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Role of Maturation Cleavage in Infectivity of Picornaviruses: Activation of an Infectosome

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Maturation of picornaviruses involves assembly of a "provirion," which undergoes an autocatalytic cleavage of VP0 to VP2 plus VP4. RNA transcripts from a cDNA clone of human rhinovirus 14 mutated at asparagine 68, one of the residues in the maturation cleavage site, generated normal yields of 150S particles which were noninfectious in the plaque assay because they were unable to initiate a second cycle of infection. These cleavage-defective provirions were otherwise indistinguishable from mature virions in sedimentation coefficient, binding affinity to monoclonal antibodies against neutralization sites IA, II, and III, attachment to HeLa cell receptors, and rate of cell-mediated conformational changes to form 125S A-particles and 80S empty capsids. These results suggest that maturation cleavage is required for the function of a previously undescribed intermediate which transfers packaged RNA across the membrane and into the cytosol. For this hypothetical intermediate, we propose the name infectosome. Since the native virus has a particle/PFU ratio of about 800, such an intermediate will be difficult to find. Mutations at serine 10 in VP2 reduced maturation cleavage to a rate sufficiently slow to show that the infectivity of virus particles increased with the degree of cleavage of VP0 to VP4 and VP2. This article describes the first characterization of a pure form of a picornaviral provirion, and hence the first direct evidence that provirions of picornaviruses lack infectivity.

Provirions of picornaviruses are particles that contain more VP0 than mature virions (43). These particles were first observed by labeling Mahoney type 1 poliovirus-infected HeLa cells by a protocol designed to enrich for newly synthesized virus particles (13). Provirions were reported initially to sediment at 125S (13) and later to cosediment with the mature 150S virions (17). However, the 125S particles could not be isolated again (17). Unlike mature virions, these particles were dissociable by treatment with 20 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), or 3 M cesium chloride (13, 17).

The general concept that provirions are not infectious was suggested by the lack of infectivity of the 125S poliovirus particles (17). It will be shown here, however, that 125S particles are transition forms homologous to A-particles from mature virions and would therefore not be expected to be infectious. It was not possible to demonstrate whether 150S provirions were similarly noninfectious because they cosedimented with infectious mature virions at 150S (17). Provirions of bovine enterovirus, sedimenting at 145S rather than the 165S of mature virions, could be enriched by repeated sedimentation on sucrose gradients (22). These 145S fractions, with about 50% of the VP0 cleaved, were 24% as infectious as the 165S fractions (22). A cleavage-defective provirion-like particle has also been observed in cells infected at 39.5°C with a temperature-sensitive mutant of Mahoney type 1 poliovirus in which arginine 76 in VP2 was changed to glutamine; in this case, the 125S particles were only 1/10 as infectious as wild-type virions (7). Thus, despite wide acceptance of the notion that provirions are not infectious, the evidence is inconclusive.

We report here the construction of a full-length cDNA clone of human rhinovirus 14 (HRV14), pWR3.26, which produces highly infectious transcripts in vitro with an infectivity that is half of that of genomic RNA from virions. Site-directed mutations for inhibiting maturation cleavage were introduced at asparagine 68 of VP4, one of the residues in the maturation cleavage site, or serine 10 of VP2, the proposed catalytic site, in HRV14 by using a mutagenesis cassette vector and the full-length cDNA clone. With the aid of a highly efficient transfection system, optically quantifiable amounts of 150S true provirions were generated for studies on their infectivity and the location of the infection defect.

MATERIALS AND METHODS

Virus, cells, and media. HRV14 (strain 1059) and H-HeLa cells were gifts from V. V. Hamparian of Ohio State University. HRV14 was passaged in HeLa cells, which were grown in complete growth medium A as a monolayer culture and in complete growth medium B as a suspension culture (33, 49).

H-HeLa cells (33), originally contaminated with mycoplasmas, were cured by treatment with minocycline (5 μg/ml) and tiamulin (10 μg/ml) (46) in suspension culture. The elimination of mycoplasmas was indicated by survival of treated cells in medium containing 6-methylpurine deoxyribose (32) and by the absence of mycoplasmas in cells stained with Hoechst dye (4). The cells were certified free of mycoplasmas by the American Type Culture Collection, from whom the cells are now available as cell line ATCC CRL 1958 (H1-HeLa).

Bacteria and plasmid vectors. *Escherichia coli* JM101 and MV1193 were the hosts for most of the recombinant DNAs. *E. coli* DH5α was the host for isolating recombinant plasmid clones when high transformation efficiency was required. The *E. coli dut ung* mutant strain CJ236 was the host for isolating uracil-enriched single-stranded DNA (ssDNA) for site-directed mutagenesis (29). M13KO7 was the helper phage for ssDNA packaging (53).

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The M13 vectors pBS$^-$ and pBS$^+$ were from Stratagene Inc., La Jolla, Calif. Vector pMJ3, also an M13 vector, is a derivative of vector pUC118 constructed by M. Janda (Institute for Molecular Virology, University of Wisconsin, Madison) to contain a T7 promoter and a unique restriction site for *Stu*I, which cuts immediately after the essential part of the T7 promoter and thereby eliminates extra bases normally added to the cDNA insert when it is ligated to other vectors.

Preparation of viral RNA for cDNA synthesis. HRV14 was grown in HeLa cell suspension as described previously (49). After 8 h of incubation at 35°C, 4×10^8 infected cells were concentrated to 10 ml, frozen, and thawed to release virus particles. To disrupt ribosomes, the supernatant fluid, clarified by centrifugation to remove cell debris, was supplemented with 200 μg of RNase A and incubated at 35°C for 15 min. The virus was pelleted through a 30% (wt/vol) sucrose cushion as described previously (48). The virus pellet was resuspended in 2 ml of extraction buffer (100 mM Tris-Cl [pH 8.0], 300 mM NaCl, 1 mM EDTA) and extracted with phenol. RNA was precipitated, and the dried pellet was dissolved in diethylpyrocarbonate-treated water. The typical RNA yield was about 60 μg, assuming 40 μg per unit of optical density at 260 nm (OD_{260}).

Construction of HRV14 full-length duplex cDNA clone which produces highly infectious transcripts. Details of the construction of the full-length duplex cDNA clone have been described by Lee (30). In brief, the first-strand cDNA was synthesized on a viral RNA template with avian myeloblastosis virus reverse transcriptase as described by Gubler and Hoffman (16) except that sodium PP_i was omitted from the synthesis buffer so that synthesis of the second-strand cDNA was much improved. Second-strand cDNA synthesis was carried out on the RNA-DNA hybrid with *E. coli* DNA polymerase I (16) in the presence of an oligonucleotide corresponding to the first 20 HRV14 genomic bases.

The almost-full-length duplex cDNA (missing the first two viral genomic bases) was originally cloned into the pBS$^-$ vector, but the yield and infectivity of the virion RNA sense transcripts, synthesized by T3 RNA polymerase, were low, and extra 5′-terminal bases have been reported to have an adverse effect on the infectivity of the transcripts (52). The HRV14 cDNA was transplanted into vector pMJ3 at the *Stu*I site. The two missing viral bases were replaced by site-directed mutagenesis, and a unique restriction site for *Mlu*I was placed after the poly(A) tail. This plasmid, containing the full-length HRV14 cDNA, was named pWR3.26 (Fig. 1). It reproducibly yielded 5- to 10-fold more full-length transcripts with T7 RNA polymerase than the previous clones with T3 RNA polymerase. Moreover, the construction (Fig. 1) of this plasmid predicted that its transcripts would have only two extra bases (GG) at the 5′ end and four extra bases (CGCG) after the poly(A) tail of 42 bases.

Site-directed mutagenesis of VP0 with a mutagenesis cassette vector and synthetic oligodeoxyribonucleotide primers. In order to facilitate the packaging of uracil-enriched ssDNA by M13 phage and to reduce the labor required to confirm the sequences of the mutants isolated, a mutagenesis cassette system was adopted. The cassette vector pRVP4 (Fig. 1) was constructed by ligating the *Hin*dIII-*Bal*I (nucleotides 128 to 949) fragment of plasmid pWR3.26 DNA with *Hin*dIII- and *Bal*I-digested DNA from a pBS$^+$ plasmid whose polylinker site had been modified with a *Bal*I linker (GTGGCCAC; BRL Life Technologies, Inc., Gaithersburg, Md.) inserted at the *Sma*I site.

By using the method of Kunkel et al. (29), vector pRVP4 yielded M13-packaged uracil-enriched ssDNA with the same sense as the virion RNA. The mutagenic oligonucleotides were synthesized in the Biotechnology Center, University of Wisconsin, and purified through SepPak C_{18} cartridges (30). Phosphorylation of primers, synthesis of the mutated strands with T4 DNA polymerase, ligation, and transformation were performed as described by Kunkel et al. (29). The cassette regions of selected mutant plasmids were completely sequenced to confirm that no nonspecific mutation had occurred during in vitro DNA synthesis.

For generating full-length clones with a site-directed mutation at VP0, the *Afl*II-*Ava*I fragments of pWR3.26 plasmids were replaced with homologous fragments from mutated pRVP4 plasmids.

