JOURNAL OF VIROLOGY, Jan. 1995, p. 247–252 Vol. 69, No. 1
0022-538X/95/$04.00+0


# Defective RNA Replication by Poliovirus Mutants Deficient in 2A Protease Cleavage Activity

SHUYUARN F. YU, PATRICIA BENTON, MICHAEL BOVEE, JAMES SESSIONS, AND RICHARD E. LLOYD*

*Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190*

Received 27 June 1994/Accepted 28 September 1994

**2A protease ($2A^{pro}$) catalyzes the initial cleavage of the poliovirus polyprotein which separates the P1 structural protein precursor from the P2-P3 nonstructural protein precursor. In addition, $2A^{pro}$ indirectly induces cleavage of the p220 component of eukaryotic initiation factor 4F, which is thought to contribute to the specific inhibition of host cell protein synthesis observed in virus-infected HeLa cells. However, it is unclear whether the *trans* function of $2A^{pro}$ which induces host cell shutoff is essential or merely facilitates efficient poliovirus replication. In this study, three point mutations in $2A^{pro}$ (D38E, Y88L, and Y89L [S. F. Yu and R. E. Lloyd, Virology 182:615–625, 1991]) which cause specific loss of *trans* but not *cis* cleavage function were independently introduced into the full-length poliovirus cDNA. In addition, mutations which caused only partial loss of both *cis* and *trans* cleavage activities (Y88S) or resulted in a wild-type phenotype (Y88F) were individually introduced. When each of these mutant poliovirus cDNAs was transcribed and translated in vitro, normal proteolytic processing of the viral polyprotein was observed, and p220 was not cleaved in those reactions containing proteases defective in *trans* function, as expected. Surprisingly, Northern (RNA) blot analysis and reverse transcriptase-PCRs performed after transfection of COS-7 or HeLa cells with these viral RNAs revealed that Y88S and Y88L RNAs replicated at only very low levels. RNA replication could not be detected at all in cells transfected with D38E and Y89L RNAs. Taken together, the results suggest a correlation between the function of $2A^{pro}$ and productive poliovirus RNA replication in vivo that may be independent of the ability to cause p220 cleavage.**

Gene expression and replication of poliovirus are controlled by a complex cascade of proteolytic processing events which are mediated mostly by two viral gene products, 2A protease ($2A^{pro}$) and 3C protease ($3C^{pro}$) (15). $3C^{pro}$ is an active enzyme which catalyzes the bulk of the viral polyprotein processing, cleaving the viral polyprotein at eight distinct sites in highly controlled reactions which ultimately result in the production of over 21 processed polypeptides and precursors. In this scheme of regulation, the protease is catalytically active in different forms, and it has been shown that $3C^{pro}$ and 3CD polypeptide catalyze cleavages at different sites (12, 34). In addition, it has now been demonstrated that the $3C^{pro}$ domain of 3CD polypeptide functions (probably in a nonproteolytic mechanism) (1, 2) in RNA replication and may possess an RNA-binding site. Moreover, $3C^{pro}$ has also been shown to use certain cellular transcription factors as substrates (6–8, 13). These data demonstrate that $3C^{pro}$ performs multiple functions for the virus.

Similarly, $2A^{pro}$ is emerging as a multifunctional polypeptide. $2A^{pro}$ was originally described as catalyzing an essential cleavage of the viral polyprotein at a tyrosine-glycine pair at the 1D-2A junction (24, 31) and another, nonessential cleavage within the viral 3D polymerase sequence in some strains of poliovirus (16). Poliovirus $2A^{pro}$ is also known to indirectly cause shutoff of host cell protein synthesis (30) via a mechanism involving activation of an uncharacterized 57-kDa cellular proteinase which cleaves the p220 component of eukaryotic initiation factor 4 (14, 32, 33). Recent studies also showed that purified rhinovirus or coxsackievirus $2A^{pro}$ produced in *Escherichia coli* can cleave p220 directly, suggesting that $2A^{pro}$ may contribute to p220 cleavage both through direct and indirect mechanisms in vivo (17, 29). Mutagenesis studies have suggested that $2A^{pro}$-mediated activation of this cellular proteinase is catalytic in nature (11, 36). More recently, $2A^{pro}$ has been proposed to enhance translation of poliovirus RNA (10, 27); however, the absolute importance of this function is unclear, since virus RNA translates efficiently in HeLa cell extracts and translation is catalyzed during infection before $2A^{pro}$ has been synthesized.

Although it is reasonable to speculate that $2A^{pro}$ has evolved primarily for the dual functions of primary processing of the viral polyprotein by *cis* cleavage of its own amino terminus and specific inhibition of host cell protein synthesis by *trans* activation of p220 cleavage, it is possible and has been suggested that the latter activity is dispensable. Thus, restriction of host cell translation may not be essential for productive poliovirus replication and may merely facilitate virus yields and pathogenesis in vivo. Bernstein et al. (3) and then O'Neill and Racaniello (25) first suggested that host cell shutoff might not be essential for virus replication in tissue culture when they each described a viable mutant virus containing a small amino acid insertion in $2A^{pro}$ which was defective in p220 cleavage and host cell shutoff phenotypes. However, understanding of these mutant viruses was complicated because alterations in $2A^{pro}$-mediated *cis* cleavage of the viral polyprotein were not assessed.

Other reported studies of $2A^{pro}$ functions in vivo utilized either catalytically inactive protease or large deletions and thus did not allow separate analysis of the *trans* function of $2A^{pro}$ since all activities were lost. In contrast, while our current work was in progress, Molla et al. (22) reported the first specific

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, P.O. Box 26901, Oklahoma City, OK 73190.

analysis of $2A^{pro}$ *trans* functions. Using dicistronic viruses in which the requirement for 2A-mediated polyprotein *cis* cleavage was removed by insertion of an encephalomyocarditis internal ribosome entry sequence flanked by stop and start codons between the P1 segment and 2A of poliovirus, they demonstrated a dramatic decrease in RNA replication by 2A-deficient viral RNAs (22), thus implicating $2A^{pro}$ function in a step in viral RNA replication.

