assay; Bio-Rad), and lysates were stored at −70°C until used in enzymatic analysis. For the β-galactosidase assay, lysate containing 50 μg of protein was brought to 100 μl with lysis buffer and then 100 μl of 2× assay buffer (200 mM sodium phosphate buffer [pH 7.3], 100 mM β-mercaptoethanol, 20 mM $MgCl_2$, 16.0 mM chlorophenol red β-D-galactopyranoside substrate [Boehringer-Mannheim Biochemicals]) was added. Reaction mixtures were incubated at 37°C for 30 min, and 300 μl of 1 M sodium carbonate was added. Then 200 μl of each reaction mixture was transferred to a 96-well plate, and the A_{570} was read in a model EL309 microtiter plate reader (BioTek Instruments Inc., Winooski, Vt.). For the luciferase assay, 5 μl of lysate (or lysate diluted 1:10 with lysis buffer) was mixed with 100 μl of luciferase substrate mixture (Promega). The samples were immediately monitored for light output by using a BioOrbit model 1250 luminometer (LKB-Wallac, Turku, Finland). Luciferase activities were normalized to the protein content of individual cell lysates, while the β-galactosidase activities are reported as optical density units at 570 nm per 50 μg of lysate protein.

RPA. The RNase protection assay (RPA) was carried out with reagents and protocols supplied with the lysate RNase protection kit (United States Biochemicals). Transfected-cell monolayers in 60-mm dishes were lysed by addition of 100 μl of a solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate, and 0.5% sarcosyl. Lysates were stored at −70°C until used for hybridization. Riboprobes were prepared from a plasmid (pSPBP) containing HRV-14 genome sequences from nt 5180 to 5446 inserted at the *Pvu*II and *Xba*I sites in pSP64 (Promega). A bacteriophage SP6 promoter is situated 27 nt upstream of the insert, resulting in production of a 290-nt runoff transcript following linearization of the DNA with *Bst*EII (nt 5183). A 263-nt stretch of this transcript represents HRV-14 minus-strand RNA sequence which is protected from RNase digestion following hybridization to plus strand-virus RNA. A control probe included in hybridization reaction mixtures contained 100 nt of antisense human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequence. A plasmid containing this sequence under the control of the bacteriophage promoter T7 produces a 139-nt unprotected runoff transcript (United States Biochemicals). For riboprobe synthesis, linearized template DNA (200 ng) was mixed with 2 μl of 10× transcription buffer (400 mM Tris-HCl [pH 7.6], 60 mM $MgCl_2$, 50 mM dithiothreitol, 20 mM spermidine-HCl), 1 μl each of ATP, GTP, and UTP at 10 mM, 1 μl of CTP at 0.5 μM, 5 μl of [α-^{32}P]CTP (50 μM, 800 Ci/mmol; Dupont NEN, Wilmington, Del.), 1 μl of RNasin (Promega), 2 μl (40 U) of RNA polymerase, and diethylpyrocarbonate-treated H_2O to 20 μl. The reaction mixtures were incubated for 1 h at 37°C for T7 polymerase (Promega or New England Biolabs) or 40°C for SP6 polymerase (Promega). Two units of RQ1-DNase (Promega) was added, and the reaction mixtures were incubated at 37°C for an additional 30 min. The reaction mixtures were brought to 100 μl with diethylpyrocarbonate-treated H_2O and purified over a Sephadex G-50 RNA spin column (Boehringer-Mannheim Biochemicals). A 10^6-cpm portion of each probe was added to 45 μl of lysate, and hybridization was carried out directly in lysate solutions at 37°C for 18 h (overnight) as described by the manufacturer (United States Biochemicals). The hybridization mixture was subsequently diluted 10-fold and digested with RNase and proteinase, and protected RNA fragments were separated by electrophoresis in 6% polyacrylamide–6 M urea gels and visualized by autoradiography.

Metabolic labeling and immunoprecipitation of reporter proteins. After RNA (or mock) transfection, cells were plated in complete medium and incubated at 34°C for 1 h. The monolayers were washed five times with PBS and fed with 2 ml of MEM containing 2.5 μg of actinomycin D per ml–5% dialyzed fetal calf serum. Following incubation at 34°C for 2 h, the monolayers were washed five times with methionine-free MEM and refed with 1 ml of methionine-free MEM containing actinomycin D and fetal calf serum, as above, and 100 μCi of [^{35}S]methionine (Dupont NEN). Following incubation for 5 h at 34°C, the cultures were washed five times with PBS and incubated at room temperature in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) for 15 min. The lysate was centrifuged for 2 min in a microcentrifuge, and the supernatant was collected for immunoprecipitations. Immunoprecipitations with rabbit polyclonal anti-β-galactosidase or anti-luciferase antibodies were carried out as previously described (7). Precipitated complexes were boiled in gel electrophoresis buffer for 3 min and analyzed by autoradiography following SDS-polyacrylamide gel electrophoresis (PAGE).

RESULTS

Construction of candidate HRV-14 replicons. The HRV-14 genome is a polyadenylated 7,212-nt, positive-sense RNA molecule that contains a 5′NTR of 628 nt, a large ORF encoding a virus polyprotein of 2,179 amino acids, and a short 3′NTR of 47 nt (8). As with other enteroviruses and rhinoviruses, the ORF can be divided into three segments (P1, P2, and P3) based on principal cleavages of the encoded polyprotein (for a review, see reference 27), with the P1 segment encoding the four capsid proteins. The HRV-14 5′NTR contains elements required for internal entry of ribosomes on uncapped viral RNA (1) and for formation of RNA replication complexes (44).

Candidate HRV-14 replicons were constructed from the plasmid pWR3.26, which contains a full-length cDNA of rhinovirus type 14 under control of a T7 promoter (28). Since several reports describe replication-competent poliovirus (RNAs with large deletions including almost the entire P1 segment of that virus (3, 13, 24, 41), we initially constructed two plasmids in which the pWR3.26 cDNA was altered to include the bacterial β-galactosidase (pΔP1βgal) or firefly luciferase (pΔP1Luc) coding sequences in lieu of almost the entire P1 segment of HRV-14 (Fig. 1A). These plasmids were constructed by PCR amplification of the reporter gene sequences by using oligonucleotide primers with in-frame flanking HRV-14 sequences and insertion of the amplified DNA into appropriate restriction sites (*Bcl*I and *Avr*II) in pWR3.26 (see Materials and Methods). In each plasmid, the 5′ end of the reporter sequence was fused directly to the authentic initiator AUG of the HRV-14 polyprotein. In an effort to ensure that recognition and processing at the VP1/2A^{pro} cleavage was not hampered by the presence of upstream foreign sequences, the reporter sequences were inserted 7 codons upstream of this Tyr-Gly dipeptide (Fig. 1). This configuration thus retained the downstream amino acid residues that have been suggested to be important for autocatalytic processing of the poliovirus polyprotein by 2A^{pro} (2, 21) and resulted in reporter-ΔVP1 fusion proteins containing the carboxyl-terminal 7 amino acids of VP1 (Fig. 1B). The predicted mass of the β-galactosidase fusion protein was approximately 118 kDa, while that for luciferase was 63 kDa. The HRV-14 RNA transcribed from pΔP1βgal was 462 bases (6.4%) longer than full-length rhinovirus RNA, while ΔP1Luc RNA was 831 bases (11.5%) shorter.

Functional reporter proteins are expressed and processed from ΔP1βgal and ΔP1Luc candidate replicon RNAs. To confirm that candidate replicon polyproteins were processed as expected and that fusion of the reporter gene sequences had not destabilized upstream RNA secondary structure required for efficient cap-independent initiation of translation under control of the HRV-14 internal ribosomal entry site (1), RNA was transcribed in vitro from the plasmids pΔP1βgal and pΔP1Luc by bacteriophage T7 RNA polymerase. This RNA was transfected into H1-HeLa cells by an efficient electroporation method which results in successful transfection of 60 to 70% of cells (see Materials and Methods). Transfected cells were pulse-labeled with [^{35}S]methionine from 3 to 8 h following transfection. Labeled reporter proteins present in cell lysates were immunoprecipitated with rabbit polyclonal antibodies directed against β-galactosidase or luciferase and subjected to SDS-PAGE (Fig. 2). Bands containing immunoreactive proteins of the expected molecular mass were present in lysates of cells transfected with either ΔP1Luc (Fig. 2, lane 3) or ΔP1βgal (lane 4). These bands were not present in mock-transfected or virus-infected cells (lanes 1 and 2, respectively). Thus, both candidate replicons expressed a protein product in which 2A^{pro} accurately processed the reporter-VP1 fusion protein from the remainder of the polyprotein.

