

Inhibition of Translation in Cells Infected with a Poliovirus 2A^{pro} Mutant Correlates with Phosphorylation of the Alpha Subunit of Eucaryotic Initiation Factor 2

ROBERT E. O'NEILL AND VINCENT R. RACANIELLO*

Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, New York 10032

Received 28 April 1989/Accepted 2 August 1989

A poliovirus type 2 Lansing mutant was constructed by inserting 6 base pairs into the 2A^{pro} region of an infectious cDNA clone, resulting in the addition of a leucine and threonine into the polypeptide sequence. The resulting small-plaque mutant, 2A-2, had a reduced viral yield in HeLa cells and synthesized viral proteins inefficiently. Infection with the mutant did not lead to specific inhibition of host cell protein synthesis early in infection, and this defect was attributed to a failure to induce cleavage of the cap-binding complex protein p220. At late times after infection with the mutant virus, both cellular and viral protein syntheses were severely inhibited. To explain this global inhibition of protein synthesis, the phosphorylation state of the alpha subunit of eucaryotic initiation factor 2 (eIF-2 α) was examined. eIF-2 α was phosphorylated in both R2-2A-2- and wild-type-virus-infected cells, indicating that poliovirus does not encode a function that blocks phosphorylation of eIF-2 α . The kinetics and extent of eIF-2 α phosphorylation correlated with the production of double-stranded RNA in infected cells, suggesting that eIF-2 α is phosphorylated by P1/eIF-2 α kinase. When HeLa cells were infected with R2-2A-2 in the presence of 2-aminopurine, a protein kinase inhibitor, much higher virus titers were produced, cleavage of p220 occurred, and host cell protein synthesis was specifically inhibited. Since phosphorylation of eIF-2 α was not inhibited by 2-aminopurine, we propose that 2-aminopurine rescues the ability of R2-2A-2 to induce cleavage of p220 by inhibition of a second as yet unidentified kinase.

Infection of cells with poliovirus results in the selective inhibition of host cell protein synthesis (for recent reviews, see references 9 and 35). Translation of host cell mRNA is specifically blocked at the initiation step of polypeptide synthesis (17, 21). A protein synthesis initiation factor, the cap-binding protein (CBP) complex, is both structurally and functionally altered in poliovirus-infected cells (4, 10, 11, 19, 36). The 220-kilodalton subunit of the CBP complex, p220, is absent from poliovirus-infected cells and is replaced by two or three antigenically related polypeptides of 100 to 130 kilodaltons, which are presumably p220 degradation products. Inactivation of the CBP complex has been proposed as the mechanism of specific inhibition of host cell translation. Poliovirus RNA is not capped (13, 25), and its translation occurs by a cap-independent mechanism in cells lacking functional CBP.

Several experimental results support the idea that the poliovirus protease 2A^{pro} is involved in cleavage of p220. 2A^{pro} expressed in reticulocyte lysate in the absence of other viral polypeptides can induce cleavage of HeLa cell p220 in vitro (18). A poliovirus mutant, B1-2A-1, that fails to inhibit host translation early after infection and does not induce p220 cleavage was recently isolated (1). This mutant contains a 1-amino-acid insertion in 2A^{pro}, providing genetic evidence that 2A^{pro} is involved in p220 cleavage. However, 2A^{pro} can be physically separated from p220 cleavage activity in infected cells, and in vitro cleavage of p220 cannot be inhibited by antiserum against 2A^{pro} which inhibits 2A^{pro}-mediated cleavage of the poliovirus polyprotein (22). Cleavage of p220 is therefore believed to be mediated indirectly by 2A^{pro}.

To further understand the role of 2A^{pro} in poliovirus replication, we have isolated a viral mutant containing a

2-amino-acid insertion in this polypeptide in a location different from that of the insertion in B1-2A-1. Mutant R2-2A-2, hereafter abbreviated as 2A-2, forms small plaques on HeLa cells, does not inhibit host cell translation early in infection, and does not induce cleavage of p220. Like mutant B1-2A-1, 2A-2 induces a global inhibition of protein synthesis at late times after infection, such that translation of both viral and cellular mRNAs is inhibited. It was proposed that the global inhibition of protein synthesis is due to the phosphorylation of the alpha subunit of eucaryotic initiation factor 2 (eIF-2 α) (1). We show here that the global inhibition of protein synthesis in cells infected with 2A-2 correlates with the phosphorylation of eIF-2 α . However, phosphorylation of eIF-2 α was also observed in wild-type-poliovirus-infected cells. Our results show that poliovirus does not encode a function which inhibits eIF-2 α phosphorylation. We suggest that poliovirus must synthesize its structural proteins before protein synthesis is globally inhibited by eIF-2 α phosphorylation and that this is accomplished by eliminating competition with cellular mRNA for the translation machinery.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells were grown in suspension culture in Joklik minimal essential medium containing 5% horse serum. For growth in monolayers, HeLa cells were plated in Dulbecco minimal essential medium containing 10% horse serum. CV1 cells (African green monkey kidney cells) were maintained in Dulbecco minimal essential medium containing 10% calf serum. The wild-type poliovirus type 2 Lansing used in these experiments was derived by transfection of HeLa cells with viral RNA synthesized in vitro with T7 RNA polymerase, using the plasmid pT7L as template (24).