The studies reported here also separate *cis* and *trans* cleavage functions of $2A^{pro}$ by a different strategy. We previously generated a panel of point mutations in $2A^{pro}$ by site-specific mutagenesis and characterized the cleavage function of these mutant enzymes by several in vitro assays (36, 37). Three $2A^{pro}$ mutants, D38E, Y88L, and Y89L (in our nomenclature D38E indicates that the aspartic acid at position 38 was changed to glutamic acid), were fully active in autocatalytic *cis* processing yet failed to activate p220 cleavage in *trans*. Since these mutants were deficient in only one of the two known cleavage functions of $2A^{pro}$, in theory it became possible to test the biological importance of p220 cleavage and host cell shutoff for viral replication in vivo without altering normal processing of the viral polyprotein. Thus, in this study, several of these point mutations in $2A^{pro}$ were introduced into full-length poliovirus RNAs which were then characterized in vitro and in vivo. Unexpectedly, viral replication was found to be defective upon transfection of cells with several mutant viral RNAs, suggesting that a critical function of $2A^{pro}$ in RNA replication had been blocked.

## MATERIALS AND METHODS

**Construction of plasmids.** Five mutant plasmids of pEP2A (D38E, Y88L, Y88F, Y88S, and Y89L) were previously constructed (36); each plasmid contained a single-base (for D38E, Y88S, or Y88F) or double-base (for Y88L and Y89L) change in the 2A gene of type 1 poliovirus cDNA. The cDNA segment containing the mutation site was cloned into a full-length poliovirus transcription vector by inserting the 596-bp *Kpn*I fragment of the mutagenized plasmid pEP2A into the 9,803-bp *Kpn*I fragment of plasmid pT7-1 (35). Three full-length mutant poliovirus cDNAs [pT7(D38E), pT7(Y88L), and pT7(Y89L)] were thus generated. Alternatively, the 1,341-bp *Spe*I fragment from pEP2A (spanning the 2A gene, nucleotides 2650 to 3991) was ligated into the *Spe*I site of pGEM5zf(+), forming plasmid pGP2A. The internal 596-bp *Kpn*I fragment was then replaced with the same *Kpn*I fragment purified from pEP2A, pEP2A(Y88S), or pEP2A(Y88F). The 1,341-bp *Spe*I fragment from these new plasmids was then ligated into the 9,058-bp fragment resulting from a *Spe*I digest of pT7-1, thus recreating the wild-type (WT) full-length poliovirus cDNA pT7-1(SWT) or generating the mutants pT7-1(Y88F) and pT7-1(Y88S). The sequence of each plasmid was verified through the 2A gene region by using a Sequenase (United States Biochemicals) kit according to the manufacturer's directions.

**In vitro transcription and translation.** DNAs from pT7 plasmids were linearized with *Sal*I and used as the template for transcription reactions, which were performed with T7 RNA polymerase as specified by the manufacturer (Promega). pEP2A (36) and pT7-3916 (19) DNAs were linearized with *Hin*cII and used as templates similarly. After incubation, an aliquot of the reaction mixture containing RNA transcripts was used for transfection experiments without further purification. Alternatively, RNA products were then subjected to phenol-chloroform extraction and ethanol precipitation and used for in vitro translation reactions. Translation reactions were carried out in HeLa cell-free lysates as previously described (23).

**Immunoblot analysis.** Induction of p220 cleavage in vitro was assayed directly from HeLa cell-free lysates containing the translation products, which were subjected to immunoblot analysis with p220-specific rabbit antiserum (20).

**RNA transfection.** Monolayers of HeLa S3 or HEp-2 cells grown in Eagle minimum essential medium supplemented with 10% calf serum were used for RNA transfection by the DEAE-dextran method described previously (4). Cells were then overlaid with liquid medium or with semisolid medium containing 0.45% agarose (SeaKem) and incubated at 28, 32, or 37°C for 2 to 14 days for development of cytopathic effect (CPE) or plaques. If no CPE or plaques were detected after 10 days, the media and cell lysates collected at 3 or 5 days posttransfection were used to infect fresh HeLa and HEp-2 cells for detection of subsequent CPE and plaques.

**Northern (RNA) blot hybridization.** Total cytoplasmic RNAs from transfected cells were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (5) or lysis in detergent followed by phenol extraction. For the latter procedure, cells were scraped off dishes with a rubber policeman, washed once with cold Earles balanced salt solution, and then resuspended in 10 cell pellet volumes with 10 mM NaCl–10 mM Tris (pH 7.4)–1.5 mM $MgCl_2$–1% Nonidet P-40 for 30 s before sedimentation at 13,000 × *g* for 30 s. Cell lysates (240 μl) were removed, combined with 100 μl of PVS solution (2% sodium dodecyl sulfate [SDS], 0.1 mM polyvinylsulfate, 20 mM EDTA), and then extracted with equal volumes of phenol saturated with TNE (150 mM NaCl, 10 mM Tris [pH 8.4], 5 mM EDTA). The aqueous phase was reextracted with TNE-saturated phenol, and then RNA was precipitated with sodium acetate and ethanol. Five micrograms of RNA from each sample was denatured with 2.2 M formaldehyde and 50% formamide and fractionated on 1% formaldehyde–agarose gels. RNA was then passively transferred onto nitrocellulose paper and hybridized with a DNA probe, which was obtained by digestion of pT7-1 DNA with *Bam*HI. The resulting 1,429-bp fragment encompassing most of the P1 gene region was gel purified and labeled with [$^{32}$P]dATP by the random-priming method (Prime-a-Gene kit; Promega). Prehybridization and hybridization procedures were performed as described previously (28).

