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Analysis of Picornavirus 2A^{pro} Proteins: Separation of Proteinase from Translation and Replication Functions

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The poliovirus (PV) genome was manipulated by replacing its 2A-encoding sequence with the corresponding sequence of coxsackie B4 virus (CBV4) or human rhinovirus type 2 (HRV2). In vitro translation of the resulting chimeric PV genomes revealed a normal *cis*-cleavage activity for both heterologous 2A^{pro} proteinases in the chimeric PV polyproteins. However, only the genome containing the 2A-encoding sequence of CBV4 (PV/CBV4-2A) yielded viable virus in transfected cells, producing a mixture of large and small plaques on HeLa cell monolayers. The large-plaque variants were found to contain single-amino-acid mutations at a specific site near the C terminus of the CBV4 2A^{pro} protein. When the same single-amino-acid mutations were directly introduced into the parental PV/CBV4-2A genome, chimeric viruses with a large-plaque phenotype and a wild-type PV-like growth pattern were obtained upon transfection, an observation demonstrating that these point mutations alone had a drastic effect on the growth of the PV/CBV4 chimeric virus. On the other hand, the chimeric genome containing the 2A-encoding sequence of HRV2 (PV/HRV2-2A) produced a null phenotype in transfected HeLa cells, although low-level replication of this chimeric genome was evident. We conclude that only 2A^{pro} of the more closely related enterovirus CBV4 is able to functionally substitute for that of PV in vivo, and a subtle genetic modification of the CBV4 2A^{pro} protein results in a drastic improvement in the growth of the chimeric PV/CBV4-2A virus. In addition, this chimeric cDNA approach enabled us to dissect multiple biological functions encoded by the 2A^{pro} proteins.

Picornaviridae, a family of small, nonenveloped RNA viruses, is divided into the five genera *Enterovirus*, *Rhinovirus*, *Cardiovirus*, *Aphthovirus*, and *Hepatovirus* (36, 43). The genus *Enterovirus* includes polioviruses (PV), coxsackie A viruses, coxsackie B viruses (CBV), and echoviruses, while the genus *Rhinovirus* is divided into major and minor receptor group rhinoviruses and consists of more than 100 distinct serotypes. Despite their great divergence in tissue tropism and in the disease syndromes that they produce, the enteroviruses and the rhinoviruses share a striking similarity in virion structure, gene organization, and replication cycle (36).

Typical of all the picornaviruses, the PV genomic RNA is a positive-sense RNA of about 7,500 nucleotides, with a small viral protein (VPg) and a poly(A) tail present at its 5′ and 3′ termini, respectively (20). Striking features of the 5′ nontranslated region of the viral RNA are that it is unusually long (approximately 750 nucleotides) and highly structured and possesses multiple noninitiating AUGs. Apart from a characteristic 5′-terminal cloverleaf structure, this region contains a *cis*-acting element termed the internal ribosomal entry site (IRES), which directs viral RNA translation in a 5′-end- and cap-independent manner (Fig. 1A) (16, 17, 33, 34, 43). The virus expresses its genetic information from a single large open reading frame that encodes a 247-kDa polyprotein, which is divided into regions for the capsid precursor, P1, and the noncapsid precursors, P2 and P3 (Fig. 1A). The initial cleavage of the polyprotein occurs at the P1*P2 junction in *cis* by 2A^{pro} located at the N terminus of the P2-P3 precursor (41). Other proteolytic cleavages are largely carried out by 3CD^{pro} and/or its processing product 3C^{pro} (12, 19, 31, 46), except the maturation cleavage, which occurs by an unknown mechanism at the VP4*VP2 junction during virus morphogenesis (3, 11). Significantly, in addition to their cleavage activities, both 2A^{pro} and 3CD^{pro} are found to play important roles in viral RNA replication (1, 2, 9, 13, 29, 47). It is not known, however, whether the different functions of these viral proteins are accomplished by independent functional domains or by domains involved in proteolysis. This question is particularly perplexing in the case of the viral 2A^{pro} proteins, which are relatively small in size and yet accommodate multiple essential functions during virus proliferation (9, 27, 29, 47).

The 2A^{pro} proteins of the enteroviruses and the rhinoviruses are cysteine proteinases which share significant sequence homology to the trypsin-like small serine proteases (4, 8, 12). Both genetic and biochemical studies suggest that 2A^{pro} also plays an essential role in the proteolytic degradation of the large subunit eIF-4γ (also known as p220) of the eukaryotic translational initiation factor 4 (eIF-4), thereby causing a shutoff of cap-dependent translation of cellular mRNAs (5, 21, 23, 24, 44). The inactivation of the cellular cap-dependent translational machinery facilitates the IRES-driven translation of the viral open reading frame (43). In addition, recent studies from a number of groups suggest that the 2A^{pro} protein of PV is involved in viral RNA replication (29, 47) and in the *trans*-activation of IRES-driven translation (9). Moreover, the 2A-encoding sequence of PV type 1, strain LS-a, contributes to the mouse-neurovirulent phenotype of the virus (27). Apparently, the 2A^{pro} proteins of the enteroviruses and the rhinoviruses have multiple functions that are essential at different stages of viral proliferation. It has been difficult, however, to dissect these functions from one another because the 2A^{pro} proteins are relatively small and their tertiary structures are unknown. Sequence alignment of the enteroviruses and the rhinoviruses reveals that the viral 2A^{pro} proteins share significant overall similarity while diverging in detailed structures (32). It is likely that subtle structural differences can fine-tune the common

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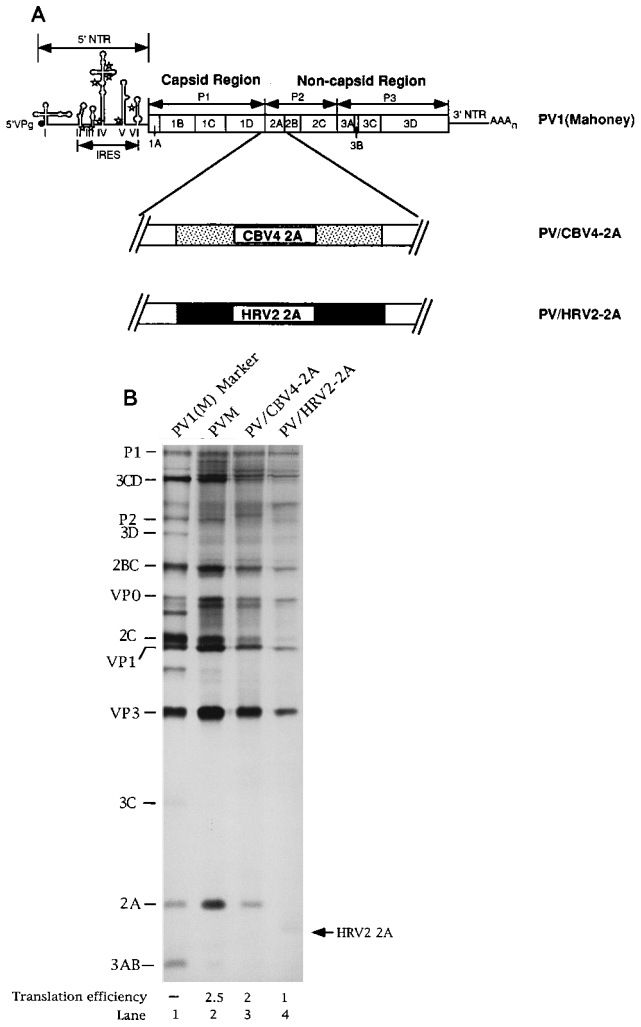