Construction of mutant 2A-2. pT7L was partially digested

* Corresponding author.

with restriction endonuclease *StuI* (sites at bases 3765 and 6512 of poliovirus type 2 Lansing cDNA) and ligated with T4 DNA ligase (New England BioLabs, Inc.) to 6-base *HpaI* linkers. After cleavage with *HpaI*, full-length linear DNA was isolated from a 1% agarose gel by a modification of the glass powder method (38), recircularized, and used to transform *Escherichia coli* DH5 α . RNA derived from one cDNA containing a linker inserted after position 3767 (pHST26) was infectious after transfection into HeLa and CV1 cells. The presence of the *HpaI* linker was confirmed by direct sequence analysis of viral RNA. Preliminary characterization of this mutant, 2A-2, has been reported elsewhere (7).

Virus infections. Analysis of viral replication was performed by infecting subconfluent monolayers of 7.5×10^5 HeLa cells at a multiplicity of infection of 20. After absorption at room temperature for 45 min, medium was added and monolayers were incubated at 37°C. At different times postinfection, monolayers were frozen and thawed three times. The medium was clarified by centrifugation, and virus titer was determined by plaque assay as described previously (24). For analysis of viral replication in the presence of 2-aminopurine (Sigma Chemical Co.), medium containing 10 mM 2-aminopurine was added to monolayers 1 h before infection and was replaced after absorption.

Analysis of protein synthesis. Synthesis of protein in virus-infected cells was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of [³⁵S]methionine-labeled cytoplasmic extracts. At various times postinfection, the medium was removed and replaced with Dulbecco minimal essential medium with D-glucose, without D-glutamine, without methionine (GIBCO Laboratories), and supplemented with 25 μ Ci of [³⁵S]methionine per ml (ICN Pharmaceuticals Inc.). After 15 min of incubation, the medium was removed and cytoplasmic extracts were prepared by lysing cells in ice-cold Nonidet P-40 lysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5], 120 mM KCl, 1 mM DTE, 1 mM magnesium acetate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) followed by removal of nuclei by centrifugation. A portion of the cytoplasmic extract was separated on a 12.5% SDS-polyacrylamide gel. After electrophoresis, gels were treated with Autofluor (National Diagnostics), dried, and exposed to X-ray film at -70°C.

Analysis of viral RNA synthesis. Viral RNA synthesis was examined by incorporating [5,6-³H]uridine into RNA in the presence of actinomycin D. Duplicate cell culture dishes were infected as described above, and at 15 min postinfection, actinomycin D was added to the medium to a final concentration of 5 μ g/ml. At 2 h postinfection, [5,6-³H]uridine was added to the medium to 20 μ Ci/ml. At hourly intervals, cytoplasmic extracts were prepared as described above. A portion of these extracts was precipitated with 5% ice-cold trichloroacetic acid, and the resulting precipitates were collected on glass fiber filters. Filters were washed with 5% trichloroacetic acid, dried, and counted by liquid scintillation.

For examination of double-stranded RNA (dsRNA), cytoplasmic extracts were prepared from [5,6-³H]uridine-labeled cells as described above. One-tenth volume of 10% SDS was added to the extracts, which were then extracted twice with phenol-chloroform (1:1) and ethanol precipitated. Pellets were suspended in 100 mM Tris hydrochloride (pH 7.5)-150 mM NaCl-0.5% SDS and fractionated on a 1% agarose gel in 40 mM Tris acetate (pH 7.8)-2 mM EDTA. The gel was treated with Autofluor, dried, and exposed to X-ray film.