**Reverse transcription and PCR.** For experiments in which PCR was employed, RNA transcription products were treated with 5 U of RQ1 DNase (Promega) before transfection of cells. Total RNA was prepared from transfected cells and subjected to reverse transcriptase (RT)-PCRs. The oligonucleotide primer H (5′ ggattcggacaccaaaacaaagcgg 3′) (plus strand, nucleotides [nt] 3385 to 3409) was used for synthesis of cDNA on the negative-strand viral RNA template. cDNA was synthesized for 45 min at 42°C in 25-μl reaction mixtures containing 40 mM KCl, 50 mM Tris (pH 8.3), 7 mM MgCl, 10 mM dithiothreitol, 200 μM deoxynucleoside triphosphates (dNTPs), 12 μg of cytoplasmic RNA, 0.5 μmol of primer H, 15 U of avian myeloblastosis virus RT (Promega), and 25 U of RNAsin (Promega). An additional oligonucleotide, primer G (5′ gcggccccaagtgactctatg 3′) (complementary to nt 3843 to 3863), was used for PCRs. Briefly, 10-μl portions of RT reaction products were added to 100-μl reaction mixtures containing 20 mM Tris (pH 8.3) (25°C), 20 mM KCl, 0.2 mM dNTPs, 3 mM MgCl, 0.5 μmol (each) of primer G and primer H, and 2 U of *Taq* polymerase (Gibco/BRL). Each cycle consisted of a denaturation step at 94°C for 1 min, annealing at 62°C for 2 min, and extension at 72°C for 3 min. DNA synthesis was carried out for 30 cycles with a DNA thermal cycler (Perkin-Elmer Cetus).

**RNA sequencing.** Cytolytic viruses appearing in cells after transfection were sequenced directly with RT through the $2A^{pro}$ gene. Briefly, virus plaques were picked from primary transfection cultures and expanded by growth in $1 \times 10^6$ HeLa cells for 3 days, and then supernatants were used to infect $2 \times 10^7$ HeLa cells at a multiplicity of infection of 10. Infected cells were harvested at 8 h postinfection, and total RNA was hybridized with the DNA oligonucleotide primer G (complementary to nt 15 to 31 of the 2B sequence) or with primer A (5′ tgc gaa tcc atg gcc 3′), which binds internally in the $2A^{pro}$ region (nt 3679 to 3693; $2A^{pro}$ codons 100 to 104). Sequencing reactions were carried out with the avian myeloblastosis virus RT (RNA sequencing kit; Boehringer-Mannheim) and dideoxynucleotides as described by the manufacturer. Alternatively, low-yield viral RNA sequences in primary transfected cells were amplified by RT-PCR before sequencing of cDNA products. The 482-bp product was gel purified and sequenced by using primer G or A and the Sequenase kit (United States Biochemicals) according to the manufacturer's instructions.

## RESULTS

**Effects of the mutations on in vitro processing of the viral polyprotein.** Five single-site mutations (D38E, Y88L, Y88F, Y88S, and Y89L) in $2A^{pro}$ were previously introduced by site-specific mutagenesis into the parental plasmid pEP2A, which contained a subgenomic segment of type 1 poliovirus cDNA (36). The predicted translation product of the plasmid pEP2A was a precursor polypeptide containing the carboxyl-terminal portion of poliovirus VP3 (1C), all of VP1 (1D), and $2A^{pro}$. Since the intact 1D-2A junction was preserved in this construct, it provided an immediate assay for the autocatalytic *cis* cleavage function of $2A^{pro}$. When these RNA transcripts were translated in rabbit reticulocyte lysates, the resultant mutant $2A^{pro}$ retained the active *cis* cleavage function at the 1D-2A junction. However, three $2A^{pro}$ mutants (D38E, Y88L, and Y89L) failed to activate p220 cleavage in *trans* when incubated with uninfected HeLa cell cytoplasmic extracts (36). Two other point mutants with mutations at position 88 displayed WT (Y88F) or partial *trans* (Y88S) cleavage activity. Since these mutants exhibited a range of *trans* cleavage activities in vitro and since three were defective only in the p220ase activation function of $2A^{pro}$, they were used in this study to examine the biological importance of other $2A^{pro}$ functions,

such as host protein shutoff, in the life cycle of poliovirus. Therefore, we introduced each of these point mutations into full-length poliovirus cDNA, generating five full-length mutant viral cDNAs which were used in all subsequent experiments.

The effect of these 2A$^{pro}$ mutants on proteolytic processing of the complete viral polyprotein was first examined in vitro. Mutant or WT pT7 plasmids were linearized and transcribed with T7 RNA polymerase, resulting in RNA transcripts which contained the mutation in 2A$^{pro}$ or WT sequence, 19 extra nucleotides at the 5′ terminus, and 31 extra nucleotides at the 3′ terminus following the poly(A) tract but which were otherwise identical to poliovirus mRNA. Figure 1 shows the products of in vitro translation reactions in HeLa cell lysates programmed with several viral RNAs. Reactions programmed with T7 RNA polymerase-transcribed WT RNA or reconstructed wild-type (SWT) RNA (lanes c and i) as well as the mutant poliovirus RNAs (lanes d, e, f, j, and k) yielded protein products that were indistinguishable from those synthesized by cell-free translation of purified virion RNA (lanes b and h). In particular, the equivalent appearance of P1, VP1, and 2A$^{pro}$ in processed products of the WT and mutant polyproteins indicated the existence of an active autocatalytic *cis* cleavage function of these 2A$^{pro}$ mutants. Although 2A$^{pro}$ is difficult to visualize in the weaker translation reaction programmed with Y88F RNA (Fig. 1, lane j) (caused by partial degradation of RNA), we judged its *cis* cleavage activity to be efficient from other translation experiments (not shown) and by the lack of unique unprocessed proteins larger than P1. Further, with all the constructs, no secondary modulation in the efficiency of 3C$^{pro}$-catalyzed processing was observed. Thus, all five 2A$^{pro}$ mutants tested (D38E, Y88L, Y88F, Y88S, and Y89L) catalyzed normal proteolytic processing of the viral polyprotein when assayed in vitro.