FIG. 1. (A) Genotypes of the wild-type PV and the chimeric constructs containing the heterologous 2A genes. Domains of the 5′ nontranslated region (NTR) are indicated by Roman numerals, and the noninitiating AUGs are denoted by stars. P1 is the precursor of the capsid proteins; P2 and P3 are the precursors of the noncapsid proteins. 1A, 1B, 1C, and 1D are capsid proteins VP4, VP2, VP3, and VP1, respectively. Enlargements show the exchanged 2A-encoding region in the chimeric constructs. (B) In vitro translation directed by the wild-type and chimeric PV RNA transcripts. The translations were performed in HeLa cell extracts by using the optimal RNA concentration for each transcript, i.e., 16 ng/ml for the wild-type PV RNA, 40 ng/ml for PV/CBV4-2A RNA, and 80 ng/ml for PV/HRV2-2A RNA. The translation products were analyzed on SDS–12.5% polyacrylamide gels. Relative efficiencies of in vitro translations of the three RNA transcripts, presented on the bottom of the autoradiogram, were estimated by densitometry of the total translated proteins.

functions encoded by these 2A proteins for each viral species. Thus, comparison of the 2A^{pro} proteins of different viruses by a genetic approach may enable us to dissect the multiple functions associated with these small proteins. Here we present results concerning the engineering of chimeric PV constructs in which the 2A-encoding sequence was replaced by those of either coxsackie B4 virus (CBV4) or human rhinovirus type 2 (HRV2). Our data suggest that 2A^{pro} of CBV4 could functionally replace that of PV, whereas that of the more distantly related HRV2 could not. We show that PV genomes containing heterologous 2A-encoding sequences displayed different translational activities in vitro, while the kinetics of proteolytic processing of their translation products were indistinguishable from those of the wild-type PV. In addition, point mutations in the region encoding the CBV4 2A^{pro} protein which conferred a wild-type PV-like growth pattern to the PV/CBV4 chimeric virus were identified.

MATERIALS AND METHODS

Cells, virus, and plasmids. HeLa R19 cell monolayers were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 5% bovine calf serum (BCS) at 37°C. PV type 1 Mahoney [PV1(M)] and its derivatives were used in this study. For virus amplification, HeLa cells were infected at a multiplicity of infection of 10 PFU per cell. The infected cells were incubated in DMEM–5% BCS and harvested upon the appearance of the cytopathic effect (CPE). After three cycles of freezing and thawing, the released virus was clarified by low-speed centrifugation and concentrated by spinning at 30,000 rpm in a Beckman 50.2 Ti rotor at 22°C for 3 h. The virus pellet was resuspended in phosphate-buffered saline (PBS).

pT7HRV2, a full-length HRV2 cDNA clone (39), and pCB4-P, a cDNA clone of CBV4 (35), were generously provided by E. Kuechler and A. Ramsingh, respectively. The 2A-encoding sequences of HRV2 and CBV4 were PCR amplified from the corresponding cDNAs. pT7PVM was a derivative of pT7XL, a full-length cDNA clone of PV1(M) constructed in our laboratory.

Oligonucleotide primers. The primers used in this study were as follows: primers 996 (5′-GCG TGA CCA TTA TGA CCG-3′), 16 (5′-TGG TGA GCT CCT TGG TG-3′), 14 (5′-CCA AGG AGC TCA CCA C-3′), 3455 (5′-GTT ATA ATA ACT AGT GAG-3′), 4578 (5′-GCG GAG CTC ACT ACA GCT GGC CCC-3′), 4579 (5′-GGC AGG CCT TGT TCT TCA GCA C-3′), 4559 (5′-CTT TCA GGC CTT GTT CCA TTG CAT CAT C-3′), 4560 (5′-CAA GGA GCT CAC CAC CTA TGG CCC CTA TGG ACA TC-3′), 4919 (5′-CTA TAA ATA AGG TTA CCT ACA TGA ACA TAC ATG TC-3′), 2436 (5′-GCA AAA AGC GCT AGC ACA GGG G-3′), 28 (5′-GGC CAA GTG GTA GTT GC-3′), and 2007 (5′-GAT CAC AAC CTG ACC AAG-3′).

DNA manipulation. *Escherichia coli* DH5α was used for plasmid transformation and propagation. DNA cloning was accomplished by standard procedures (37). The cloning enzymes and reagents were purchased from either New England Biolabs, Inc. (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

For the construction of a mutagenesis cassette for the 2A protein, pT7PVM was modified to introduce a unique *Sac*I site immediately upstream of the 2A-encoding sequence. Specifically, PCRs were carried out with oligonucleotide primers 996 and 16 and 14 and 3455 to amplify the PV1(M) sequences corresponding to nucleotides 2736 to 3381 (with a *Sac*I site at the 3′ end) and 3381 to 3997 (with a *Sac*I site at the 5′ end) of the PV genome, respectively. The two PCR products were gel purified, digested with *Sac*I, and ligated. The ligated DNA fragment was further digested with *Bst*EII and returned to pT7PVM, giving rise to plasmid pT7S/2A/S. The *Sac*I site and a previously introduced *Stu*I site (immediately downstream of the 2A-encoding sequence) constituted a 2A-mutagenesis cassette.

To construct a chimeric PV genome containing the 2A-encoding sequences from HRV2, the region encoding HRV2 2A was PCR amplified with primers 4578 and 4579, and the product was digested with *Sac*I and *Stu*I and cloned into pT7S/2A/S to yield the chimeric cDNA pPV/HRV2-2A. Similarly, the CBV4 2A sequence was generated by PCR with primers 4560 and 4559, digested with *Sac*I and *Stu*I, and returned to pT7S/2A/S to produce pPV/CBV4-2A.

In vitro transcription, translation, and RNA transfection. For the production of infectious RNA transcripts in vitro, 0.7 μg of the full-length construct was linearized with *Pvu*I downstream of the viral genome. RNA transcripts were synthesized from the linear template DNA by use of T7 RNA polymerase in 100 μl of an in vitro reaction mixture described previously (42). RNA transcripts were purified by phenol-chloroform extraction and ethanol precipitation. For each RNA transcript, the optimal RNA concentration for in vitro translation was determined as described previously (30). In vitro translation was performed at the optimal RNA concentration in a 12.5-μl reaction system containing HeLa S-10 extract, a translational master mixture, and 10 μCi of ^{35}S translabel (Amersham Corp., Arlington Heights, Ill.) (30). The reactions were carried out at 34°C for up to 7 h. Sodium dodecyl sulfate (SDS)–12% polyacrylamide gels were used to analyze the translation products according to the method of Laemmli (22). To estimate the translational efficiency, autoradiograms of the SDS-polyacrylamide gel electrophoresis (PAGE) gels were generated by use of a densitometer scan program [Scan Analysis (68000); Biosoft].

To perform transfection, HeLa R19 cells cultured on a 35-mm-diameter plate were incubated with up to 10 μg of RNA transcript in the presence of 0.5 mg of DEAE-dextran per ml in HeBSS buffer at room temperature for 30 min (26). The transfection mixture was then removed, and the cells were cultured in 2 ml of DMEM–2% BCS at 37°C and harvested at the time points indicated in the text.