Labeled, nonspecifically precipitated proteins were present in lysates of mock-transfected cells and cells transfected with the two candidate replicons (Fig. 2, lanes 1, 3, and 4). The absence of these protein bands in lysates of virus-infected cells (lane 2) most probably was due to 2A^{pro} shutoff of host cell protein synthesis. The absence of 2A^{pro} shutoff in the transfected cultures reflects the fact that only 60 to 70% of cells were successfully transfected. Expression of the β-galactosidase reporter protein from ΔP1βgal appeared to be at a lower

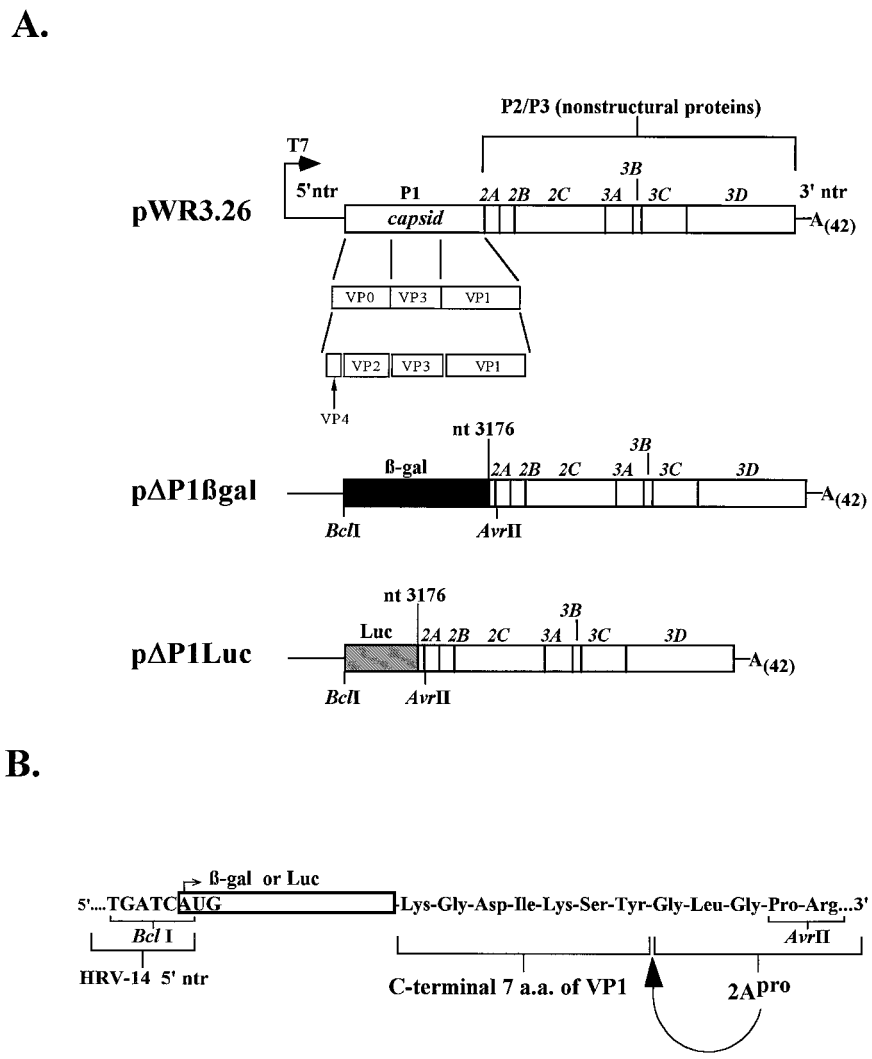

FIG. 1. Construction of the ΔP1βgal and ΔP1Luc candidate replicons. (A) Full-length HRV-14 cDNA contained in pWR3.26 is under control of a T7 promoter (28). The ΔP1βgal and ΔP1Luc replicons contain the β-galactosidase or luciferase sequences inserted in frame and replacing HRV-14 sequences between nt 628 and 3176. (B) The 5′ and 3′ in-frame fusions of the reporter sequences. At the 5′ terminus, the reporter sequences are inserted at a *Bcl*I site which overlaps the authentic AUG initiator for the HRV-14 polyprotein. At the 3′ terminus, the reporter sequence is fused in frame to the carboxyl-terminal 7 amino acids of VP1, just upstream of the $2A^{pro}$ cleavage site. Constructions were done as described in Materials and Methods.

level than luciferase expression from ΔP1Luc (compare lanes 3 and 4). This might be explained by different avidities of the antibodies used in the immunoprecipitations. However, minor immunoreactive protein products, which migrated more rapidly in SDS-PAGE than the intact β-galactosidase fusion protein did, were noted in cells transfected with ΔP1βgal upon longer exposure of the gel shown in Fig. 2 (31). This suggested the possibility that the somewhat lower expression levels observed with ΔP1βgal reflect either aberrant translation or degradation of the β-galactosidase reporter protein in vivo.

Lysates of cells transfected with the candidate replicons were also examined for expression of reporter enzyme activity. Figure 3 depicts the levels of β-galactosidase (Fig. 3A) or luciferase (Fig. 3B) activities present in H1-HeLa cell lysates prepared at various times following transfection of candidate replicon RNAs. The temporal patterns of reporter protein expression were similar for the two candidate replicons. While measurable quantities of functional enzyme were present in lysates prepared 3 to 12 h following transfection of either RNA, enzyme activities did not increase over time as expected from previous descriptions of successful poliovirus replicons expressing heterologous reporter proteins (9, 36). The expression of β-galactosidase from ΔP1βgal appeared to peak at a later time (9 h posttransfection [Fig. 3A]) than the luciferase activity from ΔP1Luc (3 h [Fig. 3B]). This is probably a result of the longer half-life of β-galactosidase than of luciferase (3) and the potential for accumulation of this reporter product (31). Furthermore, the functional β-galactosidase enzyme is a tetramer, whose assembly may be delayed after translation, while the luciferase enzyme functions as a monomer protein. However, of greater importance, the activities of both reporter proteins declined between 12 and 24 h, suggesting that less RNA was available for translation and hence that there was a failure of RNA replication.

To formally determine whether any of the enzyme activity demonstrated in Fig. 3 could reflect replication of the input RNAs, a frameshift mutation was created within the $3D^{pol}$ coding region of both candidate replicons. This resulted in

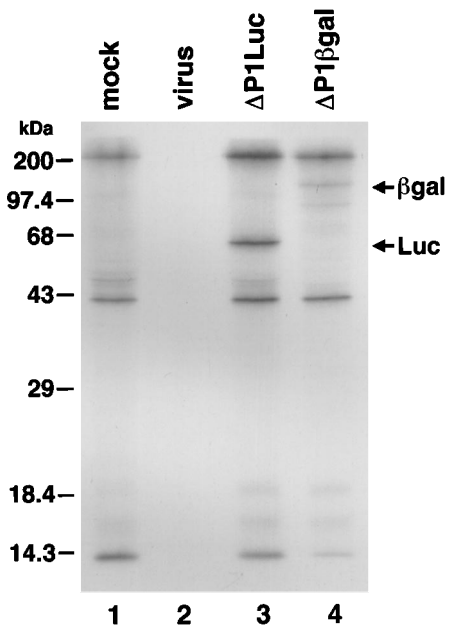

FIG. 2. Processing of reporter proteins expressed from ΔP1βgal and ΔP1Luc. $2A^{pro}$ processing of β-galactosidase–VP1 and luciferase-VP1 fusion proteins resulted in immunoreactive proteins with predicted masses of 118 and 63 kDa (arrows) in methionine-labeled cells transfected with ΔP1βgal RNA and ΔP1Luc RNA. Similar proteins were absent from mock-transfected or virus-infected cell extracts. Cell lysates were subjected to immunoprecipitation with anti-β-galactosidase or anti-luciferase antibodies followed by electrophoresis on an SDS–12% polyacrylamide gel and autoradiography.

pol^- mutant replicons, ΔP1βgal(pol^-) and ΔP1Luc(pol^-), which encoded a truncated version of the viral $3D^{pol}$ RNA polymerase lacking the conserved YGDD motif. There was no reduction in the expressed enzyme activities following transfection of these $3D^{pol}$-mutated RNAs into H1-HeLa cells compared with the reporter enzyme activities present following transfection with intact candidate replicons (31). These results indicated that the expression of reporter enzyme activity was due to translation of the input RNA only and provided further evidence that the ΔP1βgal and ΔP1Luc candidate replicons were not capable of replication.