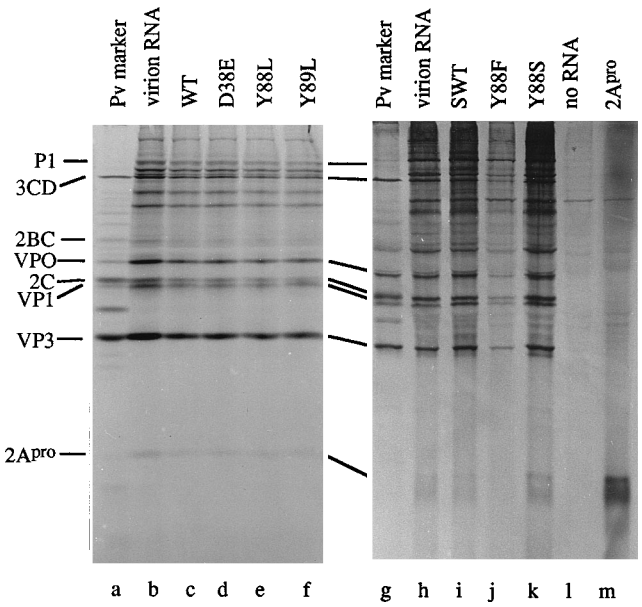


FIG. 1. In vitro translation and proteolytic processing of the mutant poliovirus polyproteins. Full-length RNA transcripts prepared from pT7-1 (lane c), reconstructed pT7-1 (SWT) (lane i), mutagenized derivatives of this plasmid (lanes d to f, j and k), or pEP2A (lane m) were translated in HeLa cell-free lysates for 3 h at 30°C. Translation reactions were also programmed with no RNA (lane l) or poliovirus virion RNA isolated from infected cells (lanes b and h). Lanes a and g contain radiolabeled poliovirus-infected-cell extracts. After translation reactions were complete, radiolabeled proteins were separated on SDS–12.5% polyacrylamide gels and detected by autoradiography. The positions of poliovirus proteins are indicated on the left.

**Effects of the mutations on in vitro induction of p220 cleavage.** To determine whether the 2A$^{pro}$ mutants were capable of activating p220-specific proteinase in *trans*, HeLa cell-free lysates containing translation products of the mutant viral RNAs were analyzed by immunoblotting with p220-specific antiserum. Translation products of WT virus RNAs and mutants Y88F and Y88S (Fig. 2, lanes d, j, k, and l) induced extensive cleavage of p220 into a set of smaller polypeptides, which comigrated with the p220 cleavage products from poliovirus-infected cells (Fig. 2, lanes b and i). The relative activity of Y88S 2A$^{pro}$ appears somewhat higher under these conditions than in previous studies, in which it was expressed in reticulocyte lysates from a subgenomic construct (36) but, in repeat experiments, was not as active as WT 2A$^{pro}$. Meanwhile, translation products from virion RNA usually induced the greatest amount of p220 cleavage, probably because of a higher level of translational efficiency (Fig. 2, lane c). In contrast, no new specific p220 cleavage products were generated in HeLa cell-free lysates programmed with the mutant viral RNAs D38E, Y88L, and Y89L (Fig. 2; compare lane a with lanes e, f, and g). We conclude that when assayed in vitro, three 2A$^{pro}$ mutants (D38E, Y88L, and Y89L) were still defective in induction of p220 cleavage but were fully active in the autocatalytic *cis* cleavage function. Further, each 2A$^{pro}$ mutant displayed similar phenotypes whether expressed in full-length genomic constructs or in subgenomic constructs, as previously observed.

**Effects of 2A$^{pro}$ mutations on virus viability.** The five point mutations (D38E, Y88F, Y88S, Y88L, and Y89L) in 2A$^{pro}$ were then tested for their effects on virus viability. The full-length poliovirus RNAs were transfected into HeLa, HEp-2, or COS-7 cells and incubated at 28, 32, or 37°C to test their infectivities by plaque development. Since three mutants did not activate p220 cleavage, defective host protein shutoff and perhaps a less cytopathic virus were anticipated for some constructs. As expected, transfection of cells with WT viral RNA or Y88F (which exhibited the WT phenotype) viral RNA consistently produced many plaques, within 24 h at 37°C or 96 h at 32°C (Fig. 3). Cells transfected with Y88L viral RNA often produced a few plaques by 50 h at 37°C or 96 h at 32°C. In contrast, no CPE or plaques developed in cells transfected with Y88S, D38E, or Y89L viral RNA at any temperature following nine independent transfections (Table 1). The type of cell transfected had no observable influence on the absolute recovery of cytopathic virus, except transfection efficiencies seemed to be about fivefold higher in COS-7 cells than either HeLa or HEp-2 cells (data not shown). Additionally, no CPE or plaques were detected when cell lysates or culture media obtained 3 or 10 days posttransfection with D38E, Y88S, or Y89L RNA were used to infect fresh HeLa or HEp-2 cells at 32 or 37°C. Therefore, two of three point mutations in 2A$^{pro}$ which abrogated only *trans* cleavage activity prevented production of cytopathic poliovirus. Interestingly, Y88S RNA, which was determined to produce 2A$^{pro}$ active in both *cis* and *trans* cleavage activities in the full-length construct, also did not produce detectable cytopathic virus.