Plaque assay and one-step growth curve. The infectivities of all the virus stocks were titrated by plaque assay on HeLa R19 cell monolayers seeded on six-well plates. Serial dilutions (10-fold) of virus stocks were made with PBS. Three hundred microliters of each dilution was added to the monolayers and incubated at room temperature for 30 min to allow binding of virus to the cells. The cells

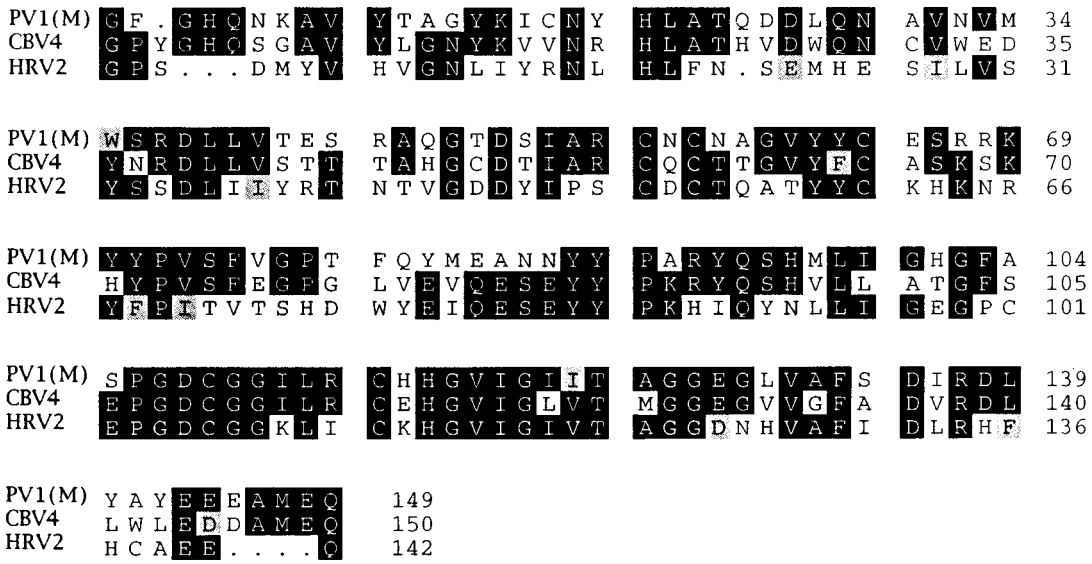

FIG. 2. Amino acid alignment of the 2A proteins of PV1(M), CBV4, and HRV2. Identical residues are shaded with black, and conserved residues are shaded with gray. Missing amino acids are indicated by black dots. Amino acid positions are given at the end of each line. The alignment is in accordance with that of Palmenberg (32).

were then overlaid with 2 ml of 1.4% Noble agar containing modified Eagle medium–5% BCS and were incubated at 37°C for 48 h. Virus plaques were developed by staining with 1% crystal violet.

To measure one-step growth kinetics, virus was loaded onto 35-mm-diameter HeLa R19 plates at a multiplicity of infection of 10 PFU per cell and the plates were incubated at room temperature for 30 min to allow virus binding. Unbound virus particles were removed by three rounds of washing with PBS. The infected cells were cultured in 2 ml of DMEM–5% BCS at 37°C and lysed at 0, 1, 2, 4, 6, 8, and 10 h postinfection. The virus titer in each cell lysate was determined by plaque assay.

RNA isolation and reverse transcription PCR (RT-PCR). A 35-mm-diameter plate of HeLa R19 cells, infected with PV or its derivatives at a multiplicity of infection of 10 or transfected with up to 10 μg of RNA transcripts, was harvested in 200 μl of lysis buffer (10 mM Tris, 1 mM EDTA, 0.5% Nonidet P-40, 100 mM NaCl [pH 7.5]). Cellular debris was removed by spinning the lysate in a microcentrifuge for 2 min. The supernatant was mixed with 2 μl of 10% SDS, extracted with phenol-chloroform, and precipitated with ethanol. Thirteen micrograms of the isolated total RNA was reverse transcribed in 20 μl of a reaction mixture containing 50 μM (each) deoxynucleoside triphosphates, 10 mM dithiothreitol, 2 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals), and 100 pmol of a backward oligonucleotide primer (negative sense). After a 60-min incubation at 42°C, 10 μl of the reaction mixture was mixed with 50 pmol of a forward primer (positive sense) and amplified with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) as described previously (26). The final products were analyzed on 1% agarose gels.

Isolation and characterization of large-plaque variants. HeLa cells were transfected with 5 to 10 μg of RNA transcripts derived from pPV/CBV-2A and lysed by three cycles of freezing and thawing upon the onset of CPE. The viral infectivity in the lysate was titrated on HeLa cell monolayers by plaque assay. Prior to staining, large plaques were individually picked up with 27G1/2 needles and inoculated onto fresh 35-mm-diameter HeLa R19 cell plates for virus expansion. The viruses harvested from the expansion were stored at −80°C as stocks of large-plaque variants. To characterize the 2A-encoding region of these variants, total RNA was isolated from cells that were infected for 6 h with the large-plaque variants of PV/CBV4-2A. RT-PCRs were performed with oligonucleotide primers 2007 and 3455 to amplify the sequences corresponding to nucleotides 3250 to 3996 of the viral genomes. The 750-bp PCR fragments were digested with *Sac*I and *Stu*I and returned to pT7S/2A/S. The 2A-encoding regions of the resulting clones were sequenced, and any mutations thus identified were further confirmed by sequencing DNA fragments obtained from independent RT-PCRs. The effect(s) of these mutations was examined by characterizing the in vitro translation of the respective RNA transcripts and the growth phenotypes of the viruses generated from these cDNA clones.

Rescue assay. Rescue of the nonviable PV/HRV2-2A RNA by the wild-type PV RNA was tested by cotransfection of HeLa cells with the two RNAs. Specifically, 35-mm-diameter HeLa cell plates were transfected with 5 μg of either the wild-type PV RNA or PV/HRV2-2A RNA or with a mixture of the two RNA species (5 μg each). The transfected cells were washed with 2 ml of DMEM three times and then cultured in 2 ml of DMEM–2% BCS at 37°C. The cells were lysed in 200 μl of the lysis buffer at 2 and 16 h posttransfection. In parallel, transfected cells were harvested at 20 h after transfection and the cell lysates were inoculated onto fresh 35-mm-diameter HeLa cell plates. At 7 h after inoculation, the cells were lysed in 200 μl of the lysis buffer. Total RNAs were extracted from the transfected or inoculated cells and used as templates for RT-PCR. Primers that specifically anneal with the 2A-encoding sequences of PV (primer 28) or HRV2 (primer 4919) were used to differentiate signals from the helper RNA and the PV/HRV2-2A RNA (see Fig. 8).

The percentage of cells simultaneously taking up two RNA species in a cotransfection assay was determined as follows. Five micrograms of the wild-type PV RNA was transfected into cells in a 35-mm-diameter HeLa cell plate, with or without the presence of 5 μg of a nonviable PV mutant. The nonviable PV mutants used in this study were PV/HRV2-2A and PVM-*Pvu*II; the latter has a 450-nucleotide truncation at the 3′ terminus of the PV genome which abrogated RNA replication. The cells were lysed at 5 h posttransfection, and the virus titer in each lysate was estimated by plaque assay. Approximately 90% reduction in wild-type PV production was observed for cotransfection of the wild-type PV RNA with either PV/HRV2-2A or PVM-*Pvu*II RNA, indicating that cotransfection was efficient in this study. The reduction in wild-type PV production was not obvious when the cells were harvested at 10 or 20 h postcotransfection.