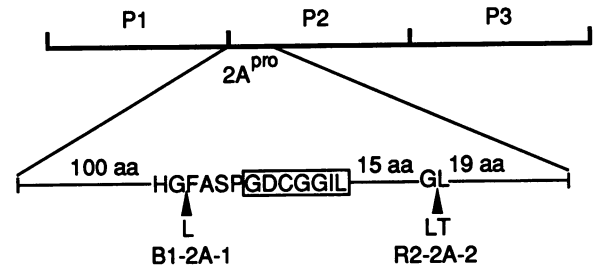


FIG. 1. Altered protein sequence of 2A^{pro} in mutant 2A-2. Poliovirus polyprotein (top) is shown. Also shown are polypeptide 2A^{pro} and locations of the 2-amino-acid (aa) insertion in 2A-2 and the 1-amino-acid insertion in previously isolated mutant B1-2A-1 (1) (bottom). Box indicates amino acids that are part of the putative protease active site (1). The single-letter amino acid code is used.

Antisera and Western blot (immunoblot) analysis. Separation of infected-cell proteins on isoelectric focusing gels and Western analysis of HeLa cell eIF-2 α was modified from the procedure of Scorsone et al. (33). Infection of HeLa cell monolayers was performed as described above. At different times postinfection, the medium was removed and cells were lysed in 3% Ampholine (pH 5 to 7:pH 3.5 to 10, 4:1; LKB Instruments, Inc.)-2% 2-mercaptoethanol-1% Nonidet P-40-9.2 M urea at 37°C. Samples were placed on ice and then heated at 37°C to redissolve the urea and loaded on an isoelectric focusing gel.

Isoelectric focusing gels contained 5.7% acrylamide, 0.3% bisacrylamide, 3% Ampholine (pH 5 to 7:pH 3.5 to 10, 4:1), and 9.2 M urea. Cathode buffer was 20 mM NaOH, and anode buffer was 30 mM H₃PO₄. The gel was prefocused for 15 min at 200 V, 30 min at 300 V, and 30 min at 400 V. After samples were loaded, the gel was run at 400 V for 5 h and then at 1,000 V for 1 h.

The transfer of proteins to nitrocellulose was performed in 25 mM Tris-192 mM glycine (pH 8.3)-20% methanol-0.1% SDS. Isoelectric focusing gels were equilibrated for 10 min in transfer buffer and transferred for 2 h at 1 amp. Immunoblotting was performed as previously described (33), except that proteins were not fixed to nitrocellulose with glutaraldehyde and the second antibody was ¹²⁵I-labeled goat anti-mouse antiserum (Amersham Corp.).

Cytoplasmic extracts made by Nonidet P-40 lysis were separated on 12.5% polyacrylamide gels and transferred to nitrocellulose as described above. Western blotting was performed as described previously (1). Monoclonal antibody against eIF-2 α was the gift of E. C. Henshaw (University of Rochester). Rabbit polyclonal antiserum against p220, originally from R. Lloyd and E. Ehrenfeld (University of Utah), was the gift of R. Schneider (New York University). Rabbit polyclonal antiserum against 2A^{pro} was provided by H. Bernstein and D. Baltimore (Massachusetts Institute of Technology). Antiserum against 3D^{pol} was described previously (15).

RESULTS

Construction of poliovirus mutant 2A-2. To study the function of poliovirus gene products, we have used linker insertion mutagenesis of infectious cDNA to create viral mutants. Poliovirus mutant 2A-2 was created by the insertion of a 6-base-pair *HpaI* linker into a *StuI* restriction site at nucleotide 3765 of the poliovirus type 2 Lansing cDNA, resulting in the insertion of 2 amino acids into protease 2A^{pro}

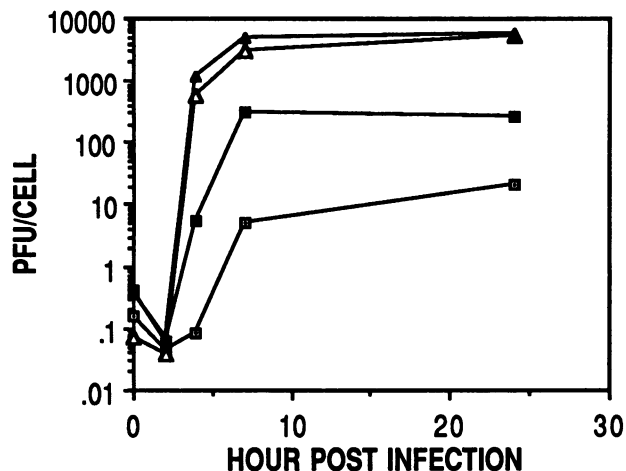


FIG. 2. One-step growth curve of 2A-2 and wild-type poliovirus in HeLa cell monolayers. Total virus production was determined at each time point. Symbols: Δ , wild-type virus; \square , 2A-2; \blacktriangle , wild-type virus in the presence of 2-aminopurine; \blacksquare , 2A-2 in the presence of 2-aminopurine.