Analysis of ΔP1βgal and ΔP1Luc RNA replication by RPA. To directly ascertain whether the candidate ΔP1βgal and ΔP1Luc replicon RNAs were undergoing amplification following transfection of H1-HeLa cells, the HRV-14 RNA content of cell lysates was quantified by RPA at various time intervals following transfection. For these measurements, equivalent amounts of cell lysate were hybridized with an excess of radiolabeled virus-specific RNA probe and then extensively digested with RNase, as described in Materials and Methods. A second probe complementary to the cellular mRNA for human GAPDH was included in each hybridization mixture to monitor the cellular RNA content of the lysates. As shown in Fig. 4, the amount of protected, virus-specific ΔP1βgal (Fig. 4A) or ΔP1Luc (Fig. 4B) replicon RNA increased minimally or not at all over the 24 h following transfection. While minimal increases in the RNA signal were often observed between 0 and 6 h following transfection of the replicon RNAs, similar minor increases were also observed with the $3D^{pol}$ mutant RNAs (Fig. 4A), indicating that this was an artifact of the system and was not indicative of RNA replication. The apparent failure of these candidate replicons to sustain efficient RNA replication was consistent with the results of reporter enzyme assays (Fig. 3), which failed to show an increase in enzyme activity indicative of increasing abundance of a replicating, messenger-active replicon RNA.

In contrast, a striking, sustained increase in the HRV-14 hybridization signal was always obtained following transfection of infectious WR3.26 RNA (shown in both Fig. 4A and 4B). In these experiments, cultures transfected with WR3.26 RNA were maintained in medium containing a concentration of neutralizing anti-HRV-14 antibody sufficient to prevent second-cycle infection of any nontransfected cells. Therefore, the RNA signal observed in these cultures should closely mimic what would be expected with a successful HRV-14 replicon RNA. The higher-molecular-weight bands that are HRV-14 specific and present only at late time points in cells transfected with the replicating virus RNA are of unknown origin. Al-

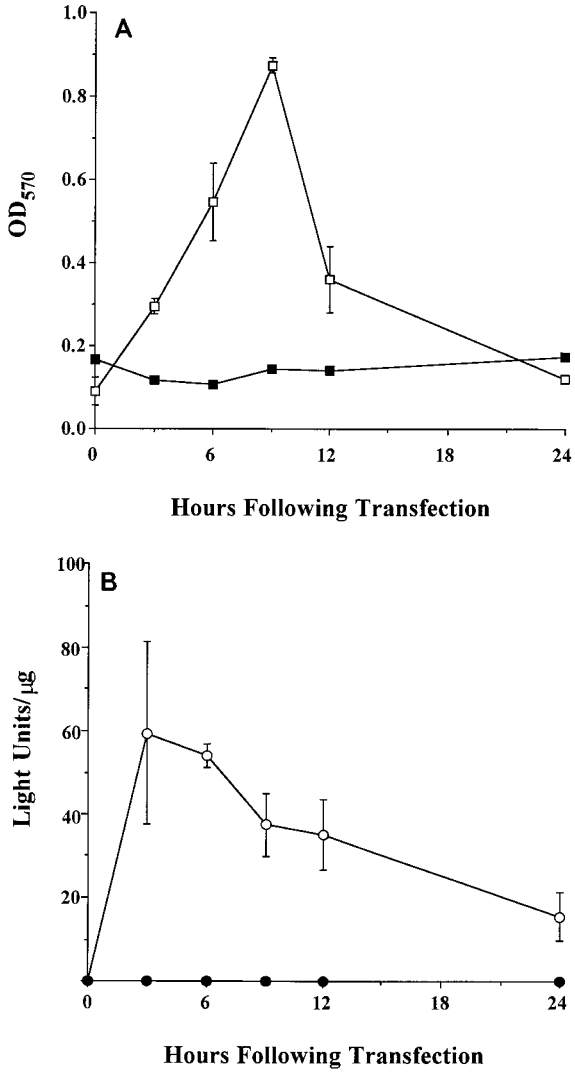

FIG. 3. Enzyme activity produced following transfection of candidate replicons. Cytoplasmic extracts were prepared at 0, 3, 6, 9, 12, and 24 h following transfection with ΔP1βgal or ΔP1Luc RNAs. (A) Cells were transfected with ΔP1βgal RNA (□) or mock transfected (■). Lysate containing 50 μg of protein was analyzed for β-galactosidase activity as described in Materials and Methods. (B) Cells were transfected with ΔP1Luc RNA (○) or mock transfected (●), and 5 μl of cell lysate was analyzed for luciferase activity. The luciferase activity was normalized for the protein content of lysates. Results shown represent means (± range) of duplicate transfections.

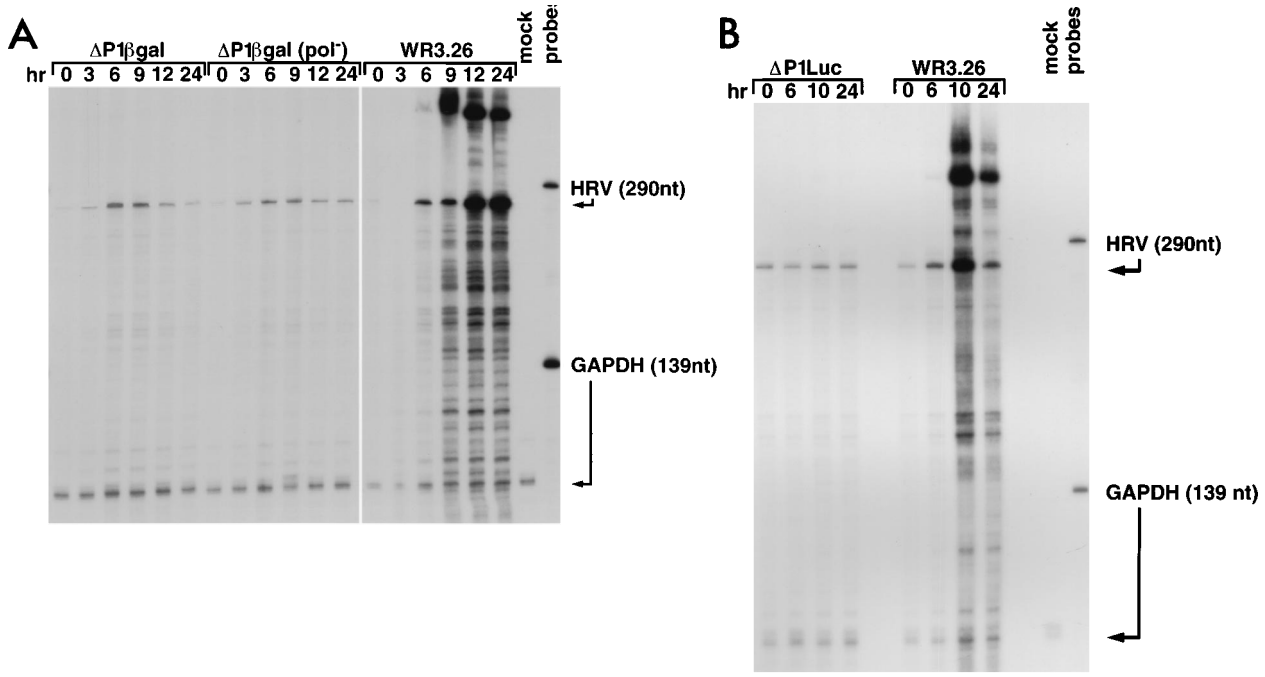

FIG. 4. RPA analysis of ΔP1βgal and ΔP1Luc RNA replication. Cell lysates were prepared at the indicated times following transfection with each candidate replicon RNA or WR3.26 infectious RNA. Virus-specific RNA was detected in lysates by hybridization with an HRV-specific probe (HRV) followed by RNase treatment and gel electrophoresis as described in Materials and Methods. An internal control probe (GAPDH) allowed monitoring of the cellular RNA content of each lysate. The extreme right lane was loaded with aliquots of each probe representing 1:100 the amount used in individual hybridization reactions. Protected fragments that are specific for virus (263 nt) and for GAPDH (100 nt) are indicated by arrows. (A) Transfection of ΔP1βgal, ΔP1βgal(pol$^-$), and WR3.26 RNA transcripts; cell lysates were prepared at 0, 3, 6, 9, 12, and 24 h following transfection. (B) Transfection of ΔP1Luc and WR3.26 RNAs prepared at 0, 6, 10, and 24 h following transfection. mock, lysate of mock-transfected cells. All WR3.26 transfected cell cultures were maintained in medium containing neutralizing anti-HRV-14 antibody.

though surprising, these larger-than-probe-sized bands appear to represent either the presence of stable duplexes which survive the denaturing gel conditions or the unexpected extension of probe molecules acting as primers and annealing to positive-strand RNAs present in the cell lysate (31).

We considered the possibility that the replicon plasmid DNAs acquired a spurious mutation during subcloning and passage in *Escherichia coli* and that this accounts for the lack of RNA replication. To exclude this possibility, we reconstructed a replicating virus genome by replacing the 5′NTR–β-galactosidase sequence (the small *Sal*I-*Avr*II fragment) in pΔP1βgal with the analogous 5′NTR-P1 sequence of HRV-14. Because the HRV-14 5′NTR/P1 sequence used in this reconstruction was derived from a pol$^-$ virus cDNA mutant, pWR3.26(pol$^-$), the replicating RNAs which were recovered from the reconstructed clone must contain the P2-P3 segments of the ΔP1βgal replicon. This result thus confirmed the integrity of the P2-P3 segments in pΔP1βgal. To ensure that there were no adventitious mutations in the 5′NTR, this region of pΔP1βgal was sequenced in its entirety.