RESULTS

The wild-type and chimeric PV genomes exhibit differential translational efficiency in vitro. The $2A^{pro}$ proteins of the enteroviruses and the rhinoviruses, originally identified as the viral proteinases involved in viral polyprotein processing, encode multiple functions that are vital to virus proliferation. It is not clear, however, if the proteinase activity of $2A^{pro}$ is indispensable for functions unrelated to proteolysis. Given the small size of these proteins, it is difficult to dissect functional domains of the $2A^{pro}$ proteins by biochemical approaches. Nevertheless, sequence alignment of the $2A^{pro}$ proteins of PV1(M), CBV4, and HRV2 revealed that the C-terminal portions of the proteins are strikingly conserved, whereas the N-terminal and central portions are more or less variable (Fig. 2). Compared with that of CBV4, $2A^{pro}$ of HRV2 is more distantly related to that of PV1(M). The structural divergence may reflect a need to interact with other cognate viral proteins even though the *cis*-cleavage function of $2A^{pro}$ remains the same. It was therefore of interest to examine whether the 2A-encoding sequence of CBV4 or HRV2 could functionally

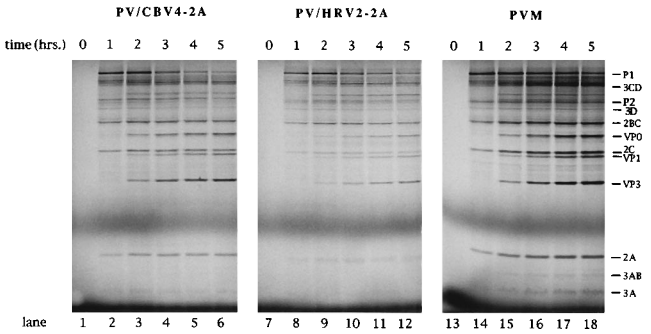

FIG. 3. Kinetics of protein synthesis and processing directed by the RNA transcripts of PV/CBV4-2A, PV/HRV2-2A, and the wild-type PV [PV(M)] in a HeLa cell-derived in vitro translation system. Translations were performed at the optimal RNA concentration for each RNA at 34°C for 1 to 5 h. The protein products were analyzed by SDS–12.5% PAGE.

replace that of PV1(M). It was anticipated that such replacements would produce viral phenotypes whose properties might reveal further $2A^{pro}$ activities.

To facilitate the genetic manipulation of PV $2A^{pro}$, a mutagenesis cassette was engineered by introducing two unique restriction sites flanking the 2A-encoding region in the full-length cDNA clone of PV1(M). The virus generated from this cassette had a growth phenotype that was indistinguishable from that of wild-type PV1(M) (data not shown), indicating that the newly introduced restriction sites did not interfere with the growth of the virus in vivo. The 2A-encoding region of PV was then replaced by the corresponding sequence of CBV4 or HRV2 (Fig. 1A). The resultant chimeric PV constructs were designated PV/CBV4-2A and PV/HRV2-2A, and they shall be abbreviated hereafter as P/C-2A and P/R-2A, respectively. In vitro translation of the RNA transcripts was performed to test whether the heterologous $2A^{pro}$ proteins would have any effects on PV RNA translation in general and on the *cis* cleavage of the viral polyprotein in particular. It was observed that in a HeLa cell-derived in vitro translation system (30), optimal translation was achieved when the RNA concentration was 16 ng/μl for the wild-type PV RNA, 40 ng/μl for P/C-2A RNA, and 80 ng/μl for P/R-2A RNA. At their respective optimal RNA concentrations, the protein profiles of the in vitro translations were very similar for the three RNA species, and as expected, differences were observed in particular in the migration of the respective $2A^{pro}$ polypeptides (Fig. 1B). However, the relative translational efficiencies of the three RNAs in vitro were significantly different; the ratio of the translation products of wild-type PV, P/C-2A, and P/R-2A RNAs was approximately 2.5:2:1, as estimated by densitometry of the total proteins translated (Fig. 1B; also see below). It is noteworthy that viral protein 3CD barely accumulated in P/R-2A translation and was disproportionally decreased in P/C-2A translation. In addition, the position at which P2 migrates also exhibited heterogeneity among the three constructs (Fig. 1B). These data imply that the exchange of $2A^{pro}$ sequences also resulted in some minor changes in proteolytic processing of the P2 and P3 proteins.

Kinetics of protein translation and processing directed by the wild-type and chimeric PV genomes in vitro. The reduced translational efficiency of P/C-2A and P/R-2A RNAs could be due either to defects in their translational initiation or to inefficient *cis* cleavage by the heterologous $2A^{pro}$. We therefore tested the possible effects of these foreign $2A^{pro}$ proteins on the kinetics of translation and proteolytic processing in the HeLa cell extract. In vitro translation was performed with the optimal RNA concentrations for each RNA. The reactions were stopped at different time points, and the translation products were analyzed by SDS-PAGE. As shown in Fig. 3, the capsid precursor P1 was one of the earliest viral proteins appearing in all three translation reactions, indicating that the $2A^{pro}$ proteinases of HRV2 and CBV4 were as efficient as that of PV1(M) in *cis* cleavage of P1 from the polyproteins. In addition, the kinetics of translation directed by the wild-type and chimeric PV RNAs were indistinguishable, whereas the quantities of the translation products were significantly different, as pointed out earlier. In all three cases, the progression of proteolytic processing by $3CD^{pro}$ or $3C^{pro}$ was largely unaffected, and the translation reactions were finished within 5 h of incubation at 34°C. These data demonstrate that the replacement of the $2A^{pro}$ of PV with that of CBV4 or HRV2 did not result in obvious alteration in the kinetics of viral protein translation or $2A^{pro}$-mediated *cis* cleavage. Instead, it appears that the predominant effect of the $2A^{pro}$ replacement was the reduction of the rate of translational initiation.

In vivo phenotypes of P/C-2A and P/R-2A. To examine whether the $2A^{pro}$ protein from HRV2 or CBV4 could functionally replace its counterpart in PV1(M) in vivo, the RNA transcripts from the wild-type PV and the two chimeric constructs were transfected into HeLa cells. CPE was scored at approximately 20 h posttransfection for the wild-type PV RNA and at approximately 24 h posttransfection for the P/C-2A RNA. Transfection with the P/R-2A RNA, however, did not induce detectable CPE even after prolonged incubation. Plaque assays of the cell lysates from each transfection revealed that both the wild-type PV and the P/C-2A RNAs produced viable viruses, an observation indicating that $2A^{pro}$ of CBV4 could functionally mimic that of PV1(M) in vivo (Fig. 4). Labeling of protein synthesized in infected cells indicated that both wild-type PV and the P/C-2A chimera completely inhibited host protein synthesis at 3.5 h postinfection (data not shown), an observation suggesting that CBV4 2A translated from the chimeric genome was capable of inducing p220 degradation. In fact, available evidence suggests that purified $2A^{pro}$ proteins of CBV4 and HRV2 are able to cleave p220 in vitro (23). We thus assume that these $2A^{pro}$ proteins, when expressed from their respective chimeric genomes, would be functional in the induction of p220 cleavage as well as the consequent shutoff of host protein synthesis. Nevertheless, the P/R-2A chimera failed to produce any viable virus in transfection assays. This phenomenon substantiates the notion that the divergence in sequence may result in functional incompatibility between the $2A^{pro}$ proteins of PV1(M) and HRV2. It is noteworthy that there is a correlation between viability and the efficiency of in vitro translation of the three viral RNA transcripts, which could be interpreted as supporting previous re-

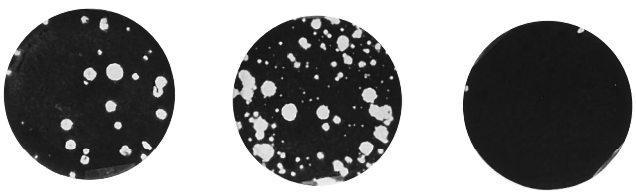

FIG. 4. Phenotypic comparison of the plaques derived from cDNA clones of PV1(M), PV/CBV4-2A, and PV/HRV2-2A. HeLa cell monolayers were infected with lysates derived from cells transfected with the respective RNA transcripts. After a 48-h incubation at 37°C, plaques were developed by staining the monolayers with 1% crystal violet.