In vitro transcription of plasmid DNA. DNA from plasmid pWR3.26 or its mutant derivatives was linearized by *Mlu*I restriction enzyme digestion. Run-off RNA transcripts were synthesized in vitro with T7 RNA polymerase under standard conditions (27, 52). The products were electrophoresed in a 0.8% agarose gel along with a known amount of virion RNA. The gel was stained with ethidium bromide to estimate the amount of full-length transcripts.

Infectivity assay of virion RNA and RNA transcripts. The infectivities of transcripts and virion RNA were assayed in 60-mm monolayers of H1-HeLa cells (2.5×10^6 cells). The monolayers were washed twice with 5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (HBS) (52) and then inoculated with diluted RNA in 0.2 ml of HBS containing 40 μg of DEAE-dextran and 16 U of RNasin (Promega Corporation, Madison, Wis.) at 23°C for 1 h. The presence of an RNase inhibitor in the transfection buffer produced a 100-fold increase in the infectivity of the transcripts over that in the control without an RNase inhibitor. The transfected monolayers were washed once with 5 ml of phosphate-buffered saline (PBS) to eliminate the DEAE-dextran, which inhibits the growth of HRV14. The subsequent overlaying, incubation, and plaque visualization steps have been described by Sherry and Rueckert (49). For temperature sensitivity tests, plates were also incubated at 31.5 and 37°C.

Plaque assay of virus infectivity. The infectivity of whole virus was assayed in 60-mm monolayers of H1-HeLa cells (2.5×10^6 cells). The monolayers were washed with 5 ml of PBS and then inoculated with diluted virus in 0.2 ml of PBSA2 (PBS with 0.1% bovine serum albumin [BSA]) at 23°C for 1 h. The subsequent overlaying, incubation, and plaque visualization steps were done as described by Sherry and Rueckert (49).

Preparation of radiolabeled NECs and mature virions. H1-HeLa cells (4×10^7/ml) in suspension were incubated with HRV14 at 10 PFU/cell at 23°C for 1 h. Cells were diluted 10-fold with medium AL (medium A lacking all amino acids except glutamine). After 4 h of incubation at 35°C, guanidine HCl was added to a final concentration of 3 mM. Radiolabel (0.5 mCi of [^{35}S]methionine [Amersham SJ.1015] or [^{3}H]leucine [Amersham TRK.510] per 10^8 cells) was added 15 min later. Virus particles were harvested at 6 h postattachment. After sedimentation through a 30% (wt/vol) sucrose cushion, the pellet was resuspended in PBSA1 (PBS with 0.01% BSA) and layered on a 12-ml 7.5 to 45% (wt/wt) sucrose gradient in PBSA1. The gradient was centrifuged in a Beckman SW41 rotor for 2 h at 274,000 × *g* and 16°C and was then fractionated. The fractions of the radioactive peak containing the naturally empty capsids (NECs) and the mature virions were pooled. The yield of mature virions was measured optically, assuming 9.4×10^{12}

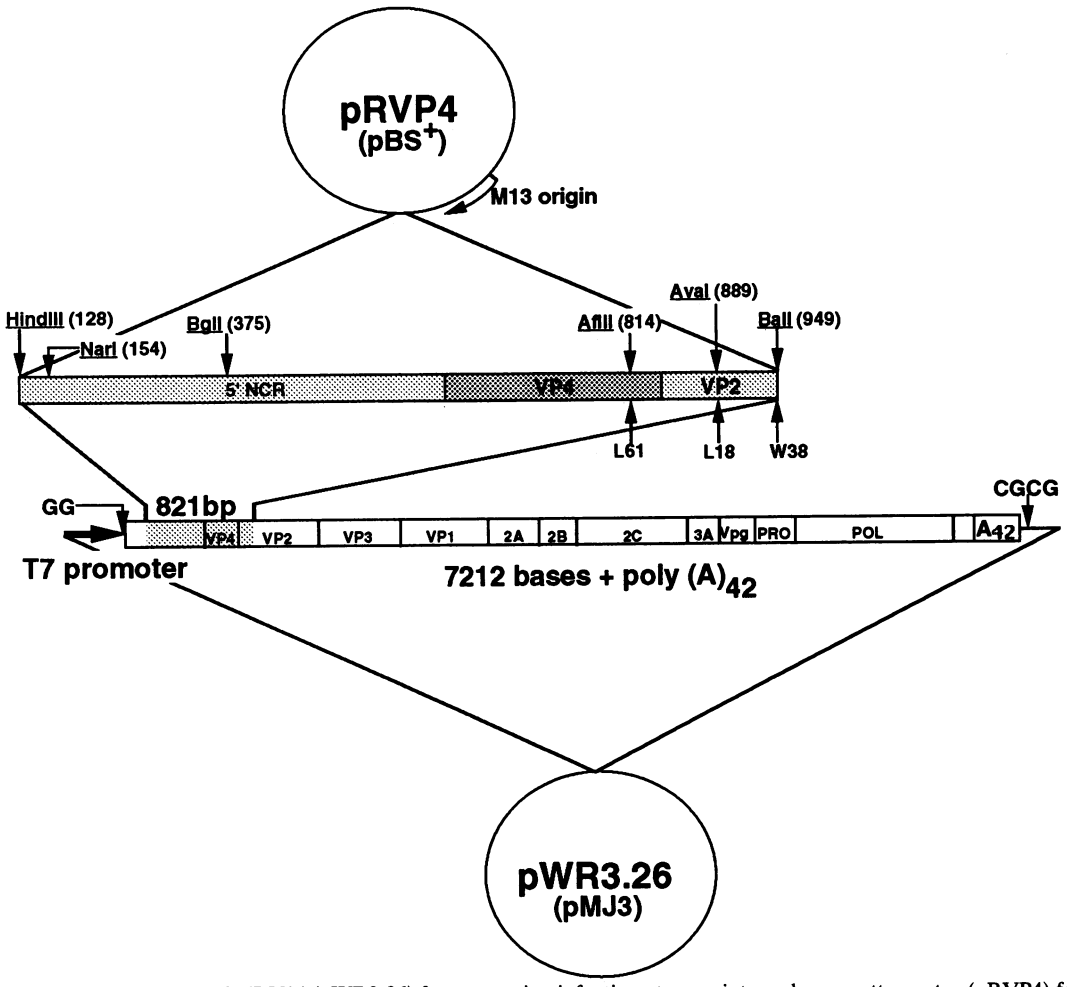

FIG. 1. Full-length cDNA clone of HRV14 (pWR3.26) for generating infectious transcripts and a cassette vector (pRVP4) for introducing site-directed mutations. The construction of the two plasmids is described in Materials and Methods. The full-length cDNA clone of HRV14 with a poly(A) tail of 42 bases was cloned into vector pMJ3. The 5′ end of the cDNA is two bases (GG) from the transcription initiation base of the T7 promoter. A unique *Mlu*I site (ACGCGT) was placed after the poly(A) tail sequence. Its T7 transcript was a half as infective as genomic RNA from virions. The cassette vector containing VP4-positive flanking segments of the 5′ noncoding region and VP2 (bases 128 to 949; shaded area) was cloned into a pBS$^+$ vector whose polylinker site had been modified by addition of a *Bal*I linker (GTGGCCAC) at the *Sma*I site.

particles per OD_{260} unit (48). The total amount of radioactivity incorporated into the NEC peak and the mature virion peak was typically similar.

Production of single-cycle virus particles from RNA transcripts. Transfection was carried out with a liposome suspension containing 0.4 mg of dimethyldioctadecylammonium bromide and 1 mg of dioleoyl-L-α-phosphatidylethanolamine (Sigma Chemical, St. Louis, Mo.) in 1 ml of deionized water, prepared as described by Rose et al. (41). Preparations that had been sonicated until clear could be stored for months in the refrigerator. Each new preparation was assayed for optimum transfection activity by diluting 100 to 300 μl of liposome suspension to 480 μl with water. Each sample was mixed with 160 μl of water containing 3 μg of infectious RNA transcripts which had been phenol extracted and ethanol precipitated three times and then with 640 μl of 2× HBS. The entire sample was pipetted onto a monolayer of 2.5×10^6 H1-HeLa cells in a 60-mm tissue culture plate that had been washed once with 5 ml of HBS. Mutant transcripts were transfected with the optimum concentration of liposomes. After 1 h at 35°C, the plates were overlaid with 2.5 ml of medium AL and then incubated for another 3 h. Virus particles were labeled by addition of 25 μCi of [^{35}S]methionine per plate. At 12 h after transfection, cells were lysed by addition of Nonidet P-40 (NP-40) to a final concentration of 0.4%. The cell debris was removed by centrifugation, and the virus particles were purified by sedimentation through a sucrose cushion and a sucrose gradient as described in the previous section.

Attachment rate and uncoating transitions of HRV14 with H1-HeLa cells. The procedure used to determine the attachment rate was similar to that of Shepard (47). H1-HeLa cell pellets, containing 6×10^7 cells, which had been washed twice with PBSA2 were resuspended in 1.5 ml of PBSA2 containing 2.4×10^{11} radiolabeled virus particles (4,000 particles per cell). The suspensions were incubated at 23°C for 1 h. The cells were pelleted at 23°C and 450 × *g* for 5 min, and supernatant fluids were removed for gradient analysis. The cell pellets were washed with PBSA2 and resuspended in 1.5 ml of PBSA2 (total, 1.75 ml). Samples of the resus-

pended cells were lysed by addition of 20 μl of 0.2 N NaOH, and radioactivity was determined.