**Viral RNA synthesis in cells transfected with mutant RNA.** To determine whether these mutant viral RNAs could replicate in transfected cells without producing marked CPE, total cytoplasmic RNAs were isolated from transfected cells at various times, fractionated on denaturing formaldehyde-agarose gels, and hybridized with a poliovirus-specific [$^{32}$P]dATP-labeled DNA probe. Figure 4 shows that after transfection with WT viral RNA, newly synthesized viral RNA was detectable in cells by 14 h posttransfection and reached the highest concen-

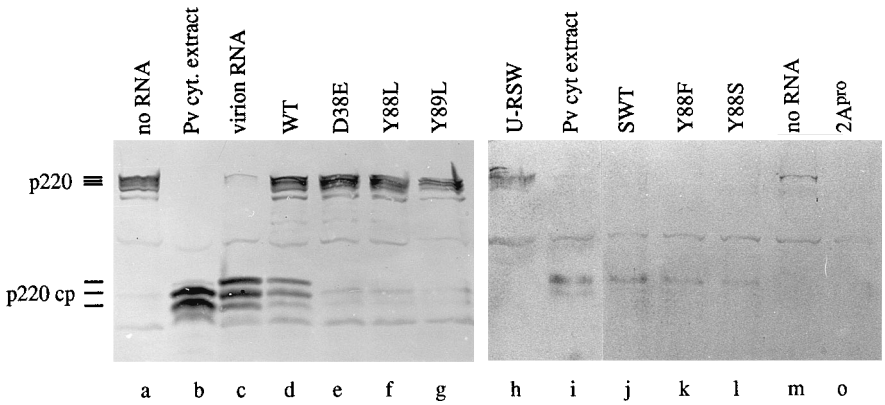


FIG. 2. Induction of p220 cleavage by in vitro translation products of mutant poliovirus RNAs. Translation reactions were programmed with full-length transcripts of WT T7-derived RNA (lanes d and j) or its mutagenized derivatives (lanes e to g, k and l), and reaction mixtures were incubated at 30°C for 3 h. Reactions were also programmed with no RNA (lanes a and m), poliovirus RNA isolated from virions (lane c), or $2A^{pro}$ RNA from pEP2A (lane o). HeLa cell-free lysates containing translation products were analyzed on SDS–7% polyacrylamide gels and immunoblotted with polyclonal antiserum specific for p220. Pv cyt extract (lanes b and i), cytoplasmic extract from poliovirus-infected HeLa cells. U-RSW (lane h), ribosomal salt wash from uninfected cells, which contains concentrated levels of p220 and is used as a standard. Positions of p220 and poliovirus-induced p220 cleavage products (cp) are indicated.

tration in cultures by 48 h posttransfection. Y88F viral RNA also replicated to high levels, with similar kinetics. In contrast, Y88L viral RNA replicated less efficiently than WT RNA at 37°C, but viral RNA was usually detectable by 48 h posttransfection. Y88S viral RNA replicated more poorly, and RNA was not found until 48 h posttransfection or, in some experiments, was undetectable by this assay. Finally, D38E and Y89L RNAs were repeatedly unable to replicate to detectable levels at any temperature tested, even when cells were assayed 5 or 7 days posttransfection (data not shown). These results strongly suggest that inhibition of RNA replication may result from different point mutations in $2A^{pro}$.

To further test the magnitude of the block in RNA replication caused by the D38E mutation, we performed RT-PCR to detect synthesis of minus-strand viral RNA sequences in transfected cells. Figure 5 shows that the expected 478-bp band was easily amplified from cells transfected with Y88L or Y88S RNA but not D38E RNA. This result confirms that RNA replication is severely inhibited by the D38E mutation in $2A^{pro}$. To determine if the other viral RNAs which did replicate still carried their original mutations, purified progeny virus RNA or RT-PCR DNA products prepared from cells 48 h posttransfection were sequenced through the $2A^{pro}$ gene region. The results are summarized in Table 1. The Y88F mutation in $2A^{pro}$ had little detectable effect on virus replication and produced a relatively stable, cytolytic virus. In contrast, in two instances when cells transfected with Y88S RNA replicated enough to be detectable by Northern analysis, sequencing of product RNAs revealed that the original TCT (Ser) mutation had been replaced with TTT (Phe, which displays a wild-type phenotype) or TGT (Cys). Another Y88S transfection in which Northern analysis did not reveal RNA replication contained Y88S negative-strand RNA which was detected by RT-PCR. Thus, the Y88S mutation was associated with a low level of RNA replication which was partially relieved by a secondary mutation at the same codon. Interestingly, the lysine mutation at the same codon (Y88L) was tolerated better in vivo, since minus-strand RNA bearing this mutation was recovered several times. Taken together, these results show that the introduction of point mutations into

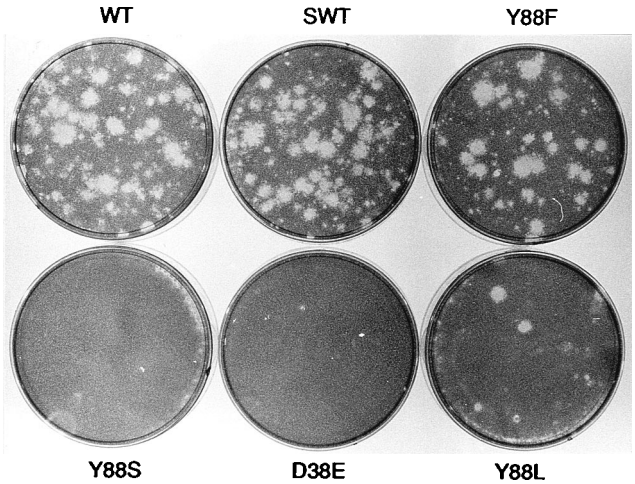


FIG. 3. Production of cytopathic poliovirus by transfection of mutant viral RNAs. COS-7 cells transfected with indicated viral RNAs were overlaid with 0.45% agarose and incubated for 96 h at 32°C before being stained with crystal violet. Small inconsistencies in the cell monolayer in Y88S and D38E were also seen in nontransfected cells (not shown) and were not associated with cells exhibiting detectable CPE under microscopic examination. SWT, reconstructed WT RNA.