Taken together, these results provide strong evidence that subgenomic HRV-14 RNAs in which foreign RNA sequences replace all but the 3′ 21 nt of the P1 segment of HRV-14 are not capable of efficient replication. This lack of replication capacity is not likely to be due to a unique feature of the inserted RNA sequence, as it was observed with two distinctly different foreign sequences inserted into the P1 region, one longer and one shorter than the excised P1 sequence.

Deletion of capsid sequences compromises HRV-14 RNA replication. The lack of replication of the candidate ΔP1βgal and ΔP1Luc replicons, despite retention of an active internal ribosomal entry site and processing of the expected reporter-VP1 fusion protein at the VP1/2A^{pro} junction, suggested that sequences within the P1 region are necessary for efficient HRV-14 RNA replication. To test this hypothesis, we constructed a series of five HRV-14 mutants which contained various in-frame deletions within the P1 segment (Fig. 5). The genomes of these mutants are similar to the genomes of purposely constructed poliovirus defective interfering particles that have been described previously (20, 24). We monitored amplification of RNAs derived from these plasmids following their transfection into H1-HeLa cells. As shown in Fig. 6, increasing amounts of HRV-14 RNA were present in lysates prepared at various intervals over the 24 h following transfection of three of the deletion mutants (CapD2, CapD3, and CapD4). Replication of the viable mutant with the largest deletion (CapD2 [Fig. 6]) appeared to proceed somewhat more rapidly than replication of RNA from pWR3.26, the full-length cDNA clone, while the increases in viral RNA seen with the other two deletion mutants were very similar to the intact RNA.

In contrast, the remaining two deletion mutant RNAs, CapD1 and CapD5, were not amplified following transfection, as evidenced by the lack of increasing amounts of protected RNA fragments in the RPA (Fig. 6). These RNAs thus resemble the nonviable ΔP1βgal and ΔP1Luc candidate replicons (Fig. 4). As shown in Fig. 5, the two replication-incompetent deletion mutants have a common deleted region, extending from nt 2121 within the VP3 coding region to nt 2724 within the VP1 coding region. It is unlikely that these deletion mutants fail to replicate because of aberrant processing at the VP1/2A site. The CapD1 and CapD5 transcripts encode different lengths of residual VP1 sequences, but in both cases the retained VP1 segment is considerably longer than the 7 resi-

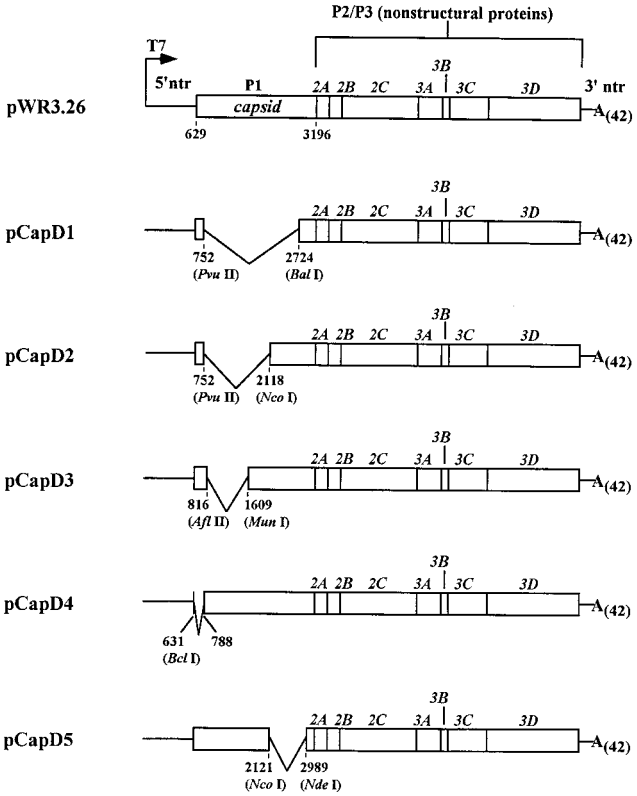

FIG. 5. Subgenomic HRV-14 constructs with P1 segment deletions. Shown is a schematic diagram of the 5 HRV-14 in-frame P1 segment deletion mutants created as described in Materials and Methods. The nucleotide positions flanking the termini of each deletion are indicated. For the pCapD4 deletion, a linker was inserted between the *Bcl*I and *Bgl*II sites to create a deletion beginning at the AUG initiator codon and extending to the first nucleotide of the Thr-54 codon at nt 788.

dues present in ΔP1βgal and ΔP1Luc, which do undergo efficient processing at this cleavage site (Fig. 2). Sequencing of plasmid DNA confirmed that the deletions in these constructs were in frame, as designed. Thus, these data strongly support the conclusion that a 603-nt sequence located near the 3′ end of the P1 segment (nt 2121 to 2724) contains an element or encodes a protein that is necessary for efficient replication of HRV-14 RNA.

Addition of sequence from the 3′ end of the P1 segment restores replication competence to the ΔP1Luc replicon. Since efficient RNA replication of P1 deletion mutants was obtained only when sequences within the 3′ region of the P1 segment were retained (Fig. 6), we determined whether the presence of this sequence would restore replication competence to the ΔP1Luc candidate replicon. This was accomplished by inserting into pΔP1Luc the sequence spanning the region from nt 2117 (the *Nco*I site in VP3) to the 3′ end of the P1 segment of HRV-14 (see Materials and Methods for details of this construction). A synthetic $3CD^{pro}$ recognition and cleavage site (Ala-X-X-Gln/Gly) was linked to the carboxyl-terminal luciferase sequence (Fig. 7A). A -Gly-Gly-Gly- hinge was placed between the luciferase sequence and this $3CD^{pro}$ recognition site to provide greater flexibility at the artificial cleavage site. Processing by $3CD^{pro}$ at this site should result in a luciferase protein with only 7 additional carboxyl-terminal amino acid residues and an expected mass of about 63 kDa. The design for this synthetic $3CD^{pro}$ cleavage site was based on $3CD^{pro}$ recognition sites found in the HRV-14 polyprotein (8, 28) and on the design of a similar functional synthetic poliovirus $3CD^{pro}$ cleavage site reported recently by others (4, 30). The synthetic cleavage site includes a Gln-Gly dipeptide with an Ala residue at the P4 position, which has been found to be important for efficient $3CD^{pro}$ processing in poliovirus (6, 16). The resulting candidate replicon was designated ΔP1Luc/VP3.

RNA transcribed from pΔP1Luc/VP3 was tested for its ability to produce properly processed luciferase protein by pulse-labeling transfected H1-HeLa cells, and then immunoprecipitating luciferase as described above. SDS-PAGE demonstrated an immunoreactive protein of the expected mass in cells transfected with ΔP1Luc/VP3 RNA (Fig. 7B, lane 4) but not in mock-transfected or rhinovirus-infected cells (lanes 1 and 2, respectively). Lack of processing at the synthetic $3CD^{pro}$ cleavage site would have resulted in a fusion protein consisting of the luciferase enzyme and 70 additional residues of VP3 (approximately 70 kDa). Immunoreactive proteins of this size

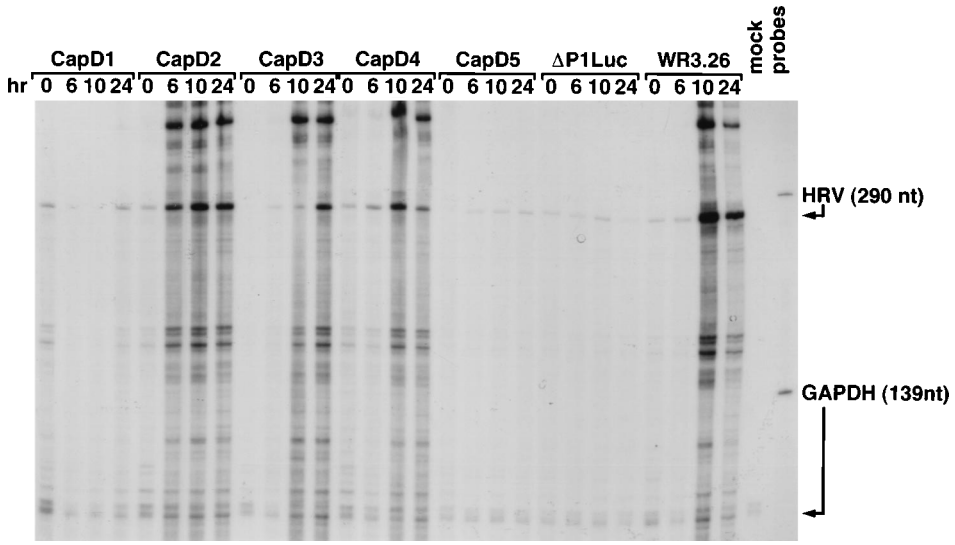

FIG. 6. Replication of P1-deleted mutant RNAs in H1-HeLa cells. Lysates were prepared at 0, 6, 10, or 24 h following transfection of cells with the indicated RNA and processed for RPA as described in Materials and Methods. See the legend to Fig. 4 for details.