For studying uncoating transitions, the virus-cell complexes (1.6 ml) were incubated at 35°C for 1 and 2 h. The cells were pelleted, and supernatant fluids (400 μl, containing the eluted particles) were layered over 5 to 30% (wt/vol) sucrose gradients and centrifuged in an SW41 rotor for 70 min at 274,000 × *g* and 16°C. The cell pellets were washed once with 8 ml of PBSA2 and resuspended in 1.4 ml of PBSA2. Cells were lysed by the addition of NP-40 to 1% and deoxycholate to 0.5%. Cell debris was pelleted, and 400 μl of supernatant fluid was analyzed on a 5 to 30% (wt/vol) sucrose gradient.

Gel electrophoresis of proteins. The coat proteins of purified radiolabeled virus particles were separated by a tricine-SDS-polyacrylamide gel electrophoresis (PAGE) system (45) which was modified by including 100 mM phosphate, pH 7.4, in all gel buffers and casting only a 28-cm-long separating gel. After the gel was run at 100 V for 20 to 24 h, it was fixed in 50% methanol–10% acetic acid for 1 to 2 h, soaked in a fluorographic reagent (Amplify, Amersham, Arlington Heights, Ill.) for 1 h, and dried for autoradiography.

Binding of maturation cleavage mutant virus particles with monoclonal antibodies against the three major neutralizing epitopes of wild-type HRV14. Diluted antibody (5.0×10^8 to 2.0×10^{10} immunoglobulin G [IgG] molecules in 10 μl of PBSA2) was added to [^{35}S]methionine-labeled virus (2.0×10^9 particles in 190 μl of PBSA2) in a microcentrifuge tube. The mixtures were incubated at 23°C for 3 to 4 h, and then 5 μl of IgGSorb (killed cells of *Staphylococcus aureus*; IGSL 10; binding capacity, 1.8 μg of IgG per μl; The Enzyme Center, Inc.) was added, incubated at 23°C for 1 h, and centrifuged at 10,000 × *g* and 8°C for 2 min. The pellet was washed once with 200 μl of RIPA buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% NP-40, 1% deoxycholate, 1% SDS), and centrifuged. The radioactivity of all the supernatant fluids and the final pellet, dissolved in NaOH, was determined. Percent virus particles bound to antibodies (radioactivity associated with the pellet divided by the total radioactivity recovered) was plotted against the number of input antibody molecules per virion. The graph was used to determine the number of input antibody molecules required to bind 50% of the virus particles.

Dideoxynucleotide sequencing of cDNA and viral RNA. Duplex plasmid DNA was sequenced by the primer extension method with Sequenase (a modified T7 DNA polymerase from United States Biochemical Corp., Cleveland, Ohio) under the conditions recommended by the supplier.

Viral RNA was sequenced by the primer extension method with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.) under the conditions described previously (20).

The gel electrophoresis, gel fixation, and autoradiography steps have been described by Sherry et al. (48).

Northern (RNA blot) analysis of viral positive-strand RNA accumulation in infected cells. Virus particles (4.8×10^{11}) were allowed to attach to H1-HeLa cells (1.2×10^8) at 23°C for 1 h. Unattached virus was removed by pelleting and washing, and cell pellets were resuspended in 30 ml of medium B at 35°C. At various times postattachment, 4 ml of cells (1.6×10^7) was removed and centrifuged. The cell pellet was resuspended in 0.4 ml of TNE (50 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA) containing 0.4% NP-40 and 2.5 U of Inhibit-ACE (an RNase inhibitor from 5 Prime → 3 Prime, Inc., West Chester, Pa.). After a 10-min incubation on ice, nuclei were removed by centrifugation. The supernatant fluid was extracted with phenol, and total cytoplasmic RNA was precipitated with ethanol. RNA (60 μg in 5 μl) was electrophoresed in a 0.8% agarose gel containing 6.6% formaldehyde and then transferred to a nylon membrane (GeneScreen Plus; Du Pont, Boston, Mass.). To detect positive-strand viral RNA, the membrane was hybridized to two ^{32}P-labeled primers complementary to bases 107 to 127 and bases 180 to 199 in the 5′ noncoding region of genomic RNA. The prehybridization, hybridization, washing, and autoradiography steps were done as described by Woods (55).

Sequence manipulation by computer. The University of Wisconsin Genetics Computer Group package (9) was used for all sequence manipulations on the computer.

RESULTS

cDNA clone of HRV14 which produces highly infectious transcripts. A full-length cDNA clone of HRV14 with a poly(A) tail of 42 bases (sequence data not shown) was cloned into vector pMJ3. The 5′ end of the cDNA is two bases (GG; sequence data not shown) from the transcription initiation base of the T7 promoter. A unique *Mlu*I site (ACGCGT) was placed after the poly(A) tail sequence. This clone was named pWR3.26 (Fig. 1). The construction of this plasmid predicted that the in vitro T7 transcript would have two extra bases at its 5′ end. This was confirmed by sequencing the transcripts (data not shown). Furthermore, viral RNA extracted from the transcript-derived virions (even only one growth cycle after transfection) had lost those two extra bases (sequence data not shown).

In vitro transcripts generated from the *Mlu*I-linearized pWR3.26 plasmid DNA by T7 RNA polymerase reproducibly produced 2×10^6 to 3×10^6 PFU/μg with DEAE-dextran as the transfection mediator. This infectivity was about half that of genomic RNA from virions (4×10^6 to 6×10^6 PFU/μg). In comparison with our pWR3.26 clone, the transcripts from HRV14 cDNA clone pRV14 (34) were only about 5 to 10% as infective as authentic viral RNA (estimated from the crystal violet-stained plates shown in reference 34). Because pRV14 had a poly(A) tail of 100 bases, the low infectivity of its transcripts was probably due to the presence of 21 nonviral 5′ bases and about 13 extra 3′ bases.

The infectivity of the transcripts was increased 10-fold by eliminating most of the extra bases carried into the 5′ and 3′ ends during the early steps of construction, i.e., by reducing the number of 5′ nonviral bases from four to two and by reducing the number of 3′ nonviral bases after the poly(A) tail from 17 to 4. This observation on HRV14 agrees with previous reports on other picornaviruses, such as Mahoney type 1 poliovirus (52), the DA strain of Theiler's murine encephalomyelitis virus (40), mengovirus (11), and coxsackie B3 virus (25); their in vitro transcripts had higher infectivity when extra sequences were removed at both ends.

The length of the poly(A) tail is also an important determinant of infectivity. Transcripts from clones with poly(A) tails of 42 bases were about six- to eightfold more infective than those from clones with poly(A) tails of 14 bases. However, transcripts from clones with poly(A) tails of up to 82 bases were indistinguishable in infectivity from those from clones with tails of 42 bases (data not shown). This result suggested that a poly(A) tail of about 40 bases was sufficient for maximum infectivity of transcripts [assuming that T7 RNA polymerase transcribes longer poly(dT) homopolymers as faithfully as the shorter one]. This result

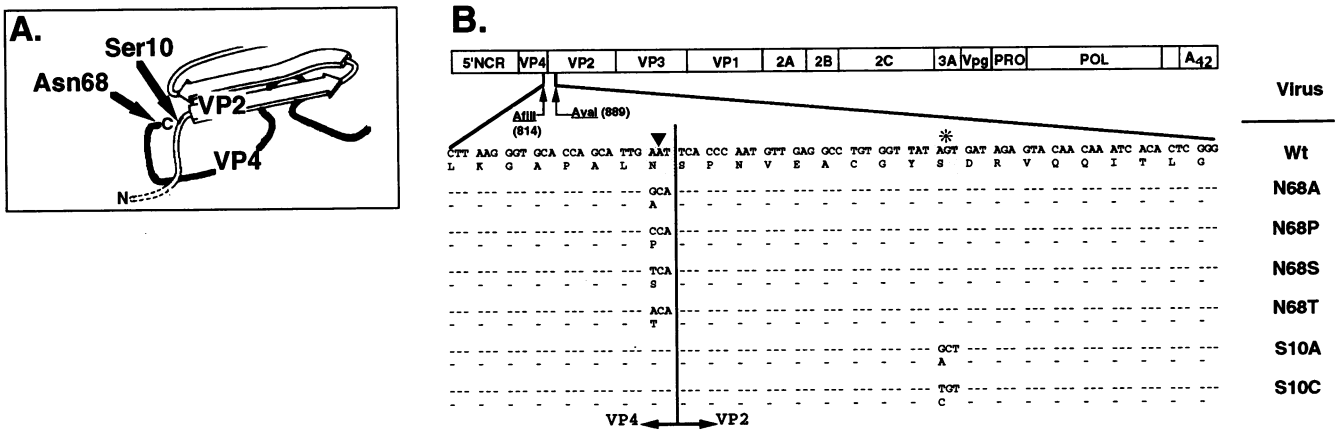

FIG. 2. (A) Ribbon diagram showing the cleaved VP4-VP2 junction (42). (B) Site-directed mutations at the cleavage site (N68) of VP4 (▼) and proposed catalytic site (S10) of VP2 (*). The mutations were introduced by using uracil-enriched ssDNA from pRVP4. The full-length mutant cDNA clones were constructed by replacing the *Afl*II-*Ava*I fragments of pWR3.26 DNA with those of mutated pRVP4 DNA. When diluted transcripts of each clone were transfected onto HeLa cell monolayers with DEAE-dextran, mutants N68A, N68S, S10A, and S10C were as infectious as the wild type (relative infectivity of 1, versus 10^{-5} for mutants N68P and N68T); however, the plaque size of mutants S10A and S10C (1 to 1.5 mm after 3 days) was much smaller than that of the wild type (wt) and mutants N68A, N68P, N68S, and N68T (6 to 7 mm). Plaques were only observed for mutants N68P and N68T when >1 μg of transcripts was used.

agreed with the earlier finding that lengthening the poly(A) tails of poliovirus type 1 transcripts from 12 to about 100 A residues by using poly(A) polymerase increased its infectivity 50-fold (44).