Virus	C-terminal portion of 2A							
PV1(M)	UAC	GAA	GAA	GAA	GCC	AUG	GAA	CAA
	Y	E	E	E	A	M	E	Q
CBV4	UUG	GAA	GAU	GAU	GCA	AUG	GAA	CAA
	L	E	D	D	A	M	E	Q
W1-P/C-2A-L_1R	UUG	GAA	GAU	G**UU**	GCA	AUG	GAA	CAA
	L	E	D	**V**	A	M	E	Q
W1-P/C-2A-L_2R	UUG	GAA	GAU	G**GA**	GCA	AUG	GAA	CAA
	L	E	D	**G**	A	M	E	Q
W1-P/C-2A-L_3R	UUG	GAA	GAU	G**UU**	GCA	AUG	GAA	CAA
	L	E	D	**V**	A	M	E	Q
W1-P/C-2A-L_4R	UUG	GAA	GAU	**UAU**	GCA	AUG	GAA	CAA
	L	E	D	**Y**	A	M	E	Q
W1-P/C-2A-L_5R	UUG	GAA	GAU	**UAU**	GCA	AUG	GAA	CAA
	L	E	D	**Y**	A	M	E	Q
W1-P/C-2A-L_6R	UUG	GAA	GAU	G**GU**	GCA	AUG	GAA	CAA
	L	E	D	**G**	A	M	E	Q
W1-P/C-2A-L_7R	UUG	GAA	GAU	G**GU**	GCA	AUG	GAA	CAA
	L	E	D	**G**	A	M	E	Q
HRV2	GCU	GAA	GAA	...	...	...	...	CAA
	A	E	E	.	.	.	.	Q

FIG. 5. Point mutations detected in large-plaque variants (PV/CBV4-2A-L_1R to -L_7R) of the PV/CBV4-2A chimera. The nucleotide sequences (upper lines) and the amino acid sequences (lower lines) corresponding to the carboxyl-terminal portions of the viral 2A proteins are aligned according to the scheme of Palmenberg (32). Point mutations are indicated by bold letters. Missing nucleotides and amino acids are represented by black dots.

sults indicating that the $2A^{pro}$ proteins play a role in cap-independent translation driven by the picornavirus IRES elements (9). However, it is unlikely that the null phenotype of the chimera P/R-2A resulted solely from inefficient translational activation of P/R-2A RNA by the heterologous HRV2 2A protein.

Rapid emergence of large-plaque variants from the P/C-2A chimera. Plaque assays showed that viruses emerging from transfection of P/C-2A RNA displayed a mixed population of plaques, the large plaques being indistinguishable from those of wild-type PV1(M) (Fig. 4). The small-plaque-phenotype virus disappeared within two additional passages of the chimeric virus through cultured cells. The rapid emergence of large-plaque variants from the P/C-2A chimera implied that certain genetic events, most likely point mutations, may have occurred during viral replication. To access the molecular mechanisms underlying the growth improvement of the P/C-2A chimera, seven of the large-plaque variants were plaque purified for further characterization. The entire 2A-encoding regions of these variants were amplified by RT-PCR. Sequence analysis of the PCR fragments revealed that each of the variants had a mutation at amino acid 146 of CBV4 2A (Fig. 5). Three of the seven variants at this position mutated from an aspartate to a glycine, two mutated to a tyrosine, and the remaining two mutated to a valine. It is interesting that the mutations occurred at a specific site in a highly negatively charged motif. In all the cases, the mutations resulted in a change of the third charged amino acid (Asp-146) of this motif to a noncharged residue. Besides one silent mutation found in variant P/C-2A-L_3R (data not shown), no other changes were observed in the 2A-encoding regions of these large plaque variants.

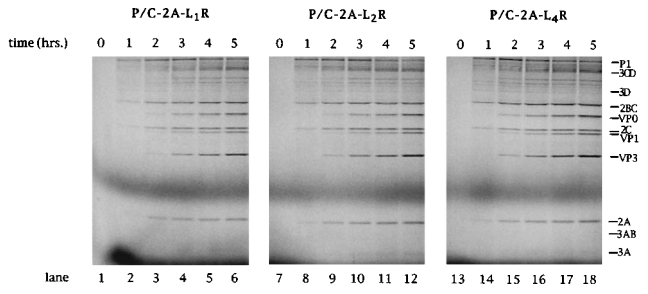

FIG. 6. Kinetic analysis of protein synthesis and processing directed by the RNA transcripts of PV/CBV4-2A-L_1R, -L_2R, and -L_4R cDNAs. The in vitro translations were performed at the optimal RNA concentration (40 ng/ml) at 34°C for 1 to 5 h in a HeLa cell extract. The translation products were analyzed on SDS–12.5% polyacrylamide gels. PV/CBV4-2A-L_1R, -L_2R, and -L_4R bear a point mutation at amino acid 146 of the CBV4 2A protein, as illustrated in Fig. 5.

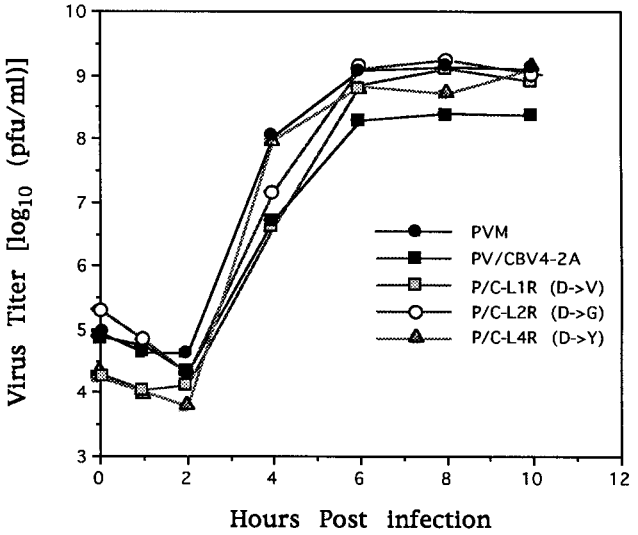

FIG. 7. One-step growth curves of wild-type PV and the PV/CBV4-2A chimera and its large-plaque variants in HeLa cells. Cells were infected at a multiplicity of infection of 10 and were harvested at 0, 1, 2, 4, 6, 8, and 10 h postinfection. The virus titer of each sample was determined by a standard plaque assay.