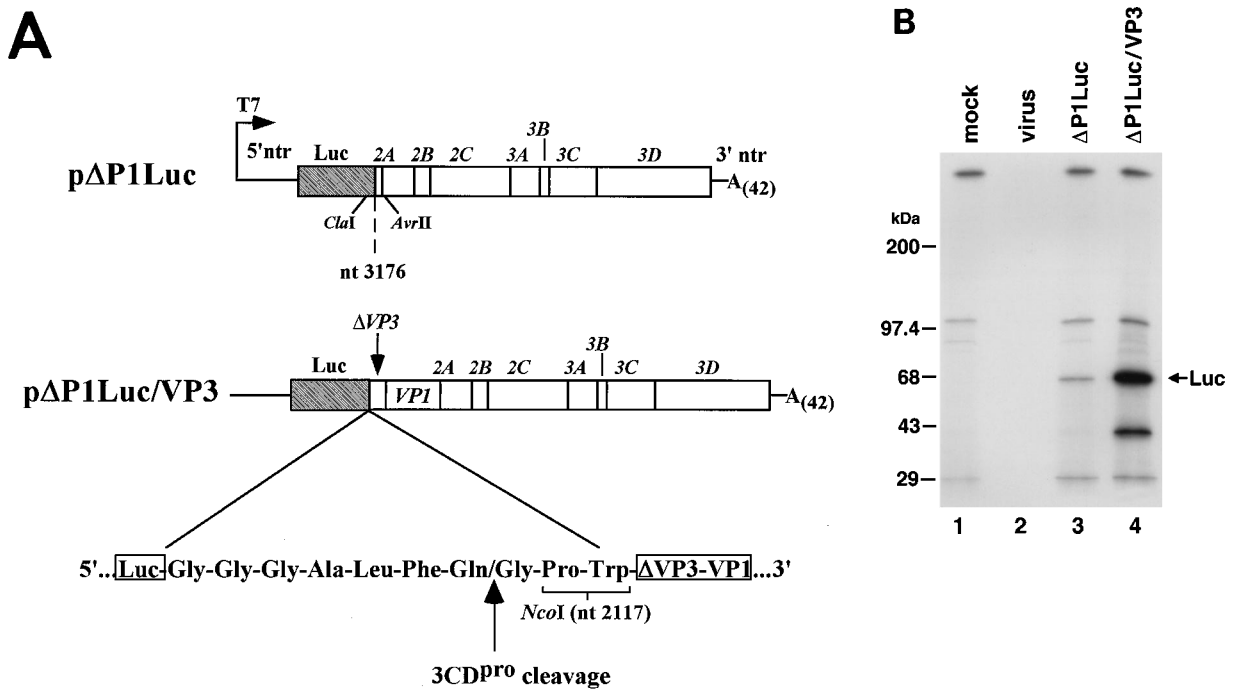

FIG. 7. Organization of the ΔP1Luc/VP3 replicon. (A) HRV-14 sequences between nt 2118 and 3176 were reinserted in ΔP1Luc to create ΔP1Luc/VP3, as illustrated. A synthetic $3CD^{pro}$ recognition and cleavage site was placed at the carboxyl terminus of the luciferase sequence. (B) $3CD^{pro}$ processing of luciferase from the ΔP1Luc/VP3 polyprotein. An immunoreactive protein (arrow) of the predicted size was present in [^{35}S]Met-labeled cells transfected with ΔP1Luc/VP3 RNA but absent in mock-transfected or virus-infected cell extracts. Luciferase generated from cells transfected with ΔP1Luc RNA is shown for comparison. Cell lysates were immunoprecipitated with rabbit antibody to luciferase and subjected to electrophoresis on an SDS–10% polyacrylamide gel. The minor immunoreactive band (at about 40 kDa) which migrated more rapidly in the ΔP1Luc/VP3 immunoprecipitation is likely a result of aberrant initiation or premature termination of translation.

were not apparent in ΔP1Luc/VP3-transfected cells (lane 4), indicating efficient *trans* recognition and utilization of the synthetic $3CD^{pro}$ cleavage site within the ΔP1Luc/VP3 polyprotein. The abundance of the luciferase protein was significantly greater in cells transfected with ΔP1Luc/VP3 RNA than in cells transfected with ΔP1Luc (compare lanes 3 and 4). Since equivalent amounts of replicon RNA were transfected in this experiment, this provided preliminary evidence for replication of the ΔP1Luc/VP3 RNA.

To confirm that ΔP1Luc/VP3 RNA was replicating, HRV-14 RNA was quantified by RPA of lysates derived from transfected cells (Fig. 8). Control transfections carried out simultaneously included WR3.26 (full-length HRV-14) and ΔP1Luc RNAs. Cell lysates were prepared as before, at various points in time following transfection. Increasing quantities of virus-specific RNA were evident in lysates of cells transfected with ΔP1Luc/VP3 (Fig. 8). The RNA levels were similar to those produced by intact HRV-14 transcripts in the same experiment. As before, no significant increase in the RNA signal was observed in cells transfected with ΔP1Luc RNA. Thus, the addition of P1 sequences extending from nt 2117 to the P1/P2 junction resulted in restoration of replication competence to the candidate ΔP1Luc replicon.

We compared the luciferase activity expressed in cells transfected with ΔP1Luc/VP3 with that expressed following transfection with ΔP1Luc (Fig. 9). Although the ΔP1Luc/VP3-transfected cells contained approximately 5-fold less luciferase activity than ΔP1Luc-transfected cells did (Fig. 9) 3 to 6 h after transfection, this activity increased exponentially in ΔP1Luc/VP3- but not in ΔP1Luc-transfected cells (Fig. 9) between 6 and 12 h. Thus, levels of luciferase at 12 and 24 h were over 100-fold greater than those present in ΔP1Luc-transfected cells. This pattern of reporter protein expression is in agreement with previous reports of successful poliovirus replicons that express foreign proteins (3, 9, 36). The early delay in luciferase expression from ΔP1Luc/VP3 compared with ΔP1Luc (Fig. 9) may reflect sequestration of input RNA in replication complexes, reducing the availability of the input RNA for translation.

Capsid sequences provided in *trans* do not rescue nonreplicating candidate HRV-14 replicons. To determine whether the replication defect in ΔP1Luc could be complemented in *trans*, cell monolayers which had been previously transfected with replicon RNAs were infected with HRV-14 at high multiplicity of infection. Under these conditions, the replicating virus genomes should provide an abundance of the capsid proteins expressed by the P1 segment. If the capsid proteins (VP1 or VP3 fragment expressed by ΔP1Luc/VP3) are important for RNA replication and can function in *trans*, a nonreplicating RNA such as ΔP1Luc might be rescued and undergo normal RNA replication. For this experiment, cells were transfected with ΔP1Luc or ΔP1Luc/VP3 RNAs and infected 6 h later with HRV-14 at multiplicity of infection of 10 PFU per cell. Monolayers were assayed for luciferase enzyme activity 18 h later. As shown in Table 1, the enzyme activity was not increased in ΔP1Luc-transfected cells that were infected with HRV-14 but was instead decreased (42% of control transfected cells). These data thus suggest that the requirement for P1 sequences in RNA replication cannot be provided in *trans*. The decrease in luciferase activity observed with HRV-14 infection probably reflects competition between the viral and candidate replicon RNAs for cellular translation factors, as well as increased cell death related to viral replication.