Transcripts of the clone selected for further study, pWR3.26, gave plaques that were twice the diameter of authentic parental virus (data not shown). Thus, the progeny virus from pWR3.26 was named LP1 (for large plaque). The mutation responsible for the large-plaque phenotype was not in the coat protein; it was mapped to a 1.8-kbp *Pfl*MI restriction fragment in the P2 and P3 regions (bases 3432 to 5184).

Sequence of HRV14 cDNA in plasmid pWR3.26. The identity of HRV14 cDNA in pWR3.26 was established by determining the complete sequence of its 7,212 bases (GenBank accession number L05355). This was accomplished by sequencing a nest of deleted plasmids generated by exonuclease III digestion of pWR3.26 (21).

Comparison with the previously determined sequence of 7,212 bases (3) revealed 36 base differences spread throughout the genome. The HRV14 sequence of Stanway et al. (50) differed at only 13 bases (5 in the 5′ noncoding region, 1 in 2A, 3 at 2C, 1 at 3A, and 3 at 3D) but, with only 7,208 bases, was 4 bases shorter than that of pWR3.26. Three of the four base deletions in the 5′ noncoding region of the sequence of Stanway et al. are plausibly attributable to misreading of the sequencing ladder, an interpretation supported by the sequence published by Callahan et al. (3).

Infectivity of mutant transcripts as measured by plaque formation on HeLa cell monolayers. The crystallographic structure of mature HRV14 virions suggested that serine 10 of VP2 might be the catalytic residue for maturation cleavage because it forms a hydrogen bond with the carboxy terminus (asparagine 68) of VP4 (1). Furthermore, the presence of adjacent aspartate (position 11) and arginine (position 12) residues and a β-pleated sheet is reminiscent of the active-site conformation found in normal serine proteases, such as trypsin (38). Although a base (e.g., a histidine residue) required to activate the serine 10 was missing from this structure, it was proposed that the genomic RNA provides this base. Accordingly, the cleavage site (asparagine 68 of VP4) and the proposed catalytic site, serine 10 of VP2, were modified in hopes of generating maturation cleavage-defective mutants.

Site-directed mutagenesis of asparagine 68 of VP4 and serine 10 of VP2 (Fig. 2) was carried out with the mutagenesis cassette vector pRVP4 (Fig. 1). Three independent clones for each site-directed mutation were sequenced through the *Afl*II-*Ava*I fragments (69 bases), and no nonspecific mutations were found. Full-length mutant cDNA clones were then constructed by replacing the *Afl*II-*Ava*I fragments of pWR3.26 DNA with those of the mutated pRVP4 DNA.

Serial dilutions of transcripts made in vitro with T7 RNA polymerase were transfected onto HeLa cell monolayers. Plaque formation was visualized after incubation for 2, 3, or 4 days at 35°C. Wild-type and mutant N68A and N68S transcripts yielded normal plaques (2 to 3 mm) at 35°C after 2 days (mutant designations consist of the wild-type residue [in the single-letter code], the position and the new residue; e.g., in mutant N68A, the asparagine at residue 68 was changed to an alanine). Transcripts from mutants S10A and S10C yielded small plaques (1 to 1.5 mm, versus 6 to 7 mm for the wild type) after 3 days of incubation at 35°C (Fig. 2). However, the specific infectivity (PFU per microgram of transcripts) of mutants S10A and S10C was indistinguishable from that of the wild type and mutants N68A and N68S. Mutants N68P and N68T yielded a few normal-sized plaques with very low infectivity (about 10^{-5} relative to wild-type infectivity) (Fig. 2), presumably because of revertant RNA produced during in vitro transcription. The plaque assay revealed no difference in temperature sensitivity when mutant transcripts were transfected onto HeLa cell monolayers.

Yield of virus particles from a single-step growth cycle initiated by transfection. HeLa cells infected with wild-type virions normally yield about 100 to 200 PFU/cell. Transfection with RNA and DEAE-dextran or Lipofectin (BRL) (31) typically afforded only 5 to 10% of wild-type yields (data not shown). However, by adapting the DNA transfection method of Rose et al. (41), it was possible to achieve RNA

transfection yields of 50 to 80 PFU/cell with wild-type transcripts. We attribute this slightly lower infectivity yield from transfected cells, roughly half that obtained from cells infected with intact virus, to the cytotoxic effects of the transfection procedure. Nevertheless, this high transfection efficiency made it practical to produce purified particles in amounts sufficient for optical quantification, even from mutants not capable of initiating a second round of infection.

The yields of mutant (23, 25, and 15 μg for mutants S10A, S10C, and N68T, respectively) particles from transfected cells were similar to that of wild-type particles (24 μg). This result suggested that each of those mutations had no significant effect on viral replication and assembly; therefore, the poor plaquing efficiency of these mutant transcripts on HeLa cell monolayers may be due to inefficient spread of the mutant particles from transfected cells to adjacent cells. In contrast, no particles were detected in cells transfected with mutant N68P transcripts.

That mutants S10A, S10C, and N68T are synthesized and assembled as efficiently as wild-type virus was supported by other experiments involving recovery of radiolabeled particles on sucrose density gradients (data not shown). No radioactivity was recovered in particles of mutant N68P (data not shown), suggesting that it was assembly defective.

The identity of the mutants was verified by sequencing viral RNA recovered from the virus particles (data not shown). Furthermore, no nonspecific mutation was observed in the region sequenced (asparagine 15 to asparagine 68 of VP4 and serine 1 to aspartate 49 of VP2).

Identification of VP0 in the electrophoretic profile of HRV14. The coat proteins of HRV14 were particularly difficult to separate by SDS-PAGE. The best result achieved is illustrated in Fig. 3. As shown in Fig. 3, lane 1, authentic wild-type virus, i.e., the parental stock from which the cDNA was prepared, displayed five bands: a double one near the top of the gel, one strong and one weak; another pair of incompletely separated strong bands; and a rapidly moving band near the bottom of the gel. The genetic sequence indicates the following sizes: VP0, 35.7 kDa; VP1, 32.4 kDa; VP2, 28.5 kDa; VP3, 26.2 kDa; and VP4, 7.2 kDa. Surprisingly, the weak band (second from the top), expected to be VP0, was not the slowest one. To further identify the bands, NECs were prepared by treating infected cells with 3 mM guanidine at 4 h postattachment, and [^{35}S]methionine was added 15 min later. Since HRV14 is less sensitive to guanidine than Mahoney type 1 poliovirus (data not shown), the formation of mature virions was only partially blocked at this drug concentration, and a significant amount of NECs accumulated in the infected cells. Labeled NECs (80S) were separated from the mature virions (150S) on a sucrose gradient. As shown in Fig. 3, lane 2, the second band in the NECs was now strong, while the third and fifth bands were missing. As expected, the profile of the guanidine-treated 150S particles (Fig. 3, lane 3) was same as that of untreated virions (Fig. 3, lane 1). Because VP0 in picornaviruses is a precursor of VP2 and VP4 (43), this identifies the second, third, and fifth bands as VP0, VP2, and VP4, respectively. Similar results were found with virus LP1, the wild-type version generated from transcripts of the cDNA clone (Fig. 3, lanes 4 and 5).