TABLE 1. Effects of point mutations in $2A^{pro}$[a]

| Viral RNA | Polyprotein cleavage | p220 cleavage | Cytopathic virus recovery | RNA replication (Northern blot) | $2A^{pro}$ sequence recovered[b] |
|---|---|---|---|---|---|
| SWT | ++ | ++ | +++ | +++ | WT |
| D38E | ++ | − | − | − | ND |
| Y88F | ++ | ++ | +++ | +++ | Y88F |
| Y88S[c] | ++ | ++ | − | + | Y88F |
| | | | − | − | Y88S |
| | | | − | + | Y88C |
| Y88L | ++ | − | + | + | Y88L |
| Y89L | ++ | − | − | − | ND |

[a] SWT, reconstructed WT RNA; +++, activity ≥90% of WT level; ++, activity 10 to 90% of WT level; +, activity 1 to 10% of WT level; −, activity undetectable; ND, not determined.

[b] Sequences were determined from plaque-purified progeny virus in infected cells (SWT and Y88F) or from RT-PCR cDNA products (Y88S and Y88L) at 48 h posttransfection.

[c] Results of three separate transfection experiments are listed.

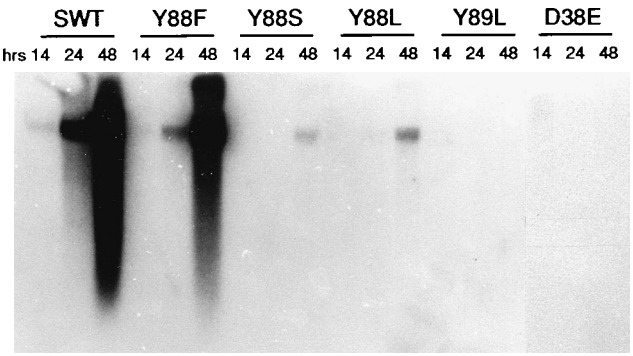


FIG. 4. Northern blot analysis of RNA from transfected cells. Total cellular RNA was prepared at 14, 24, and 48 h after transfection of HeLa cells with WT or mutant viral RNA. RNA was then fractionated in a 1% agarose–formaldehyde gel, transferred to nitrocellulose, hybridized with a 1,429-bp $^{32}$P-labeled poliovirus cDNA probe, and autoradiographed. SWT, reconstructed WT RNA.

2A$^{pro}$ at three loci strongly influences the ability of viral RNA to replicate in vivo.

## DISCUSSION

Until recently, only two distinct functions had been described for 2A$^{pro}$: processing of the viral polyprotein, in particular, catalyzing the initial cleavage of the polyprotein which releases the P1 structural protein precursor; and inhibition of host cell protein synthesis (3, 30), through activation of a cellular proteinase which cleaves the p220 component of eukaryotic initiation factor 4F (33) as well as via direct cleavage of p220 (17, 29). Although shutoff of host cell protein synthesis is generally believed to be important for a productive poliovirus infection, it is still unclear whether this is an essential function to support replication in cells. Previously, two mutant viruses with small-plaque phenotypes were generated by small insertions in the 2A gene (3, 25). Although each of these viruses was defective in the host cell shutoff phenotype, interpretations of the contribution of this defect to the small-plaque phenotype were complicated by uncertain phenotypes of polyprotein processing and/or leakiness of the mutants. Nevertheless, the relative viabilities of these viruses and the ability of WT virus to replicate in erythroblastoid cells without inhibiting host translation (18) suggested that host cell shutoff may not be requisite for virus replication in tissue culture cells.

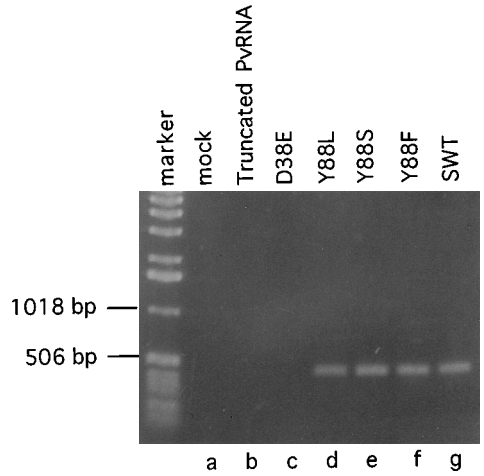


FIG. 5. Detection of minus-strand poliovirus RNA by RT-PCR. RT-PCR was performed on total cytoplasmic RNA samples taken from COS cells 48 h posttransfection with the indicated viral RNAs (lanes c to g). In addition, mock-transfected cells and cells transfected with a nonreplicating incomplete poliovirus RNA (spanning viral nucleotides 1 to 3916) were included in the analysis (lanes a and b, respectively). Product cDNAs were analyzed on a 0.9% agarose gel stained with ethidium bromide. SWT, reconstructed WT RNA.