Compared with ΔP1Luc, the fractional decrease in luciferase activity was substantially greater following virus infection of cells which had been transfected previously with the ΔP1Luc/

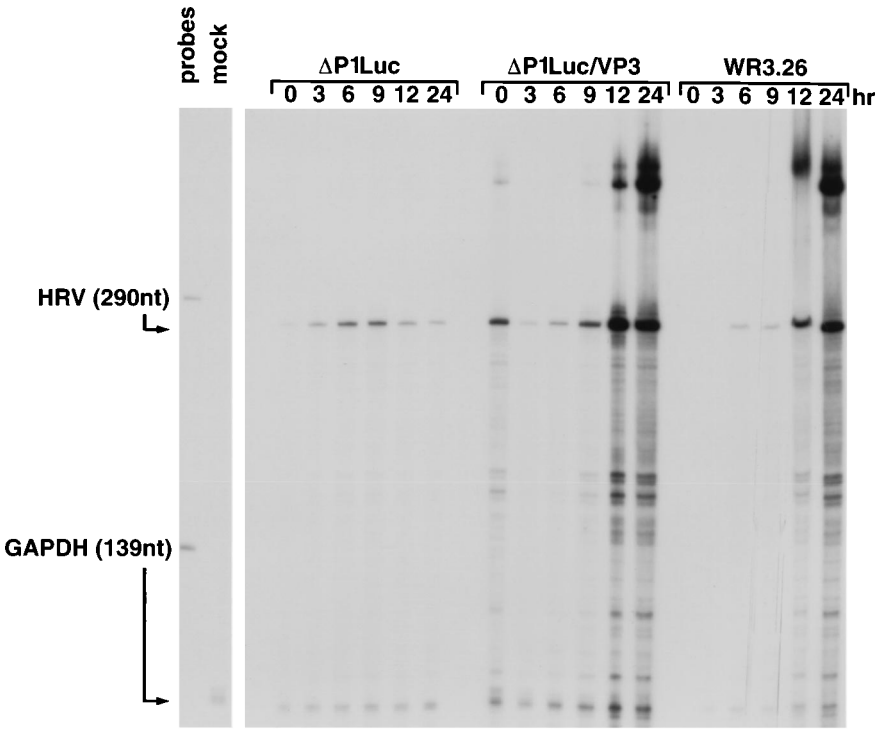

FIG. 8. RPA analysis of ΔP1Luc/VP3 RNA replication. Cell lysates were prepared at 0, 3, 6, 9, 12, and 24 h following transfection of the indicated RNA. See the legend to Fig. 4 for details.

VP3 replicon (4% of uninfected control cells; Table 1). This decrease could not be related to removal of RNA from the pool of translating RNAs as a result of packaging of the replicon, because a similar decrease was observed following cotransfection with CapD2 RNA (Fig. 5), which does not encode a complete set of capsid proteins (Table 1). This decrease in luciferase expression is reflected by reduced ΔP1Luc/VP3 RNA replication, probably owing to competition from a more efficiently replicating CapD2 or HRV-14 RNA (31). The apparent difference in the replication efficiency of these RNAs could be related to differences in their length (7,389 nt for ΔP1Luc/VP3 versus 7,212 nt for HRV-14 and 5,846 nt for CapD2). Importantly, ΔP1Luc/VP3 and CapD2 RNAs contain similar HRV-14 P1 sequences (Fig. 5 and 7A).

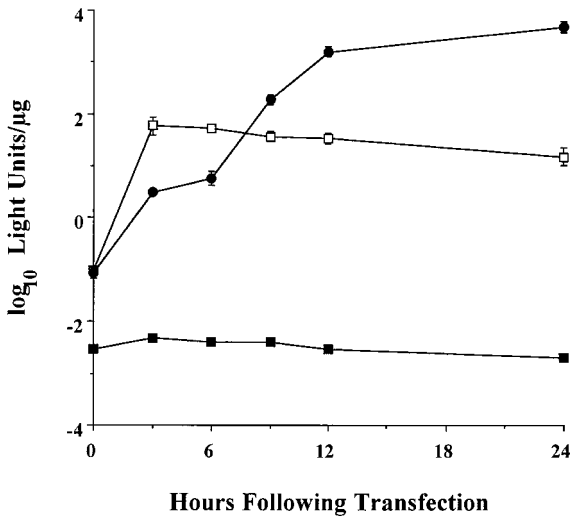

FIG. 9. Luciferase activity in cells transfected with ΔP1Luc/VP3 RNA. Cytoplasmic extracts were prepared at 0, 3, 6, 9, 12, and 24 h following transfection with ΔP1Luc/VP3 RNA (●) or ΔP1Luc RNA (□) or mock transfection (■). Results shown represent means (± range) of duplicate transfections.

ΔP1Luc/VP3 RNA is encapsidated during HRV-14 infection. To determine whether ΔP1Luc/VP3 replicon RNA could be packaged by capsid proteins provided in *trans*, transfected cell monolayers were infected with HRV-14 as described above. Virus harvests were prepared 18 h postinfection. Prior to inoculation onto fresh H1-HeLa cells, this first-passage virus was treated with 1 mg of RNase A per ml to remove any free replicon RNA. As shown in Table 2, cell lysates prepared from monolayers inoculated with this virus harvest contained small but readily detectable amounts of luciferase enzyme activity. The expression of this luciferase activity could be completely

TABLE 1. Luciferase activity in H1-HeLa cells transfected with candidate replicon RNAs and coinfected with HRV-14 or cotransfected with CapD2 RNA

Replicon RNA	Helper genome[a]	Luciferase activity (light units) (%)[b]
ΔP1Luc	None	164 (100)
	HRV-14	69 (42)
ΔP1Luc/VP3	None	19,985 (100)
	HRV-14	802 (4)
	CapD2	781 (4)

[a] Helper genome supplied by infection with HRV-14 6 h following replicon transfection or by simultaneous cotransfection of replicon and CapD2 RNAs.

[b] Luciferase values represent means of duplicate cultures assayed 24 h following transfection (percent luciferase expression in the absence of helper genome is given in parentheses).

TABLE 2. Encapsidation of candidate replicon RNAs by HRV-14 helper virus infection

Replicon RNA	Helper virus	Luciferase activity (light units) on passage to fresh H1-HeLa cells[a]	
		Nonneutralized	Postneutralization
ΔP1Luc	None	0.01	ND[b]
	HRV-14	0.03	ND
ΔP1Luc/VP3	None	0.07	ND
	HRV-14	109	0.09

[a] Luciferase expression in H1-HeLa cells infected with RNase-digested lysates of cells which had been transfected with replicon RNAs and infected with helper virus as indicated. Lysates (first-passage virus harvests) were inoculated onto fresh cells with or without prior incubation with anti-HRV-14 neutralizing antibody.

[b] ND, not determined.

blocked by pretreatment of the virus harvest with neutralizing antibody to HRV-14. In contrast, no luciferase activity was present in cells inoculated with virus harvested from ΔP1Luc-transfected cultures that were infected with HRV-14 or from ΔP1Luc/VP3-transfected cultures that were not infected with helper virus (Table 2). These results confirm that ΔP1Luc/VP3 replicon RNA is packaged by helper virus during infection. However, no evidence for survival of the replicon RNA could be obtained on subsequent passage of the virus-replicon mixture (31), indicating that the packaged ΔP1Luc/VP3 genomes were not stable in the helper virus population.

DISCUSSION

We have shown that an HRV replicon RNA, ΔP1Luc/VP3, in which sequences encoding the firefly luciferase protein replaced the 5′ 1,488 nt of the P1 capsid-coding sequence of HRV-14, replicated efficiently following its transfection into H1-HeLa cells (Fig. 8). The luciferase activity expressed from ΔP1Luc/VP3 RNA was dependent on replication of the RNA genome (Fig. 9). In contrast, a similar candidate replicon, ΔP1Luc, in which all but 21 nt of the HRV-14 P1 segment were removed from the genome, failed to undergo efficient RNA replication (Fig. 4B) and expressed only very low levels of luciferase (Fig. 3B). While it is not possible to exclude a very low level of replication of ΔP1Luc or the structurally related ΔP1βgal candidate replicons, these RNAs did not undergo amplification that was detectable in a sensitive RPA following transfection of HeLa cells (Fig. 4). Intracellular RNA levels remained similar to those present following transfection with candidate replicon RNAs containing a lethal frameshift mutation within the $3D^{pol}$ RNA polymerase coding region. These results were consistent with the analysis of a series of subgenomic HRV-14 RNAs containing various in-frame deletions within the P1 segment of the genome, which suggested that the inclusion of sequence between nt 2121 and 2724 was essential for replication of subgenomic HRV-14 RNAs (Fig. 6). However, the minimal sequence within this 603-nt segment which is required for replication has yet to be determined.

Inefficient or aberrant processing of the HRV-14 polyprotein at the P1/P2 junction seems unlikely to account for the lack of replication of ΔP1βgal, ΔP1Luc, or the capsid sequence deletion mutants CapD1 and CapD5. Pulse-labeling experiments demonstrated efficient processing of the $2A^{pro}$-directed cleavage at the carboxyl terminus of the Luc-VP1 fusion protein expressed by ΔP1Luc (Fig. 2). Similarly, an immunoreactive β-galactosidase protein of the expected size was present in cells transfected with ΔP1βgal (Fig. 2). These observations indicate the retention of autocatalytic processing of the polyprotein of these replication-defective candidate replicons by $2A^{pro}$ and are consistent with the retention of proteolytic activity when poliovirus $2A^{pro}$ was similarly fused to heterologous sequences (2, 34, 41). Processing of the remaining P2/P3 portion of the replicon polyprotein was not examined, because it is expected to proceed normally after the primary $2A^{pro}$ cleavage. Although we did not examine the P1/P2 cleavage in cells transfected with the capsid deletion mutants shown in Fig. 5, the replication-defective mutants CapD1 and CapD5 encode polyproteins with carboxyl-terminal fragments of VP1 that are substantially longer than the 7 residues present in the ΔP1Luc and ΔP1βgal polyproteins and are thus likely to be effectively processed.