Evidence for progressive increase in specific infectivity with increasing degree of VP0 cleavage. Figure 4A shows the electrophoretic profile of coat proteins from purified mutant and wild-type particles harvested 12 h after transfection of HeLa cells with transcripts from cDNA clones. All three mutants displayed a marked reduction in VP4 content. The

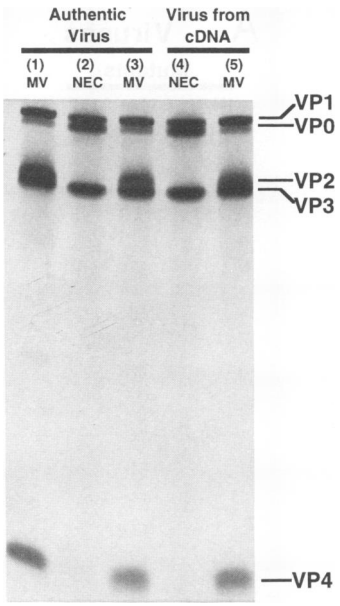

FIG. 3. Electrophoretic profile of coat proteins from mature virions (MV) and NECs of HRV14. Lanes 1 to 3 are from authentic wild-type virus, and lanes 4 and 5 are from "wild-type" virus generated from the cDNA clone pWR3.26. [^{35}S]methionine-labeled NECs were produced in HRV14-infected H1-HeLa cells treated with guanidine HCl (Materials and Methods). Because the 3 mM concentration was suboptimal, radiolabeled mature virions were also produced in this culture. Mature virions and NECs were purified and separated on sucrose gradients. Lane 1, mature virion produced in the absence of guanidine; lanes 2 and 4, NECs produced in the presence of 3 mM guanidine; lanes 3 and 5, mature virions produced in the presence of 3 mM guanidine. Coat proteins of purified particles were separated by SDS-PAGE and visualized by autoradiography as described in Materials and Methods. VP, viral coat protein.

same results were obtained in three independent transfection experiments. Such preparations could be stored for at least a month at −70°C without further cleavage.

However, further cleavage did occur when the particles were incubated at 35°C, but the rate of cleavage varied with the mutation, with the N68T mutant having the slowest rate (Fig. 4B). The S10A mutant had the fastest cleavage rate, but the rate declined markedly after 24 h. At time zero, the VP4 content of mutants S10A and S10C was abut 20% of the wild-type level (Table 1 and Fig. 4B); their infectivities were 4 to 5% of the wild-type level (Table 1 and Fig. 4C). RNA extracted from these two mutant particles was as infectious as that from wild-type virions (Table 1).

As shown in Fig. 4B and C, the infectivity of the two serine 10 mutants increased with increasing VP4 content. Thus, by 24 h, when the VP4 content of S10A had increased fourfold, its infectivity increased eightfold. Similarly, a doubling in the VP4 content of S10C was accompanied by a fourfold increase in infectivity. The apparent lack of a direct relationship between degree of cleavage and infectivity can be attributed, at least in part, to a simultaneous decay in the infectivity of mature virions at 35°C (Fig. 4C). No attempt was made to determine whether this spontaneous thermodecay of wild-type infectivity was due to the known fragmentation of the HRV14 genome (15) or to denaturation of its coat protein.

Mutant N68T had no measurable infectivity except that

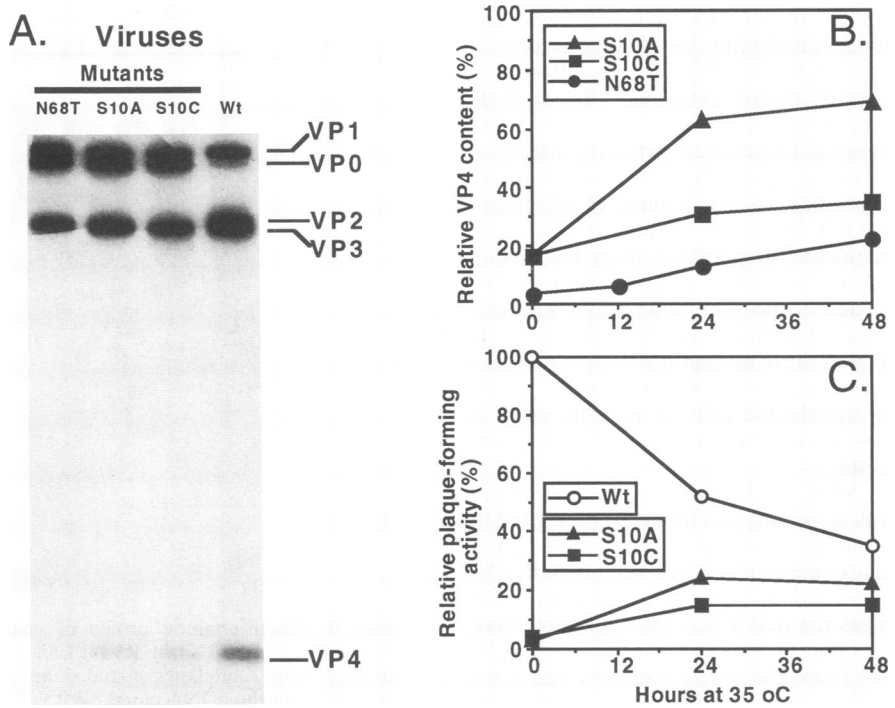

FIG. 4. Evidence for progressive increase in specific infectivity with increasing degree of VP0 cleavage. (A) Autoradiogram of proteins from mutant and wild-type (wt) particles after electrophoretic separation on SDS-polyacrylamide gels. Particles were prepared as described in the text. (B) Differential effect of mutations on rate of VP0 cleavage. Preparations containing 23,000 cpm of purified particles (wild type, 0.75 μg; S10A, 0.7 μg; S10C, 0.6 μg; and N68T, 0.5 μg) were incubated in 30 μl of PBSA1 for the indicated times (hours) at 35°C. Each sample was then divided into two portions; 25 μl was used for electrophoretic analysis and 5 μl was used for titering infectivity by the plaque assay. The intensity of VP4 bands on autoradiograms was quantified by densitometry and normalized to that of the wild-type virus, whose VP4 content remained constant over time. (C) Infectivity curves. Relative infectivity is specific infectivity (PFU per microgram) normalized to that of wild-type (wt) virions at time zero (7.3×10^7 PFU/μg in this experiment). No corrections were made for decay in the infectivity of wild-type virions at 35°C. Plaques were counted for the wild type (2 to 3 mm in diameter) and mutants S10A and S10C (1 to 1.5 mm in diameter) after 2 and 3 days, respectively. Mutant N68T showed no plaque-forming activity even after 5 days in HeLa cell monolayers, while mutants S10A and S10C gave plaques of 4 to 5 mm in diameter.

due to reversions (Table 1). In this case, the increase in VP4 content from 4% at time zero to 20% after 48 h at 35°C was not accompanied by any measurable increase in plaque-forming activity (see legend to Fig. 4C). This does not necessarily mean that partially cleaved particles lack infectivity, as measured by the ability to infect a cell, but it may mean that maturation cleavage occurs too slowly to permit the development of detectable plaques.

TABLE 1. Correlation between extent of maturation cleavage and specific infectivity

Virus	Virus particles[a]			Relative infectivity of virion RNA[c]
	Relative VP4 content[b]	Specific infectivity (PFU/μg)	Relative infectivity	
Wild type	1.0	8.9×10^{7}[d]	1.0	1.0
N68T	0.04	3,200[e]	4×10^{-5}	ND[f]
S10A	0.19	3.8×10^{6}	0.04	1.2
S10C	0.23	4.7×10^{6}	0.05	1.0

[a] A frozen (−70°C) stock of each virus was thawed and diluted in PBSA2, and virus were counted as described in Materials and Methods.
[b] Intensity of mutant VP4 bands on an autoradiogram (see Fig. 4) relative to that of the wild type.
[c] RNA was extracted from the purified particles and transfected into HeLa cell monolayers with DEAE-dextran. The specific infectivity of wild-type virion RNA was 6×10^6 PFU/μg.
[d] Eight hundred particles per PFU.
[e] Probably revertants.
[f] ND, not determined.

Physical properties of cleavage-defective mutants. Provirions of poliovirus are variously reported to sediment at 125S (7, 13) or to cosediment with 150S virions (17); provirions of bovine enterovirus have been reported to sediment at 145S, whereas virions sediment at 165S (22). As shown in Fig. 5A, the sedimentation profile of mutant N68T particles was indistinguishable from that of authentic mature wild-type virions, with a reported sedimentation velocity of 150S (43). The maturation cleavage was accompanied by no detectable change in size. A difference in position of one fraction should have been detected easily; with a peak centered at fraction 36, and assuming similar masses for the virion and mutant provirion, we conclude that their hydrodynamic radii were identical, with an uncertainty of less than 3%. The same conclusion applied to mutants S10A (Fig. 5B) and S10C (data not shown).

Using monoclonal antibodies (48), we were also unable to detect any differences from wild-type virions in the immunological reactivity of neutralization sites IA, II, and III in any of the cleavage-defective mutants (Table 2).