Point mutations in CBV4 2A promote the replication of the P/C-2A chimera. To determine whether the point mutations found in CBV4 $2A^{pro}$ of the large-plaque P/C-2A variants are the sole cause for the improved replication properties of the chimeric virus, the 2A-encoding regions of the seven large-plaque P/C-2A variants were separately used to replace the equivalent sequence in the wild-type PV1(M) cDNA. All the resulting reconstructed chimeric PV clones (P/C-2A-L_1R to -L_7R) contained a CBV4 2A-encoding sequence plus point mutations at the 146th codon of CBV4 2A. In vitro translation of the resulting RNAs revealed that none of the point mutations significantly affected translational efficiency or the kinetics of protein translation and processing (Fig. 6). RNA transcripts derived from these constructs were transfected into HeLa cells. It was observed that all of them produced CPE at approximately 20 h posttransfection. This was identical to the time at which wild-type PV RNA produces CPE, but it was 4 h faster than the time for the original P/C-2A RNA. The viruses derived from these constructs produced wild-type PV-like large plaques on HeLa cell monolayers (data not shown), in contrast to the mixed plaques produced by the parental P/C-2A chimera. In addition, one-step growth analysis demonstrated that these point mutations promoted the growth rate of the P/C-2A chimera to a level which was similar to that of PV1(M)

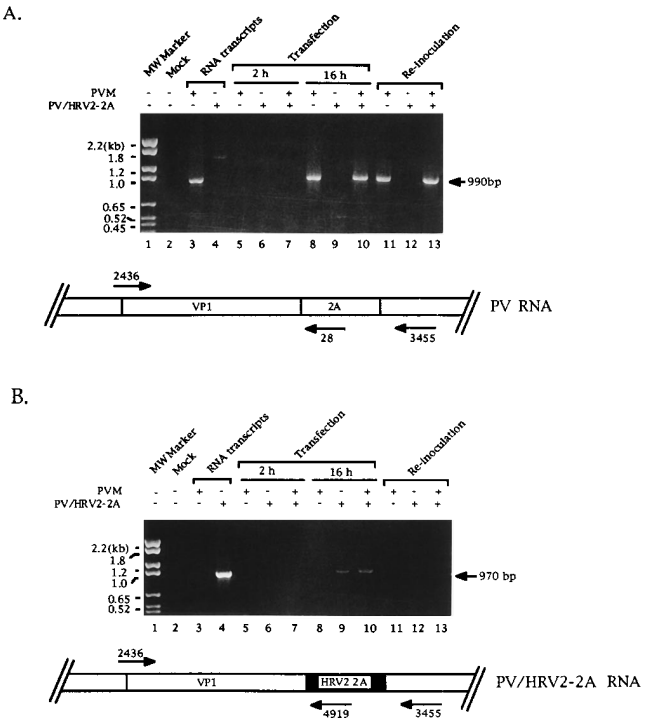

FIG. 8. Attempt to rescue nonviable mutant PV/HRV2-2A by cotransfection with the wild-type PV RNA. The cells were transfected with 5 μg of either PV/HRV2-2A RNA or the wild-type PV RNA or with a combination of the two RNA species. Cells were lysed at 2 h (lanes 5 to 7) or 16 h (lanes 8 to 10) after transfection or at 7 h after inoculation with the lysates of the transfected cells (lanes 11 to 13). Total RNA was purified and reverse transcribed by using primer 3455. A PV-specific primer (A) and an HRV2 2A-specific primer (B) were used in PCRs to selectively amplify wild-type PV RNA and PV/HRV2-2A RNA, respectively. The RT-PCR products were analyzed on 1% agarose gels, and sizes of the products are indicated.

(Fig. 7). These observations strongly argue that the point mutations in CBV4 $2A^{pro}$ were necessary and sufficient to confer the improved growth property of the P/C-2A variants.

P/R-2A could not be rescued by cotransfection with the wild-type PV RNA. We have shown that the exchange of the 2A-encoding sequence of PV with that of HRV2 significantly reduced the translation efficiency of the viral RNA in vitro and yielded a null phenotype upon transfection. It was of interest to know whether the nonviable P/R-2A RNA could be rescued and encapsidated by cotransfection with wild-type PV RNA. If the 2A proteins can be supplemented in *trans*, then the $2A^{pro}$ protein of wild-type PV would be able to promote the replication of P/R-2A RNA, presumably by a mechanism of intergenomic complementation (6, 43). We therefore cotransfected cells with equal amounts (5 μg) of the RNAs of the wild-type PV and the P/R-2A chimera, and in control experiments, cells were transfected with the RNA of either species. The transfected cells were lysed at 2 and 16 h posttransfection. In parallel, to examine any possible encapsidation of the P/R-2A RNA, the transfected cells were harvested at 20 h posttransfection and the lysates were subsequently inoculated onto fresh HeLa cells which were lysed at 7 h after the inoculation. Total RNA of each sample was analyzed by RT-PCR using either PV 2A-specific or HRV2 2A-specific primers. Amplification of viral sequences using a common primer (oligonucleotide 2436 anneals with the VP3-VP1 junction of PV) and a PV 2A-specific primer (oligonucleotide 28) revealed that there was no detectable level of PV RNA accumulation at 2 h posttransfection (Fig. 8A, lanes 5 to 7) but a significant level of PV RNA replication at either 16 h posttransfection (lanes 8 and 10) or 7 h after inoculation with the transfected cell lysates (lanes 11 and 13). RT-PCR using primer 2436 and an HRV2 2A-specific primer (oligonucleotide 4919), on the other hand, revealed no detectable level of the P/R-2A chimeric RNA at 2 h posttransfection (Fig. 8B, lanes 5 to 7) and a weak band of the expected size (970 bp) at 16 h posttransfection with either the P/R-2A RNA alone or a mixture of the P/R-2A RNA and the wild-type PV RNA (lanes 9 and 10). No PCR products were detected in cells inoculated with the transfected cell lysates. These observations suggest that the P/R-2A chimeric RNA was capable of replicating itself to a low, but detectable, level. However, cotransfection with the wild-type PV RNA did not significantly promote the replication of the chimera. Furthermore, low-level replication of the chimeric P/R-2A RNA did not lead to a detectable level of encapsidation by the capsid proteins that were expressed either from its own genome or from the cotransfected wild-type PV genome.

DISCUSSION

All primary translation products of PV are generated in equimolar amounts, a strategy that could be viewed as wasteful since only the capsid proteins are needed in large excess over the nonstructural proteins. However, PV, like many other RNA viruses, uses precursors as well as end products of the proteolytic processing pathway for different functions. For example, the virus-encoded proteinase $3CD^{pro}$ is not just a proteinase but also a cofactor in various steps of genome replication (1, 2, 10, 13, 19, 45).

Similarly, PV $2A^{pro}$ has numerous different functions: as a proteinase, as a translational *trans* activator, and as a component in viral RNA replication (9, 21, 27, 29, 31, 44, 47). We have used a strategy to replace the PV $2A^{pro}$-encoding sequence in the viral genome with picornavirus $2A^{pro}$ sequences that are related, but not identical, to that of PV, hoping that the genetic exchange of the proteins would reveal phenotypes indicative of one or another function of picornavirus 2A proteins. We have chosen the 2A-encoding sequences of the enterovirus CBV4 and the rhinovirus HRV2, which are 66 and 50% homologous to PV $2A^{pro}$, respectively.