In this study, we introduced five 2A$^{pro}$ point mutations into full-length virus RNA, three of which (D38E, Y88L, and Y89L) were previously shown to encode 2A$^{pro}$ specifically lacking *trans* but not *cis* cleavage function (36). Therefore, we could test the contribution of *trans* functions of 2A$^{pro}$ to virus replication without complications arising from poor or improper polyprotein processing. When the full-length viral RNAs containing these three point mutations were translated in HeLa cell lysates in vitro, the efficiency of translation was unaffected compared with that of WT viral RNA. Both 2A$^{pro}$- and 3C$^{pro}$-mediated processing were normal and p220 cleavage was not observed, thus verifying the same phenotype in full-length clones as previously observed in smaller subgenomic constructs. Thus, transfection of these mutant viral RNAs was expected to produce small plaques at delayed times or perhaps even generate a persistent infection with production of noncytopathic viruses. Indeed, cytopathic viruses were not recovered after transfection with two of these mutant viral RNAs. Surprisingly, however, no synthesis of virus-specific RNA was detected by Northern blot analysis after transfection of the D38E and Y89L mutant viral RNAs, which caused us to conclude that viral RNA replication was severely inhibited and that noncytopathic viruses were not produced as well. In the case of Y88L, virus production was delayed and reduced in titer. Therefore, each of the three point mutations in 2A$^{pro}$ which inactivated *trans* functions proved either to be lethal or deleterious for poliovirus replication.

Interestingly, while the Y88S mutation in 2A$^{pro}$ reduced RNA replication significantly, enough replication occurred to enable mutation of input sequences by the RNA polymerase in two instances, to Y88F or Y88C. Both of these changes were associated with higher levels of RNA replication, and both changed the relatively hydrophilic serine to a more hydrophobic amino acid.

There are three interpretations for the effects that these 2A$^{pro}$ mutations have on the replication cycle of poliovirus. First, the lethality of these mutations may simply suggest that the ability to shut off host translation is critical to the virus. However, the fact that the mutant Y88S, which induced p220 cleavage in vitro, was still defective in RNA replication suggests that lack of host cell shutoff may not be the cause of the major replication defect. Also, Y88L RNA, which encodes a 2A$^{pro}$ which could not induce p220 cleavage, was able to replicate more efficiently than Y88S RNA, which does cause p220 cleavage. Further, several lines of evidence suggest that poliovirus can survive and replicate in cells without inducing host protein shutoff. These include reports of successful replication and propagation of two distinct virus mutants, containing short insertions in 2A$^{pro}$, each of which did not shut off host translation in HeLa cells and did not induce significant p220 cleavage (3, 25). Human K562 cells can be persistently infected with WT poliovirus without inducing host cell shutoff and are able to support replication at levels of 70 to 150 PFU per cell (18). Similarly, persistent infection of neuroblastoma cells proceeds without host protein shutoff (26). Thus, loss of shutoff function may cause a reduction in overall virus yield but is likely not requisite for replication in tissue culture cells.

Second, it is formally possible that the observed effects of 2A$^{pro}$ mutants could be mediated through repression of viral

RNA translation in vivo. However, the translation transactivation function of $2A^{pro}$ described previously was not nearly as dramatic in intensity as seen here (10, 27), and no differences in in vitro translation rates of viral RNAs encoding WT and mutant $2A^{pro}$ were observed.

Third, our results suggest that $2A^{pro}$ may be a multifunctional protein which mediates another unknown function(s) which is required for virus replication, as has been shown for $3C^{pro}$. Pleiotropic functions have been recently described for poliovirus $3C^{pro}$ in the precursor form 3CD, which was suggested to bind viral RNA and function in replication (1, 2). Although the specific function of $2A^{pro}$ which was adversely affected in our mutants has not yet been identified, our results imply that some $2A^{pro}$-mediated function(s) besides the initial polyprotein processing and p220 cleavage is essential for productive poliovirus replication. These results strongly support those recently reported by Molla et al. (22) and strengthen the evidence that a new function of $2A^{pro}$ is closely linked to productive poliovirus RNA replication. It is possible that $2A^{pro}$ may play a pivotal role in viral replication and translation by interacting with certain host factors, other viral proteins, or even, in keeping with the $3C^{pro}$ analogy, viral RNA. It is significant that the 2A polypeptide of encephalomyocarditis virus has been previously reported to bind RNA (9), and genetic evidence for direct interactions between $2A^{pro}$ and the 5′ nontranslated region of poliovirus RNA has been recently described (21). It is possible that the unknown role of $2A^{pro}$ in viral RNA replication is achieved through an RNA-binding function. Experiments to test this hypothesis directly are under way.

## ACKNOWLEDGMENTS

We thank E. Ehrenfeld for helpful discussions and W. S. Blair for technical suggestions. S.F.Y. gratefully recognizes the assistance and support of C. E. Yu.

This work was supported by Public Health Service grant AI27914, the Presbyterian Health Foundation, and Oklahoma Center for the Advancement of Science project HR3-082.