Stability of cleavage-defective mutants in EDTA and SDS. Both forms of the poliovirus provirion, 125S (7, 13) and 150S (17), have been reported to be unstable when sedimented

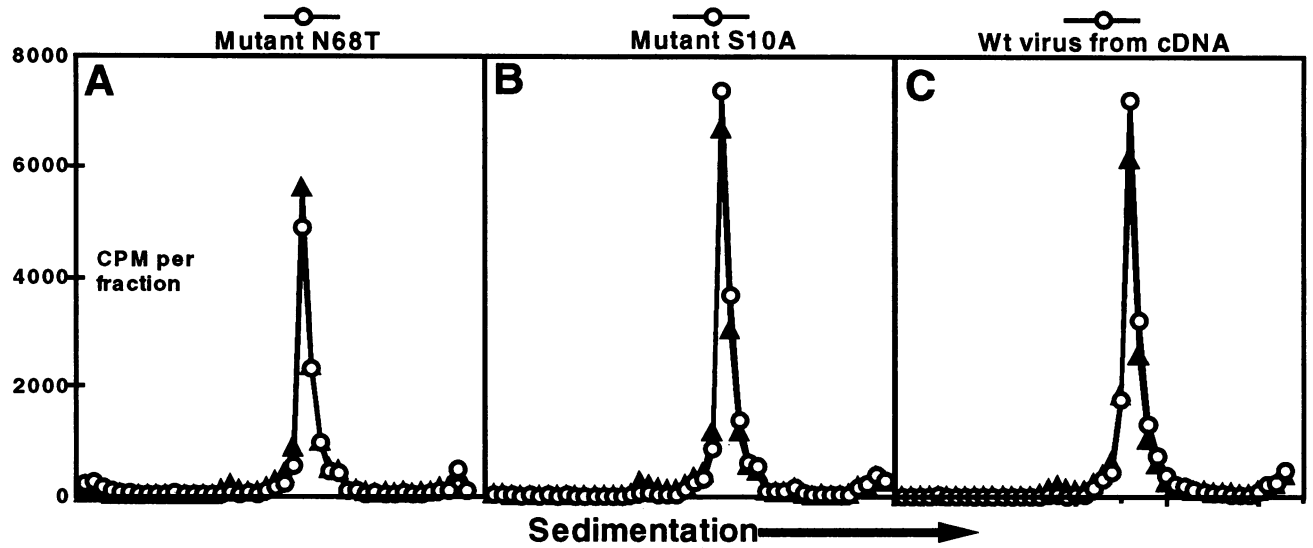

FIG. 5. Maturation cleavage-defective particles cosediment with authentic HRV14 mature virions. Authentic HRV14 mature virions (▲) were sedimented with (A) provirions from mutant N68T transcripts, (B) partially mature particles from mutant S10A transcripts, and (C) mature virions from wild-type (wt) transcripts. Purified [^{35}S]methionine-labeled virus particles recovered from transfected HeLa cells were sedimented along with [^{3}H]leucine-labeled authentic mature virions through a 7.5 to 45% (wt/wt) sucrose gradient. After 2 h of centrifugation at 16°C, the gradient was fractionated, and the radioactivity in each fraction was counted.

through a sucrose gradient containing 20 mM EDTA. By contrast, all three of the cleavage-defective HRV14 mutants were as stable as the parental virus under the same conditions. Typical results are illustrated in Fig. 6A, B, and C).

Poliovirions are stable in 0.5% SDS, while both forms of the provirion are completely labile (7, 13, 17). The cleavage-defective virions of HRV14, on the other hand, were relatively stable, although not completely so, in SDS. With wild-type HRV14 about 20% of the particles were degraded into nonsedimentable material after a 20-min exposure to detergent at 23°C (Fig. 6D); about 30% of the S10A mutant (Fig. 6E) and about 60% of the N68T mutant (Fig. 6F) were degraded under the same conditions.

Attachment of maturation-defective particles to HeLa cells was indistinguishable from that of mature virions. The immature particles were defective in plaque formation on HeLa cell monolayers, although normal yields of particles with wild-type physical properties were obtained when transcripts were transfected onto HeLa cells. Thus, they must lack a function that is required for mature virions to establish infection. Attachment, binding to the cellular receptor (ICAM-1 for HRV14), is the first step in this process.

TABLE 2. Evidence that cleavage-defective mutants are serologically indistinguishable from mature virions[a]

Neutralization site (monoclonal antibody)	No. of MAb molecules/virion required to bind 50% of virions			
	N68T	S10A	S10C	Wild type
IA (MAb17)	0.6	0.6	0.7	0.6
II (MAb18)	0.7	0.7	0.9	0.8
III (MAb22)	2	2	2	2

[a] PBSA2 (200 μl) containing 2.0 × 10^{9} [^{35}S]methionine-labeled virus particles was incubated with different amounts of monoclonal antibodies (MAbs) (0, 0.25, 1, 4, and 10 MAb molecules per virus particle) at 23°C for 3 to 4 h. The virus-antibody complexes were precipitated by addition of IgGSorb (killed cells of *S. aureus*).

Attachment of the virus particles was studied by incubating radiolabeled particles (4,000 particles per cell) with HeLa cells in suspension at 23°C for 1 h. The cells were sedimented, and unattached particles were washed away. The percent attached particles is the fraction of total radioactivity recovered present in the cell pellet. In this experiment, 37% of the input wild-type mature virions attached to HeLa cells; mutant N68T (39%) and mutant S10A particles (44%) behaved similarly. In a separate experiment, S10C particles also attached normally (data not shown). Thus, the lack of infectivity was not due to defects in binding to the cellular receptor.

Uncoating transitions of maturation-defective VP0 mutant N68T in HeLa cells were indistinguishable from those of wild-type HRV14. To further pinpoint the step at which the infectivity of the maturation-defective mutant was blocked, virus-cell complexes were prepared by attaching radioactive particles to HeLa cells for 60 min at 23°C, as described in the previous section. The cells were resuspended in PBSA2, and uncoating was then initiated by warming the suspension to 35°C. After 1 or 2 h, the cells were sedimented, and the supernatant fluid was saved to analyze for eluted particles. The sedimented cells were lysed with 1% NP-40 and 0.5% sodium deoxycholate. The cell extracts, clarified to remove debris, were then analyzed on sucrose density gradients.

It was established in preliminary experiments with wild-type virus that all of the cell-associated radioactivity was present as intact 150S particles after the 60-min attachment period at 23°C. Under these conditions, 39% of the particles (both wild type and mutant) attached; as shown in Fig. 7A and B, the unattached particles (61% of input) were still completely intact at the end of the attachment step.

Only 60% of the attached radioactivity of wild-type HRV14 remained cell associated after 1 h at 35°C (Fig. 7E); only 52% remained cell associated after 2 h (Fig. 7I). Of the remaining radioactivity, 32% was recovered as eluted subparticles after 1 h (Fig. 7C) and 43% after 2 h (Fig. 7G).

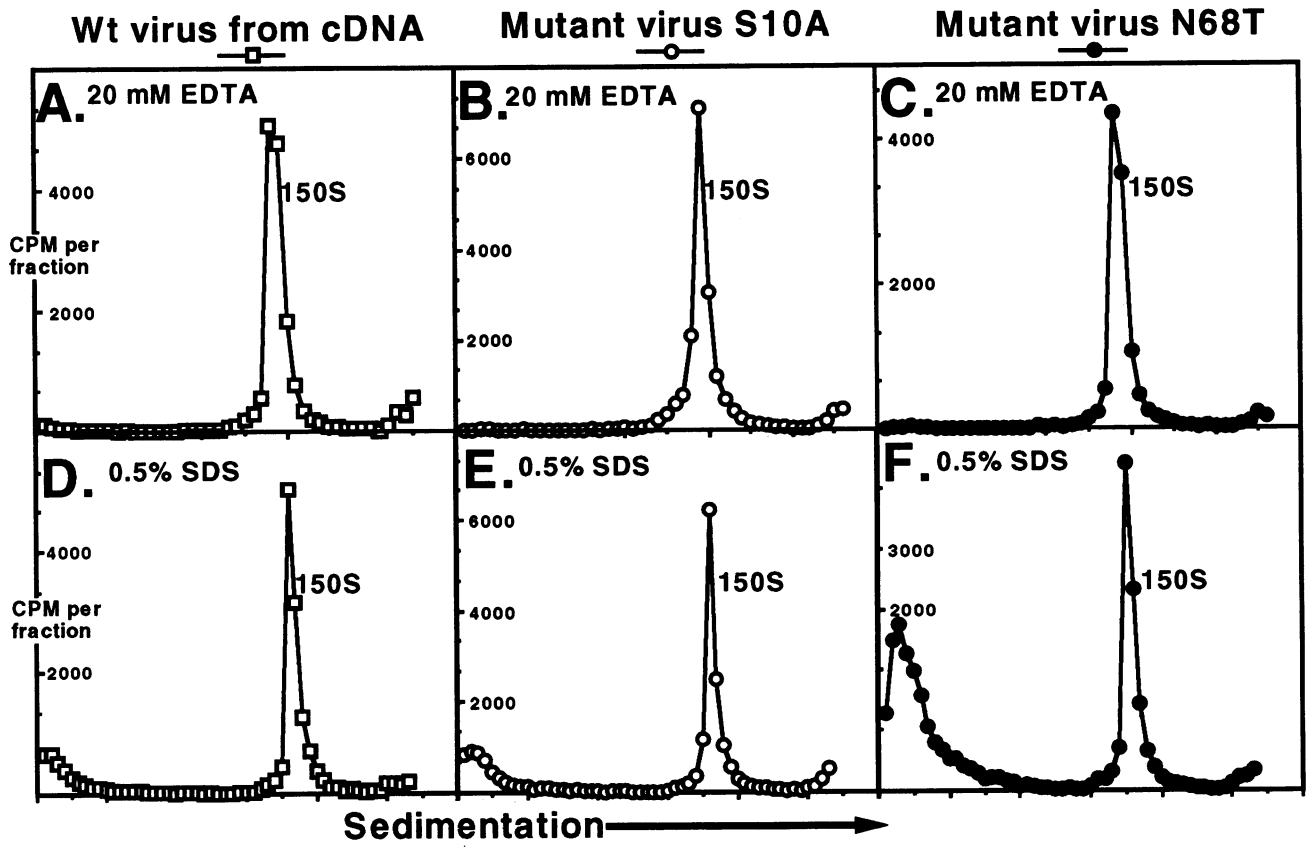

FIG. 6. Sedimentation profiles of HRV14 mature virions and maturation cleavage-defective mutant particles in the presence of 20 mM EDTA (A to C) or after treatment with 0.5% SDS (D to F). Purified 150S virus particles labeled with [^{35}S]methionine were incubated at 23°C for 20 min in a buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, and 20 mM EDTA (A to C) or 100 mM Tris-acetate (pH 7.2), 0.1% BSA, and 0.5% SDS (D to F). Treated particles were analyzed for surviving 150S particles by sedimentation of each sample through a 12-ml 7.5 to 45% (wt/wt) sucrose gradient in a buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 20 mM EDTA, and 0.01% BSA (A to C) or in PBSA1 (D to F). After 2 h of centrifugation at 16°C, the gradient was fractionated, and the radioactivity in each fraction was counted.