That P1 sequences appear to be required for efficient replication of HRV-14 RNA contrasts sharply with reports of poliovirus replicons which replicate well despite large deletions in the P1 region (3, 13, 24, 41). However, it is interesting that naturally occurring poliovirus defective interfering genomes generally retain sequence at the 3′ end of the P1 segment (20, 23, 26), although the downstream P1 sequences retained by these genomes are significantly shorter than the P1 sequence reinserted in ΔP1Luc/VP3 and encode only a truncated VP1 protein. The fact that candidate rhinovirus replicons which lacked almost all of the P1 segment failed to replicate indicates an important difference between these two otherwise quite closely related viruses.

The required P1 sequence could function in several different ways during the replication of HRV-14 RNA. The VP1 protein is expressed as an intact protein by ΔP1Luc/VP3 and in each of the replication-competent P1 deletion mutants. The analogous poliovirus VP1 protein is closely associated with the membrane-bound RNA replication complex in poliovirus-infected cells (37). While this association has been assumed to reflect the well-documented role of this protein in encapsidation of RNA (25), an accessory function associated with RNA replication would be consistent with the tendency of picornavirus proteins to fulfill multiple functions in viral replication. VP1 provided in *trans* by replicating HRV-14 helper virus did not result in the rescue and replication of nonviable RNAs such as ΔP1Luc (Table 1). While this does not rule out a *cis*-acting role for VP1 in viral RNA replication, as is known to be the case for several of the nonstructural proteins of poliovirus (43, 50), this observation does argue against a *trans*-acting role for any of the capsid proteins of HRV-14 in RNA replication. Further experiments are required to determine whether the essential P1 sequence must be expressed as protein to support viral replication. The construction of dicistronic HRV-14 replicons may be helpful in addressing this question (33).

It is also possible that this region of the P1 segment acts directly as RNA in facilitating the replication of the HRV-14 genome. While such a P1 segment function is not suggested by any current model of picornavirus RNA replication, the existence of a *cis*-acting replication signal located within this region of the viral genome would act to suppress the evolution of defective interfering particles. Such an element would prevent the amplification of RNA molecules sustaining large P1 deletions that might, because of their shorter length, be otherwise favored for replication. Interestingly, internally located, *cis*-acting RNA replication elements are not without precedent among plus-strand RNA viruses. Efficient synthesis of RNA-3 of brome mosaic virus, a positive-sense plant virus with a tripartite genome, requires the presence of internal sequences which are located within an intercistronic region of this RNA (18, 39, 42). These internal sequences may facilitate assembly

of an active brome mosaic virus RNA-dependent RNA polymerase complex required for negative-strand RNA-3 synthesis in yeast cells (42), but they may not be absolutely required for minus-strand initiation in plants, because a low level of RNA-3 replication proceeds in their absence (18). Recent work also has revealed the presence of an internal *cis*-acting replication signal located between 3.1 and 3.9 kb of the 5′ end of a murine coronavirus RNA genome (24a). Similar to the HRV artificial defective interfering genomes described in this report, this sequence was found to be required for efficient replication of coronavirus defective interfering genomes.

Studies with the bacteriophage Qβ replicase have demonstrated a requirement for binding of the replicase complex to two internal sites on the plus-strand RNA (5). Both the viral replicase and a host factor bind to these internal sites, with binding of the host factor possibly acting to bring the 3′ end of the genome into close proximity to the internal sites, thereby allowing initiation of minus-strand RNA synthesis (5). It is not difficult to imagine a similar event in the replication of picornavirus RNAs, although there are no prior data to suggest this. The existence and nature of possible RNA secondary structures within the relevant region of the rhinovirus P1 coding segment are not known, but alignment of available rhinovirus sequences indicates the presence of several strong areas of nucleotide sequence conservation (31). Multiple studies indicate that the P1 segment of poliovirus is not required for efficient RNA replication, but there are no data which would exclude the possibility of such a *cis*-acting RNA replication element within the P2/P3 segments of poliovirus. Interestingly, Novak and Kirkegaard identified a region within the P2-P3 segments of the poliovirus genome which must undergo translation in *cis* for replication of the RNA to proceed (34a). It is intriguing to speculate that the replication element we have identified in HRV-14 may be analogous to this "*cis*-translation required," element of poliovirus but located within a different region of the genome.

We found clear evidence for encapsidation of the ΔP1Luc/VP3 RNAs following infection of previously transfected cells with the HRV-14 helper virus (Table 2). However, luciferase expression was very low when helper virus harvests were used to infect fresh H1-HeLa cells, and in situ visualization of luciferase expression demonstrated only very small numbers of infected cells (31). No luciferase activity was noted on further passage of the helper-replicon mixture. At present, it is not clear whether this reflects the inhibition of replicon replication by the helper viral genome or restrictions on packaging of the longer-than-genome-length RNA. Additional experiments were carried out in which the time of HRV-14 infection was incrementally delayed up to 12 h after replicon transfection, when substantial amounts of newly replicated RNA should have been available for encapsidation. However, we observed no significant increase in encapsidation of replicon RNA over that shown in Table 2 (31). Although these results suggest that the low levels of replicon encapsidation may be related to the size or structure of RNA preferred by the HRV-14 packaging mechanism, further studies are required to determine the magnitude of any restriction to encapsidation.

The luciferase expressed by the ΔP1Luc/VP3 replicon provides a simple and sensitive marker for HRV-14 RNA replication. Since the pathogenesis of HRV infections is not well understood, this or other replicons may be useful in studying certain aspects of HRV pathogenesis, including the cell types within the respiratory mucosa that are infected by HRV and the routes by which the virus disseminates through the epithelium of the upper airway during infection. Furthermore, the unique tropism of HRV for cells of the respiratory mucosa offers the possibility that HRV replicons expressing heterologous antigens or functional proteins will ultimately prove useful as vaccine delivery systems or for targeted gene therapy.

ACKNOWLEDGMENTS

We thank Wai-Ming Lee and Roland Rueckert for their kind gift of the HRV-14 cDNA, pWR3.26. Also, much appreciation goes to Anne Mosser for her gift of the anti-HRV-14 neutralizing antibody and to Ann Palmenberg for providing us with the rhinovirus sequence alignments. We are also grateful to John Newbold for his critical review of the manuscript.

This work was supported by grants from the U.S. Public Health Service (HL51818 and AI32599) and the Cystic Fibrosis Foundation (S880).