Common to the enteroviruses and the rhinoviruses is the speed at which the P1 region is severed from the nonstructural P2-P3 regions, a reaction thought to occur cotranslationally (12, 14). Available evidence suggests that the cleavage employs a *cis* mechanism (15). The cleavage site at the P1*P2 junctions (i.e., $P1*2A^{pro}$) of the PV polyprotein is LtTY*GfGh, where the capital letters represent residues conserved among PV strains (15). In contrast, the corresponding CBV4 cleavage site is LITT*GPYG (18), and that of HRV2 is ITTA*GPSD (38). In the constructs described here, the P1*P2 junctions are chimeric, being LTTY*GPYG for P/C-2A and LTTA*GPSD for P/R-2A. It is noteworthy that the heterologous $2A^{pro}$ proteinases process the chimeric cleavage sites in P/C-2A or P/R-2A as efficiently as the homologous $2A^{pro}$ does in wild-type PV polyprotein. This observation supports previous conclusions that the *cis* cleavage signal at the P1*P2 junction does not pose stringent sequence conditions for processing (15, 38).

Whereas the general efficiencies of proteolytic processing are comparable between the wild-type and the chimeric constructs (Fig. 3), the efficiencies of translation are not (Fig. 1B). Currently, we cannot explain this phenomenon, but it is appealing to speculate that it may be attributable to the role of the $2A^{pro}$ protein as a translational activator in *trans* (9). Unfortunately, the mechanism of $2A^{pro}$-mediated translational

activation has not been studied in detail. Second site mutations mapping in PV $2A^{pro}$ have been isolated in response to mutations in domain V of the PV IRES (Fig. 1A), a result that has been interpreted as indicating possible interactions between $2A^{pro}$ and the IRES element (28). It is possible, therefore, that neither the CBV4 2A protein nor the HRV2 2A protein can perform efficient translational activation leading to wild-type PV-level protein translation from the chimeric genomes.

Transfections of PV/CBV4-2A RNA yield a progeny virus population with mixed plaque phenotypes. It has not been possible to isolate virus consisting only of the small-plaque phenotype because of rapid genetic variation. Sequence analysis of several plaque-purified viruses with the large-plaque phenotype consistently revealed a change of the amino acid which is 5 residues upstream of the chimeric 2A*2B junction (Fig. 5). These point mutations, however, did not significantly affect $3C^{pro}$-mediated cleavage at the chimeric junction. The $3C^{pro}$ proteinases of PV, CBV, and HRV recognize a consensus P5-P4-P3-P2-Q*G signal, where the residues at the P5, P3, and P2 positions are variable while the P4 position has a prevalence of alanine or other small aliphatic residues (6a, 7, 8, 25, 31). The point mutations detected in the large-plaque variants of P/C-2A mapped to the P5 position of the 2A*2B junction, and as expected, none of them significantly affected the $3C^{pro}$-mediated cleavage at this site. It is noteworthy, however, that these point mutations occurred within an acidic motif, E-E/D-E/**D**-A-M-E-Q_{COOH}, that is highly conserved among enteroviruses. Surprisingly, the aspartic acid in the large-plaque variants of P/C-2A (indicated above by the bold letter **D**) was changed to three different noncharged amino acids. Clusters of highly charged amino acids may be located at the surface of a protein and thus participate in interactions with other proteins. The mutations in CBV4 $2A^{pro}$ of the large-plaque variants of P/C-2A may modify such interactions, thereby improving the binding of CBV4 $2A^{pro}$ to its nonnative partner(s), a PV polypeptide(s). In any event, in spite of the 34% sequence heterogeneity between CBV4 and PV $2A^{pro}$ proteins, the point mutations elevate the replication of the P/C-2A chimera to the wild-type PV level in cultured cells. The wild-type-like replication phenotype of the chimera variants did not result from improved translation efficiencies of the chimeric RNA, because in vitro translation indicated that the efficiencies of translation of the large-plaque P/C-2A variant RNAs in a HeLa cell extract remained lower than that of the wild-type PV RNA. Thus, we propose that the point mutations may exert their effect, possibly via an interaction with PV polypeptides, on other biochemical processes, most likely including genome replication.

Given the extent of sequence heterogeneity, it was perhaps not surprising that P/R-2A RNA expressed a null phenotype. Sequence alignments indicate that the C termini of HRV $2A^{pro}$ proteins lack 4 amino acids compared with those of the enteroviruses, although an acidic motif has been preserved (EE - - - - Q_{COOH}). Sommergruber et al. (40) have reported that the C terminus of HRV2 $2A^{pro}$ can be truncated by up to 6 residues without losing its *cis*-cleavage activity. It is possible, therefore, that the lesion leading to the null phenotype of the P/R-2A chimera resides in macromolecular events other than 2A-mediated protein processing. In vitro translation of P/R-2A RNA revealed minor alterations in the processing of the P2 proteins, which could also contribute to the defects in replication of this chimera. Rescue of the P/R-2A construct by cotransfections with wild-type PV RNA was unsuccessful. In contrast, a PV mutant bearing a lesion in the 2A region, which is defective in the inhibition of host cell translation, can be rescued by genetic complementation (6). One possible explanation for this discrepancy is that HRV2 $2A^{pro}$ may have multiple functional defects (e.g., translational activation and RNA replication) in the replication of the P/R-2A chimera, whereas the $2A^{pro}$ mutant studied by Bernstein et al. was found to be defective only in the inhibition of host cell translation. The multiple defects of the HRV $2A^{pro}$ within a PV polyprotein may not be fully complemented by wild-type PV 2A provided in *trans*. In addition, the level of genome replication was very low in transfections with P/R-2A RNA, and so far, we have failed in numerous attempts to isolate viable variants from this construct. Nevertheless, these studies have allowed us to separate 2A-related functions, and they may allow us to engineer an HRV2 $2A^{pro}$ that will allow efficient PV genome replication and encapsidation.

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REFERENCES

1. **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.
2. **Andino, R., G. E. Rieckhof, and D. Baltimore.** 1990. A functional ribonucleoprotein complex forms around the 5′ end of poliovirus RNA. Cell **63:**369–380.
3. **Ansardi, D. C., and C. D. Morrow.** 1995. Amino acid substitutions in the poliovirus maturation cleavage site affect assembly and result in accumulation of provirions. J. Virol. **69:**1540–1547.
4. **Bazan, J. F., and R. J. Fletterick.** 1988. Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: structural and functional implications. Proc. Natl. Acad. Sci. USA **85:**7872–7876.
5. **Bernstein, H. D., N. Sonenberg, and D. Baltimore.** 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. Mol. Cell. Biol. **5:**2913–2923.
6. **Bernstein, J. F., P. Sarnow, and D. Baltimore.** 1986. Genetic complementation among poliovirus mutants derived from an infectious cDNA clone. J. Virol. **60:**1040–1049.

6a. **Cao, X. M., and E. Wimmer.** Genetic variation of the poliovirus genome with two VPg coding units. EMBO J., in press.