Recoveries of the maturation-defective mutant N68T (Fig. 7D, F, H, and J) paralleled those of wild-type virions.

The flow of radioactivity in wild-type virus-cell complexes fit the generally accepted picture that infection involves conversion of intact 150S mature virions to 125S swollen A-particles lacking VP4, followed by release of genomic RNA to form 80S empty protein shells (8). As shown in Fig. 7E, about a third of the wild-type virions survived as 150S particles; the rest were converted into subparticles sedimenting at 125S, 80S, and soluble material remaining at the top of the gradient. No 150S particles were found in the eluted fraction (Fig. 7C). By 2 h, most of the cell-associated virions had been converted into soluble material (Fig. 7I) and 43% had been eluted from the cells (Fig. 7G). Again, the sedimentation profiles of the maturation-defective mutant N68T (Fig. 7D, F, H, and J) paralleled those of wild-type virions. The compression of the 80S, 125S, and 150S peaks in Fig. 7E and F relative to those in Fig. 7C and D is due to the greater number of fractions caused by smaller drop sizes at the top of gradients containing detergent-treated cell extracts.

That intact 150S particles from mutant N68T underwent normal cell-mediated uncoating transitions indicated that formation of dissociable VP4 by cleavage of VP0 to VP4 plus VP2 was not required for the swelling of 150S particles to form 125S A-particles and subsequent release of RNA to form 80S empty capsids. However, mutant N68T particles were not infectious, even though cells transfected with its RNA transcripts generated normal yields of 150S particles, which attached to HeLa cells normally. Therefore, formation of VP4 and VP2 was somehow important for targeting the genomic RNA into the cytosol, the site of viral replication.

Evidence that genomic RNA of mutant particles was not transferred to the cytosol. The view that the genomic RNA of the mutant particles was not delivered into the cytosol was confirmed by studying viral RNA synthesis in HeLa cells. At various times after attachment, total cytoplasmic RNA was extracted, electrophoresed, blotted to a nylon membrane, and then probed for positive-strand viral RNA. Positive-strand RNA from the wild-type virus was detected at 5 h postattachment (Fig. 8, lane 7) and continued to accumulate until the end of the experiment (Fig. 8, lanes 10 and 13). By contrast, there was very little evidence of newly synthesized viral RNA in cells infected with the two mutants. No mutant N68T positive-strand RNA was detected at 8 h (Fig. 8, lane 11), even when the exposure time was extended to 48 h (data not shown). A weak signal for positive-strand RNA from mutant S10A was detected at 8 h (Fig. 8, lane 12) and suggested that only a small fraction of cells were infected. This was consistent with the data shown in Table 1, indicat-

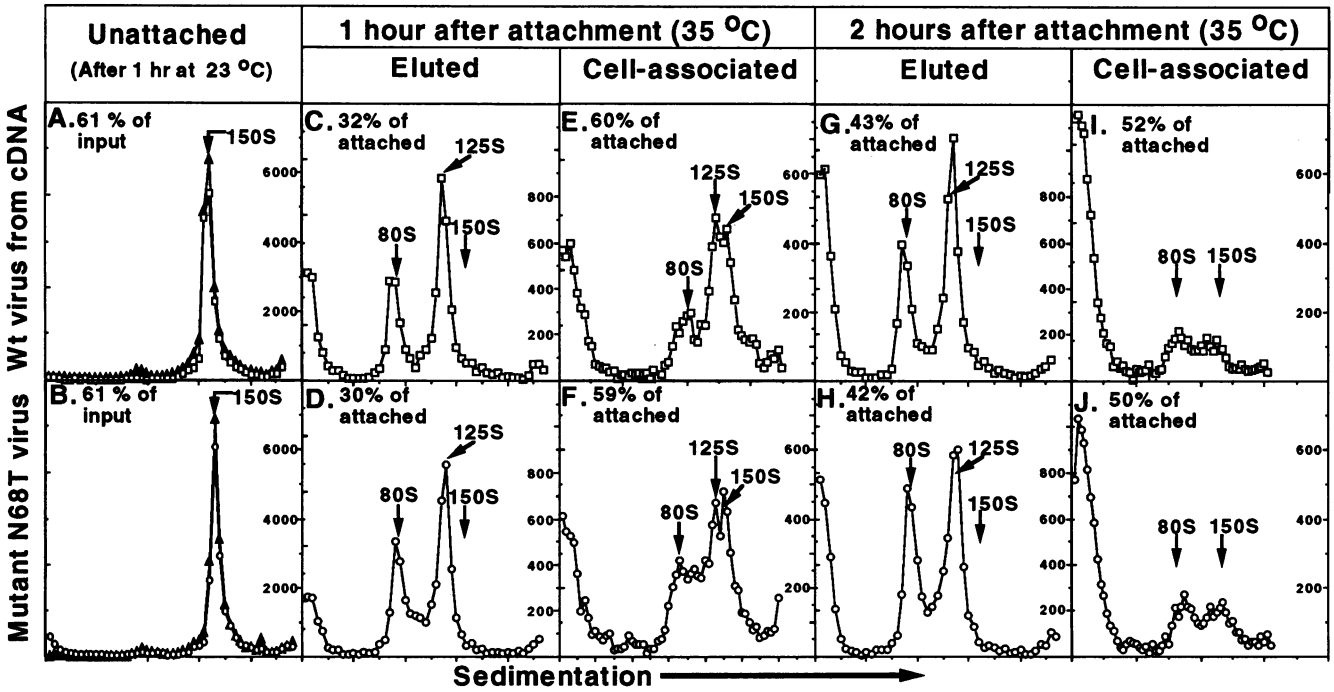

FIG. 7. Sedimentation analysis of viral and subviral particles recovered at early stages of infection. See Materials and Methods for experimental details. (A and B) Unattached virus particles after 1 h of incubation of virus particles labeled with [^{35}S]methionine with H1-HeLa cells at 23°C. (C, D, G, and H) Eluted particles after H1-HeLa cells with attached viruses were incubated at 35°C for 1 h (C and D) or 2 h (G and H). (E, F, I, and J) Cell-associated particles after 1 h (E and F) or 2 h (I and J) of incubation at 35°C. The unattached particles, eluted particles, or cell-associated particles (released by treating the cells with 1% NP-40 and 0.5% deoxycholate) were sedimented along with [^{3}H]leucine-labeled markers, authentic 80S NECs, and 150S mature virions through a 5 to 30% (wt/vol) sucrose gradient in PBSA1. After 70 min of centrifugation at 16°C, the gradient was fractionated, and the radioactivity in each fraction was counted. The sedimentation values in the figure were assigned with the aid of [^{3}H]leucine-labeled markers, whose sedimentation profiles were omitted for clarity. Symbols: ▲, [^{3}H]leucine-labeled authentic wild-type mature virion (150S); ○, [^{35}S]methionine-labeled mutant N68T provirions; □, [^{35}S]methionine-labeled mature virions of wild-type virus from the cDNA. The scales are in cpm per fraction.

ing that its particle/PFU ratio was about 25-fold higher than that for the wild type.