## 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. **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.
4. **Charini, W. A., C. C. Burns, E. Ehrenfeld, and B. L. Semler.** 1991. *trans* rescue of a mutant poliovirus RNA polymerase function. J. Virol. **65:**2655–2665.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **Clark, M. E., and A. Dasgupta.** 1990. A transcriptionally active form of TFIIIC is modified in poliovirus-infected HeLa cells. Mol. Cell. Biol. **10:**5106–5113.
7. **Clark, M. E., P. M. Lieberman, A. J. Berk, and A. Dasgupta.** 1993. Direct cleavage of human TATA-binding protein by poliovirus protease 3C in vivo and in vitro. Mol. Cell. Biol. **13:**1232–1237.
8. **Das, S., and A. Dasgupta.** 1993. Identification of the cleavage site and determinants required for poliovirus $3C^{Pro}$-catalyzed cleavage of human TATA-binding transcription factor TBP. J. Virol. **67:**3326–3331.
9. **Gorbalenya, A. E., K. M. Chumakov, and V. I. Agol.** 1978. RNA binding properties of nonstructural polypeptide G of encephalomyocarditis virus. Virology **88:**183–185.
10. **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.
11. **Hellen, C. U. T., M. Fäcke, H. G. Kräusslich, C.-K. Lee, and E. Wimmer.** 1991. Characterization of poliovirus 2A protease by mutational analysis: residues required for autocatalytic activity are essential for induction of cleavage of eukaryotic initiation factor 4F polypeptide p220. J. Virol. **65:** 4226–4231.
12. **Jore, J., B. DeGeus, R. J. Jackson, P. H. Pouwels, and B. 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.
13. **Kliewer, S., C. Muchardt, R. Gaynor, and A. Dasgupta.** 1990. Loss of a phosphorylated form of transcription factor CREB/ATF in poliovirus-infected cells. J. Virol. **64:**4507–4515.
14. **Krausslich, 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.
15. **Kraüsslich, H. G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57:**701–754.
16. **Lee, C. K., and E. Wimmer.** 1988. Proteolytic processing of poliovirus polyprotein: elimination of 2Apro-mediated, alternative cleavage of polypeptide 3CD by in vitro mutagenesis. Virology **166:**405–414.
17. **Liebig, H. D., E. Ziegler, R. Yan, K. Hartmuth, H. Klump, H. Kowalski, D. Blaas, W. Sommergruber, L. Frasel, B. Lamphear, R. Rhoads, E. Kuechler, and T. Skern.** 1993. Purification of 2 picornaviral-2A proteinases—interaction with eIF-4-γ and influence on in vitro translation. Biochemistry **32:** 7581–7588.
18. **Lloyd, R. E., and M. Bovee.** 1993. Persistent infection of human erythroblastoid cells by poliovirus. Virology **194:**200–209.
19. **Lloyd, R. E., M. J. Grubman, and E. Ehrenfeld.** 1988. Relationship of p220 cleavage during picornavirus infection to 2A proteinase sequences. J. Virol. **62:**4216–4223.
20. **Lloyd, R. E., H. G. Jense, and E. Ehrenfeld.** 1987. Restriction of translation of capped mRNA in vitro as a model for poliovirus-induced inhibition of host cell protein synthesis: relationship to p220 cleavage. J. Virol. **61:**2480–2488.
21. **Macadam, A. J., G. Ferguson, T. Fleming, D. M. Stone, J. W. Almond, and P. D. Minor.** 1994. Role for poliovirus protease 2A in cap independent translation. EMBO J. **13:**924–927.
22. **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.
23. **Morley, S. J., W.-J. Buhl, and R. J. Jackson.** 1985. A rabbit reticulocyte factor which stimulates protein synthesis in several mammalian cell-free systems. Biochim. Biophys. Acta **825:**57–69.
24. **Nicklin, M. J., H. G. Kräusslich, H. Toyoda, J. J. Dunn, and E. Wimmer.** 1987. Poliovirus polypeptide precursors: expression in vitro and processing by exogenous 3C and 2A proteinases. Proc. Natl. Acad. Sci. USA **84:**4002–4006.
25. **O'Neill, R. E., and V. R. Racaniello.** 1989. Inhibition of translation in cells infected with a poliovirus $2A^{pro}$ mutant correlates with phosphorylation of the alpha subunit of eucaryotic initiation factor 2. J. Virol. **63:**5069–5075.
26. **Pelletier, I., T. Couderc, S. Borzakian, E. Wyckoff, R. Crainic, E. Ehrenfeld, and F. Colberegarapin.** 1991. Characterization of persistent poliovirus mutants selected in human neuroblastoma cells. Virology **180:**729–737.
27. **Percy, N., G. J. Belsham, J. K. Brangwyn, M. Sullivan, D. M. Stone, and J. W. Almond.** 1992. Intracellular modifications induced by poliovirus reduce the requirement for structural motifs in the 5′ noncoding region of the genome involved in internal initiation of protein synthesis. J. Virol. **66:**1695–1701.
28. **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.
29. **Sommergruber, W., H. Ahorn, H. Klump, J. Seipelt, A. Zoephel, F. Fessl, E. Krystek, D. Blaas, E. Kuechler, H. D. Liebig, and T. Skern.** 1994. 2A proteinases of coxsackie- and rhinovirus cleave peptides derived from eIF-4 γ via a common recognition motif. Virology **198:**741–745.
30. **Sun, X.-H., and D. Baltimore.** 1989. Human immunodeficiency virus tat-activated expression of poliovirus protein 2A inhibits mRNA translation. Proc. Natl. Acad. Sci. USA **86:**2143–2146.
31. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45:**761–770.
32. **Wyckoff, E. 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.
33. **Wyckoff, E. E., R. E. Lloyd, and E. Ehrenfeld.** 1992. Relationship of eukaryotic initiation factor 3 to poliovirus-induced p220 cleavage activity. J. Virol. **66:**2943–2951.
34. **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.
35. **Ypma-Wong, M. F., and B. L. Semler.** 1987. In vitro molecular genetics as a tool for determining the differential cleavage specificities of the poliovirus 3C proteinase. Nucleic Acids Res. **15:**2069–2088.
36. **Yu, S. F., and R. E. Lloyd.** 1991. Identification of essential amino acid residues in the functional activity of poliovirus 2A-protease. Virology **182:**615–625.
37. **Yu, S. Y. F., and R. E. Lloyd.** 1992. Characterization of the roles of conserved cysteine and histidine residues in poliovirus 2A-protease. Virology **186:**725–735.