REFERENCES

1. **Alsaadi, S., S. Hassard, and G. Stanway.** 1989. Sequences in the 5′ noncoding region of human rhinovirus 14 RNA that affect *in vitro* translation. J. Gen. Virol. **70:**2799–2804.
2. **Alvey, J. C., E. E. Wyckoff, S. F. Yu, R. Lloyd, and E. Ehrenfeld.** 1991. *cis*- and *trans*-cleavage activities of poliovirus 2A protease expressed in *Escherichia coli*. J. Virol. **65:**6077–6083.
3. **Andino, R., G. E. Rieckhof, P. L. Achacoso, and D. Baltimore.** 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. EMBO J. **12:**3587–3598.
4. **Andino, R., D. Silvera, S. D. Suggett, P. L. Achacoso, C. J. Miller, D. Baltimore, and M. B. Feinberg.** 1994. Engineering poliovirus as a vaccine vector for the expression of diverse antigens. Science **265:**1448–1451.
5. **Barrera, I., D. Schuppli, J. M. Sogo, and H. Weber.** 1993. Different mechanisms of recognition of bacteriophage Qβ plus and minus strand RNAs by Qβ replicase. J. Mol. Biol. **232:**512–521.
6. **Blair, W. S., and B. L. Semler.** 1991. Role for the P4 amino acid residue in substrate utilization by the poliovirus 3CD proteinase. J. Virol. **65:**6111–6123.
7. **Brown, E. A., S. P. Day, R. W. Jansen, and S. M. Lemon.** 1991. The 5′ nontranslated region of hepatitis A virus: secondary structure and elements required for translation in vitro. J. Virol. **65:**5828–5838.
8. **Callahan, P. L., S. Mizutani, and R. J. Colonno.** 1985. Molecular cloning and complete sequence determination of the RNA genome of human rhinovirus type 14. Proc. Natl. Acad. Sci. USA **82:**732–736.
9. **Choi, W. S., R. Pal-Ghosh, and C. D. Morrow.** 1991. Expression of human immunodeficiency virus type 1 (HIV-1) *gag*, *pol*, and *env* proteins from chimeric HIV-1-poliovirus minireplicons. J. Virol. **65:**2875–2883.
10. **Cole, C. N.** 1975. Defective interfering (DI) particles of poliovirus. Prog. Med. Virol. **20:**180–207.
11. **Cole, C. N., and D. Baltimore.** 1973. Defective interfering particles of poliovirus. II. Nature of the defect. J. Mol. Biol. **76:**325–343.
12. **Cole, C. N., D. Smoler, E. Wimmer, and D. Baltimore.** 1971. Defective interfering particles of poliovirus. I. Isolation and physical properties. J. Virol. **7:**478–485.
13. **Collis, P. S., B. J. O'Donnell, D. J. Barton, J. A. Rogers, and J. B. Flanegan.** 1992. Replication of poliovirus RNA and subgenomic RNA transcripts in transfected cells. J. Virol. **66:**6480–6488.
14. **Colonno, R. J., R. L. Lafemina, C. M. Dewitt, and J. E. Tomassini.** 1990. The major-group rhinoviruses utilize the intracellular adhesion molecule 1 ligand as a cellular receptor during infection, p. 257–261. *In* M. A. Brinton and F. X. Heinz (ed.), New aspects of positive-strand RNA viruses. American Society for Microbiology, Washington, D.C.
15. **Couch, R. B.** 1990. Rhinoviruses, p. 607–629. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
16. **Dewalt, P. G., M. A. Lawson, R. J. Colonno, and B. L. Semler.** 1989. Chimeric picornavirus polyproteins demonstrate a common 3C proteinase substrate specificity. J. Virol. **63:**3444–3452.
17. **Duechler, M., T. Skern, D. Blaas, B. Berger, W. Sommergruber, and E. Kuechler.** 1989. Human rhinovirus serotype 2: *in vitro* synthesis of an infectious RNA. Virology **168:**159–161.
18. **French, R., and P. Ahlquist.** 1987. Intercistronic as well as terminal sequences are required for efficient amplification of brome mosaic virus RNA3. J. Virol. **61:**1457–1465.
19. **Gwaltney, J. M., Jr.** 1982. The rhinoviruses, p. 491–517. *In* E. A. Evans (ed.), Viral infection of humans: epidemiology and control. Plenum Publishing Corp., New York.
20. **Hagino-Yamagishi, K., and A. Nomoto.** 1989. In vitro construction of poliovirus defective interfering particles. J. Virol. **63:**5386–5392.
21. **Hellen, C. U. T., C.-K. Lee, and E. Wimmer.** 1992. Determinants of substrate recognition by poliovirus 2A proteinase. J. Virol. **66:**3330–3338.
22. **Hofer, F., M. Gruenberger, H. Kowalski, H. Machat, M. Huettinger, E.**

Kuechler, and D. Blass. 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. Proc. Natl. Acad. Sci. USA **91:**1839–1842.
23. **Kajigaya, S., H. Arakawa, S. Kuge, T. Koi, N. Imura, and A. Nomoto.** 1985. Isolation and characterization of defective-interfering particles of poliovirus. Sabin 1 strain. Virology **142:**307–316.
24. **Kaplan, G., J. Lubinski, A. Dasgupta, and V. R. Racaniello.** 1985. *In vitro* synthesis of infectious poliovirus RNA. Proc. Natl. Acad. Sci. USA **82:** 8424–8428.
24a. **Kim, Y.-N., and S. Makino.** 1995. Characterization of murine coronavirus defective interfering RNA internal *cis*-acting replication signal. J. Virol. **69:** 4963–4971.
25. **Kirkegaard, K.** 1990. Mutations in VP1 of poliovirus specifically affect both encapsidation and release of viral RNA. J. Virol. **64:**195–206.
26. **Kuge, S., I. Saito, and A. Nomoto.** 1986. Primary structure of poliovirus defective-interfering particle genomes and possible generation mechanisms of the particles. J. Mol. Biol. **192:**473–487.
27. **Lawson, M. A., and B. L. Semler.** 1990. Picornavirus protein processing-enzymes, substrates, and genetic regulation, p. 49–87. *In* V. R. Racaniello (ed.), Picornaviruses. Springer-Verlag KG, Berlin.
28. **Lee, W.-M., S. S. Monroe, and R. R. Rueckert.** 1993. Role of maturation cleavage in infectivity of picornaviruses: activation of an infectosome. J. Virol. **67:**2110–2122.
29. **Liljestrom, P., S. Lusa, D. Huylebroeck, and H. Garoff.** 1991. In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. J. Virol. **65:**4107–4113.
30. **Mattion, N. M., P. A. Reilly, S. J. DiMichele, J. C. Crowley, and C. Weeks-Levy.** 1994. Attenuated poliovirus strain as a live vector: expression of regions of rotavirus outer capsid protein VP7 by using recombinant Sabin 3 viruses. J. Virol. **68:**3925–3933.
31. **McKnight, K. L., and S. M. Lemon.** Unpublished data.
32. **Mizutami, S., and R. J. Colonno.** 1985. In vitro synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14. J. Virol. **56:**628–632.
33. **Molla, A., S. K. Jang, A. V. Paul, Q. Reuer, and E. Wimmer.** 1992. Cardioviral internal ribosomal entry site is functional in a genetically engineered dicistronic poliovirus. Nature (London) **356:**255–257.
34. **Molla, A., A. V. Paul, M. Schmid, S. K. Jang, and E. Wimmer.** 1993. Studies on dicistronic polioviruses implicate viral proteinase $2A^{pro}$ in RNA replication. Virology **196:**739–747.
34a. **Novak, J. E., and K. Kirkegaard.** 1994. Coupling between genome translation and replication in an RNA virus. Genes Dev. **8:**1726–1737.
35. **Palmenberg, A. C.** 1989. Sequence alignments of picornaviral capsid proteins, p. 211–241. *In* B. Semler and E. Ehrenfeld (ed.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, D.C.
36. **Percy, N., W. S. Barclay, M. Sullivan, and J. W. Almond.** 1992. A poliovirus replicon containing the chloramphenicol acetyltransferase gene can be used to study the replication and encapsidation of poliovirus RNA. J. Virol. **66:** 5040–5046.
37. **Pfister, T., D. Egger, and K. Bienz.** 1995. Poliovirus subviral particles associated with progeny RNA in the replication complex. J. Gen. Virol. **76:**63–71.
38. **Pilipenko, E. V., V. M. Blinov, and V. I. Agol.** 1990. Gross rearrangements within the 5′-untranslated region of the picornaviral genomes. Nucleic Acids Res. **18:**3371–3375.
39. **Pogue, G. P., and T. C. Hall.** 1992. The requirement for a 5′ stem-loop structure in brome mosaic virus replication supports a new model for viral positive-strand RNA initiation. J. Virol. **66:**674–684.
40. **Porter, D. C., D. C. Ansardi, W. S. Choi, and C. D. Morrow.** 1993. Encapsidation of genetically engineered poliovirus minireplicons which express human immunodeficiency virus type 1 Gag and Pol proteins upon infection. J. Virol. **67:**3712–3719.
41. **Porter, D. C., D. C. Ansardi, and C. D. Morrow.** 1995. Encapsidation of poliovirus replicons encoding the complete human immunodeficiency virus type 1 *gag* gene by using a complementation system which provides the P1 capsid protein in *trans*. J. Virol. **69:**1548–1555.
42. **Quadt, R., M. Ishikawa, M. Janda, and P. Ahlquist.** 1995. Formation of brome mosaic virus RNA-dependent RNA polymerase in yeast requires coexpression of viral proteins and viral RNA. Proc. Natl. Acad. Sci. USA **92:** 4892–4896.
43. **Richards, O. C., and E. Ehrenfeld.** 1990. Poliovirus RNA replication, p. 89–119. *In* V. R. Racaniello (ed.), Picornaviruses. Springer-Verlag KG, Berlin.
44. **Rohll, J. B., N. Percy, R. Ley, D. J. Evans, J. W. Almond, and W. S. Barclay.** 1994. The 5′-untranslated regions of picornavirus RNAs contain independent functional domains essential for RNA replication and translation. J. Virol. **68:**4384–4391.
45. **Rossmann, M. G., E. Arnold, J. W. Erickson, E. A. Frankenberger, J. P. Griffith, H.-J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend.** 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature (London) **317:**145–153.
46. **Rueckert, R. R., and E. Wimmer.** 1984. Systematic nomenclature of picornavirus proteins. J. Virol. **50:**957–959.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Sherry, B., and R. R. Rueckert.** 1985. Evidence for at least two dominant neutralization antigens on human rhinovirus 14. J. Virol. **53:**137–143.
49. **Sommergruber, W., H. Ahorn, A. Zöphel, I. Maurer-Fogy, F. Fessl, G. Schnorrenberg, H.-D. Liebig, D. Blaas, E. Kuechler, and T. Skern.** 1992. Cleavage specificity on synthetic peptide substrates of human rhinovirus 2 proteinase 2A. J. Biol. Chem. **267:**22639–22644.
50. **Wimmer, E., C. U. T. Hellen, and X. Cui.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353–436.