DISCUSSION

We report here the construction of a full-length cDNA clone of HRV14, pWR3.26, which produces highly infectious in vitro transcripts with an infectivity that is half as great as that of genomic RNA from virions. That infectivity, 2×10^6 to 3×10^6 PFU/μg of transcripts, is one of the highest reported for picornaviruses (Table 3). Sarnow reported a value of 1.5×10^6 PFU/μg for transcripts of Mahoney type 1 poliovirus cDNA clone; in this case, however, addition of a longer poly(A) tail was required after in vitro transcription (44). cDNAs and infectious transcripts (which yielded progeny virus when transfected into permissive cells) of poliovirus type 2 (35, 36), hepatitis A virus (6), foot-and-mouth disease virus (56), and Theiler's virus strain GDVII (51) have also been synthesized, but the specific infectivity of the in vitro transcripts was not reported. The cDNAs of some viruses were infectious but had a much lower infectivity than the in vitro transcripts when specific infectivity was reported, including poliovirus type 1 (specific infectivity, 3,600 PFU/μg [28] and 7 PFU/μg [39]), poliovirus type 2 (35, 36), poliovirus type 3 (54), coxsackievirus B3 (2 PFU/μg) (23), hepatitis A virus (6), and echovirus 6 (2). More importantly, we also showed that it was feasible to use mutant derivatives of pWR3.26 transcripts in combination

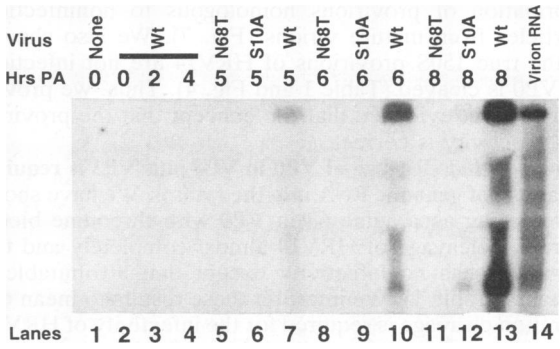

FIG. 8. Time course of viral positive-strand RNA synthesis in wild-type (wt) and maturation cleavage-defective mutant particles attached to H1-HeLa cells. Virus particles (4,000 per cell) were attached to HeLa cells in suspension (4×10^7 cells per ml in PBSA2) at 23°C for 1 h. Unattached particles were washed off. In this experiment, 43.9, 46.6, and 33.4% of input particles were attached to HeLa cells for mutant N68T, mutant S10A, and wild-type viruses, respectively. Cell pellets were resuspended in medium B (4×10^6 cells per ml) and incubated at 35°C. At various times postattachment (PA), total cytoplasmic RNA was extracted from cells (cells were lysed with 0.5% NP-40 and phenolated). RNA from 2.5×10^6 cells (60 μg) was electrophoresed in a 0.8% agarose gel in the presence of formaldehyde and then transferred onto a nylon membrane. Immobilized positive-strand viral RNA was detected with two oligonucleotides (complementary to bases 107 to 127 and bases 180 to 199 of the HRV14 genome) which were end-labeled with [γ-^{32}P]ATP. The autoradiogram was developed after exposure to the hybridized membrane for 2 h.

TABLE 3. Infectivity of virion RNA and in vitro transcripts of picornaviruses which have been cloned into cDNA

Virus (reference)	Infectivity[a] (PFU/μg)	
	Virion RNA	Transcript
Poliovirus type 1 (52)	2.1×10^6	1.0×10^5
Poliovirus type 1[b] (24)	1×10^6	4×10^4
pWR3.26 (this study)	4×10^6–6×10^6	2×10^6–3×10^6
HRV14[c] (34)	1×10^3–2×10^3	100
HRV2 (10)	100–200	20–50
Coxsackievirus B3 (25)	2×10^5	14–30
Cardioviruses		
Encephalomyocarditis virus B (37)	9.0×10^4	6.2×10^3
Encephalomyocarditis virus D (37)	5.6×10^5	2.3×10^3
Mengovirus (11, 12)	2.4×10^6	6.6×10^4
Theiler's virus strain DA (40)	3.6×10^5	1.8×10^4

[a] Infectivities were determined with DEAE-dextran as the facilitator.
[b] The infectivity of transcripts was similar to that of virion RNA when the poly(A) tails were lengthened from 12 to 100 residues by poly(A) polymerase after in vitro transcription (44).
[c] Estimated from the pictures of crystal violet-stained plates shown in Fig. 3 of reference 34.

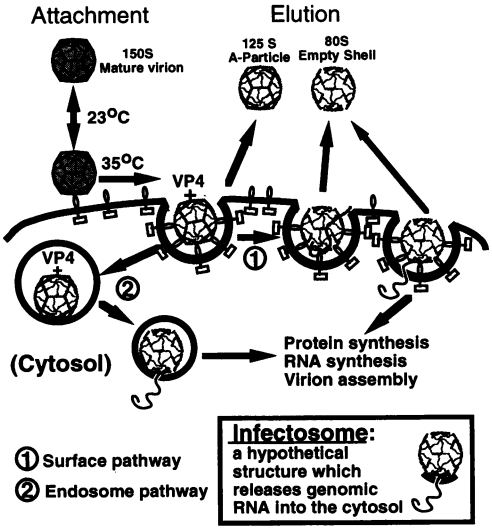

FIG. 9. Proposed stages in the establishment of infection by HRV14. Maturation-defective provirions (mutant N68T) attached to HeLa cells normally and underwent all of the known transitions thought to be involved in infection (even the rates of the transitions were similar), yet they were not infective. The mutant particles N68T (Fig. 7) and S10A (data not shown) appeared to undergo the same degradative transition as wild-type virus. We propose that maturation cleavage is required for the function of an intermediate (infectosome) which transfers its RNA through the membrane. Because only 1 of every 800 mature virions is infectious (Table 1), such an intermediate, which remains to be identified by biochemical studies, is likely rare.

with a highly efficient transfection procedure (41) to study nonviable maturation cleavage-defective mutants. This study on the functional defect of provirions suggested a new intermediate, which we term the infectosome, in picornavirus infection.

Evidence that picornaviral provirions are not infectious. The concept that picornaviral provirions are not infectious is based upon a single report (13) that 125S particles, then thought to be provirions, lack infectivity. We have shown here that the true provirion cosediments with 150S mature virions (Fig. 5) and that the noninfectious 125S particle is a conformation of provirions homologous to noninfectious A-particles from mature virions (Fig. 7). We also showed that the true 150S provirions of HRV14 are not infectious until VP0 is cleaved (Table 1 and Fig. 4). Thus, we provide the first direct evidence that the concept that the provirion lacks infectivity is correct.

Evidence that cleavage of VP0 to VP4 plus VP2 is required for transfer of genomic RNA into the cytosol. We have shown that replacing asparagine 68 in VP0 with threonine blocks maturation cleavage of HRV14 almost completely and that this mutant has no infectivity except that attributable to revertants (Table 1). We interpret these results to mean that maturation cleavage is required for the infectivity of HRV14.

That maturation cleavage itself is required for infectivity, and not a structural abnormality associated with the mutation, is supported by several lines of evidence. First, the infectivity of the S10A and S10C mutants, in which maturation cleavage is markedly slower than in the wild-type virus, increases as the VP4 content increases (Fig. 4B and C). Second, the mutants exhibit no measurable differences in either viral replication or assembly, as measured by particle yield from cells transfected with RNA transcripts, nor do they show any differences from the wild type in antigenicity (Table 2) or hydrodynamic radius (Fig. 5).

Finally, as shown in Fig. 7, the mutants exhibited no measurable differences from wild-type virions in attachment to HeLa cells or in the rate of cell-mediated conformational transitions (swelling of the protein shells and formation of RNA-free capsids) normally associated with infection by picornaviruses. These transitions may indeed be required for infection; they are not sufficient to ensure infection. We propose that cleavage of VP0 to VP4 plus VP2 is a prerequisite for the formation of an intermediate structure (infectosome) required for the transfer of genomic RNA into the cytosol, the site of viral replication (Fig. 9).

Thus, the dissociability of VP4 molecules from the protein shell appears to be essential for the function of an infectosome. There are two possible models. The first is that released VP4 molecules somehow form channels to allow the movement of genomic RNA into the cytosol (Fig. 9). This is supported by the observation that the N terminus of VP4 is myristoylated (5). Furthermore, addition of VP4 protein to HeLa cells sensitizes the cells for infection by isolated viral RNA (reference 26, p. 300 to 306). An alternative is that the presence of uncleaved VP4 protein at the pentamer vertex blocks the extrusion of the N terminus of VP1, which is thought to form a pore or to disrupt the membrane to facilitate the transport of the viral genome into the cytosol (14).

Evidence that serine 10 of VP2 is not the catalytic residue for maturation cleavage. It has been suggested (1) from structural studies on HRV14 that serine 10 of VP2 might be the catalytic residue responsible for maturation cleavage, based on its connection, through a hydrogen bond, with asparagine 68, the carboxy terminus of VP4 (1), and the presence of adjacent aspartate and arginine residues and a β-pleated sheet resembling the active site found in serine proteases, such as trypsin (38). The conservation of amino acid sequences near the amino terminus of VP2 seemed to confirm this model (38).

However, this hypothesis seems to be contradicted by our

observation that replacing serine 10 with an alanine or a cysteine residue did not block the maturation cleavage of HRV14. Harber et al. reached a similar conclusion for Mahoney type 1 poliovirus (19). Moreover, the particle yields, hydrodynamic diameter (measured by sedimentation velocity) (Fig. 5), antigenicity (Table 2), and ability to attach to HeLa cells of the mutants were indistinguishable from those of wild-type virus, suggesting that the misfolding, if it exists, is very subtle. Nevertheless, the marked slowing effect on the maturation cleavage of HRV14 suggests that mutation of serine 10 changes the environment of the catalytic center.

In agreement with our interpretation, the proposed catalytic role of serine 215 in Sindbis virus capsid protein was supported by the result that mutant Ser-215-Ala had no detectable proteolytic activity (18).

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