7. **Cordingley, M. G., P. L. Callahan, V. V. Sardana, V. M. Garsky, and R. J. Colonno.** 1990. Substrate requirements of human rhinovirus 3C protease for peptide cleavage in vitro. J. Biol. Chem. **265:**9062–9065.
8. **Dougherty, W. G., and B. L. Semler.** 1993. Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. Microbiol. Rev. **57:**781–822.
9. **Hambidge, S. J., and P. Sarnow.** 1992. Translational enhancement of the poliovirus 5′ noncoding region mediated by virus-encoded polypeptide 2A. Proc. Natl. Acad. Sci. USA **89:**10272–10276.
10. **Hanecak, R., B. Semler, C. Anderson, and E. Wimmer.** 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. Proc. Natl. Acad. Sci. USA **79:**3973–3977.
11. **Harber, J. J., J. Bradley, C. W. Anderson, and E. Wimmer.** 1991. The catalysis of the poliovirus VP0 maturation cleavage is not mediated by serine 10 of VP2. J. Virol. **65:**326–334.
12. **Harris, K. S., C. U. T. Hellen, and E. Wimmer.** 1990. Proteolytic processing in the replication of picornaviruses. Semin. Virol. **1:**323–333.
13. **Harris, K. S., W. Xiang, L. Alexander, A. V. Paul, W. S. Lane, and E. Wimmer.** 1994. Interaction of the polioviral polypeptide $3CD^{pro}$ with the 5′ and 3′ termini of the poliovirus genome: identification of viral and cellular cofactors necessary for efficient binding. J. Biol. Chem. **269:**27004–27014.
14. **Hellen, C. U. T., H.-G. Kräusslich, and E. Wimmer.** 1989. Proteolytic processing of polyproteins in the replication of RNA viruses. Biochemistry **28:**9881–9890.
15. **Hellen, C. U. T., C.-K. Lee, and E. Wimmer.** 1992. Determinants of substrate recognition by poliovirus 2A proteinase. J. Virol. **66:**3330–3338.
16. **Jang, S. K., H.-G. Kräusslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of

encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62:**2636–2643.
17. **Jang, S. K., and E. Wimmer.** 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kDa RNA-binding protein. Genes Dev. **4:**1560–1572.
18. **Jenkins, O., J. Booth, P. Minor, and J. Almond.** 1987. The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the Picornaviridae. J. Gen. Virol. **68:**1835–1848.
19. **Jore, J., B. de Geus, R. J. Jackson, P. H. Pouwels, and B. E. Enger-Valk.** 1988. Poliovirus protein 3CD is the active protease for processing of the precursor protein P1 in vitro. J. Gen. Virol. **69:**1627–1636.
20. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291:**547–553.
21. **Kräusslich, H. G., M. J. H. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer.** 1987. Poliovirus proteinase 2A induces cleavage of eukaryotic initiation factor 4F polypeptide p220. J. Virol. **61:**2711–2718.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
23. **Liebig, H.-D., E. Ziegler, R. Yan, K. Hartmuth, H. Klump, H. Kowalski, D. Blaas, W. Sommergruber, B. Lamphear, R. Rhoads, E. Kuechler, and T. Skern.** 1993. Purification of two picornaviral 2A proteinases: interaction with eIF-4γ and influence on in vitro translation. Biochemistry **32:**7581–7588.
24. **Lloyd, R. E., M. J. Grubman, and E. Ehrenfeld.** 1988. Relationship of p220 cleavage during picornavirus infection to 2A proteinase sequencing. J. Virol. **62:**4216–4223.
25. **Long, A. C., D. C. Orr, J. M. Cameron, B. M. Dunn, and J. Kay.** 1989. A consensus sequence for substrate hydrolysis by rhinovirus 3C proteinase. FEBS Lett. **258:**75–78.
26. **Lu, H.-H., L. Alexander, and E. Wimmer.** 1995. Construction and genetic analysis of dicistronic polioviruses containing open reading frames for epitopes of human immunodeficiency virus type 1 gp120. J. Virol. **69:**4797–4806.
27. **Lu, H.-H., C.-F. Yang, A. D. Murdin, M. H. Klein, J. J. Harber, O. M. Kew, and E. Wimmer.** 1994. Mouse neurovirulence determinants of poliovirus type 1 strain LS-a map to the coding regions of capsid protein VP1 and proteinase $2A^{pro}$. J. Virol. **68:**7507–7515.
28. **Macadam, A. J., G. Ferguson, T. Fleming, D. M. Stone, J. W. Almond, and P. M. Minor.** 1994. Role for poliovirus protease 2A in cap independent translation. EMBO J. **13:**924–927.
29. **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.
30. **Molla, A., A. V. Paul, and E. Wimmer.** 1991. Cell-free, de novo synthesis of poliovirus. Science **254:**1647–1651.
31. **Nicklin, M. J. H., H. Toyoda, M. G. Murray, and E. Wimmer.** 1986. Proteolytic processing in the replication of polio and related viruses. Bio/Technology **4:**33–42.
32. **Palmenberg, A. C.** 1989. Sequence alignments of picornaviral capsid proteins, p. 211–241. *In* B. L. Semler and E. Ehrenfeld (ed.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, D.C.
33. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Cap-independent translation of poliovirus mRNA is conferred by sequence elements within the 5′ noncoding region. Mol. Cell. Biol. **8:**1103–1112.
34. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334:**320–325.
35. **Ramsingh, A., A. Hixson, B. Duceman, and J. Slack.** 1990. Evidence suggesting that virulence maps to the P1 region of the coxsackievirus B4 genome. J. Virol. **64:**3078–3081.
36. **Rueckert, R. R.** 1990. Picornaviridae and their replication, p. 507–548. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Morath, and B. Roizman (ed.), Virology, 2nd ed., vol. 1. Raven Press, New York.
37. **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.
38. **Skern, T., W. Sommergruber, H. Auer, P. Volkmann, M. Zorn, H.-D. Liebig, F. Fessl, D. Blaas, and E. Kuechler.** 1991. Substrate requirement of a human rhinoviral 2A proteinase. Virology **181:**46–54.
39. **Skern, T., W. Sommergruber, D. Blaas, P. Gruendler, F. Fraundorfer, C. Pieler, I. Fogy, and E. Kuechler.** 1985. Human rhinovirus 2: complete nucleotide sequence and proteolytic processing signals in the capsid protein region. Nucleic Acids Res. **13:**2111–2126.
40. **Sommergruber, W., M. Zorn, D. Blaas, F. Fessl, P. Volkmann, I. Maurer-Fogy, P. Pallai, V. Merluzzi, M. Matteo, T. Skern, and E. Kuechler.** 1989. Polypeptide 2A of human rhinovirus type 2: identification as a protease and characterization by mutational analysis. Virology **169:**68–77.
41. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Sudier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45:**761–770.
42. **van der Werf, S., J. Bradley, E. Wimmer, F. W. Studier, and J. J. Dunn.** 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**2330–2334.
43. **Wimmer, E., C. U. T. Hellen, and X. M. Cao.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353–436.
44. **Wyckoff, E., J. W. B. Hershey, and E. Ehrenfeld.** 1990. Eukaryotic initiation factor-3 is required for poliovirus-2A protease-induced cleavage of the p220 component of eukaryotic initiation factor-4F. Proc. Natl. Acad. Sci. USA **87:**9529–9533.
45. **Xiang, W., K. S. Harris, L. Alexander, and E. Wimmer.** 1995. Interaction between the 5′-terminal cloverleaf and $3AB/3CD^{pro}$ of poliovirus is essential for RNA replication. J. Virol. **69:**3658–3667.
46. **Ypma-Wong, M.-F., P. G. Dewalt, V. H. Johnson, J. G. Lamb, and B. L. Semler.** 1988. Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor. Virology **166:**265–270.
47. **Yu, S., P. Benton, M. Bovee, J. Session, and R. E. Lloyd.** 1995. Defective RNA replication by poliovirus mutants deficient in 2A protease cleavage activity. J. Virol. **69:**247–252.