(Fig. 1). Transfection of HeLa cells with a T7 RNA polymerase-directed transcript produced infectious virus with a small-plaque phenotype. Virus stocks prepared in this way contained up to 10% large plaques, which were presumably revertants. These virus stocks displayed a wild-type plaque size on CV1 monolayers. Therefore, viral stocks for use in these studies were generated by transfection of CV1 cells, and the resulting stocks contained $\geq 1,000$ times more small-plaque mutants than large-plaque revertants when stocks were assayed on HeLa cells. Although 2A-2 is not temperature sensitive (data not shown), viral stocks prepared by incubation of transfected cells at 32°C contained fewer large-plaque revertants than did stocks prepared at 37°C (data not shown).

In a one-step growth analysis on HeLa cells, 2A-2 replicated much more slowly than did wild-type poliovirus and to a titer $10^{2.3}$ PFU per cell below that of wild-type poliovirus (Fig. 2).

To confirm that the small-plaque phenotype was due to the presence of the *Hpa*I linker, the nucleotide sequence of an *Xho*I-*Bgl*III DNA fragment from pHST26 (nucleotides 3299 through 3792) was determined by the chemical degradation method (23). No nucleotide changes, other than the inserted *Hpa*I linker, were detected. The *Xho*I-*Bgl*III restriction fragment of pHST26 was removed and substituted for the corresponding fragment of pT7L, resulting in reconstruction of pHST26. Virus stocks produced by transfection of the reconstructed plasmid had the same small-plaque phenotype as the original mutant (data not shown).

Protein synthesis in mutant-infected cells. To determine the biochemical basis for the reduced growth of 2A-2, polypeptide synthesis in infected HeLa cells was examined. Cells were pulse-labeled with [³⁵S]methionine, and cytoplasmic extracts were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Host translation was severely inhibited in wild-type-poliovirus-infected cells by 4 h postinfection, at which time only viral polypeptides were observed (Fig. 3). In 2A-2-infected cells, host protein synthesis continued until 8 h postinfection but declined substantially between 5 and 8 h postinfection. Very little viral protein synthesis was evident. Starting at 5 h postinfection, polypeptide 3CD, the precursor for the viral polymerase 3D^{POI},

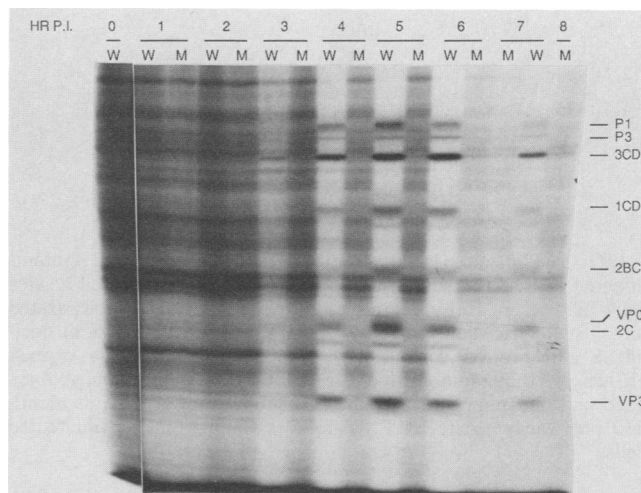


FIG. 3. Protein synthesis in wild-type- and mutant 2A-2-virus-infected cells. Cells were pulse-labeled with [³⁵S]methionine at indicated times after infection. Cell extracts were fractionated on a 12.5% SDS-polyacrylamide gel and then autoradiographed. HR P.I., Hour postinfection; M, mutant-infected cells; W, wild-type-virus-infected cells.

could be seen above the background of host protein synthesis in 2A-2-infected cells, but no other viral polypeptides were apparent. However, other viral proteins, including 2A^{PRO} and 3D^{POI}, were detected in mutant-infected cells by Western blot analysis, although these proteins were present at levels lower than those found in wild-type infected cells (data not shown).

p220 cleavage. Specific shutoff of host cell protein synthesis correlates with cleavage of p220, one of the subunits of the CBP complex (11). To determine whether p220 was cleaved in mutant-infected cells, Western blot analysis of infected-cell extracts was used. In wild-type-virus-infected cells, p220 was largely cleaved by 2 h postinfection and completely cleaved by 3 h postinfection (Fig. 4). In contrast, 2A-2 did not induce cleavage of p220. At 5 h postinfection, no p220 cleavage was seen. By 8 h postinfection, only a small amount of p220 was cleaved; this was not enough to account for the dramatic decrease in protein synthesis seen by this time. The small amount of p220 cleavage observed late in infection may be due to the presence of revertants in the virus stock, revertants arising during the course of infection or as a result of leakiness of the mutation.

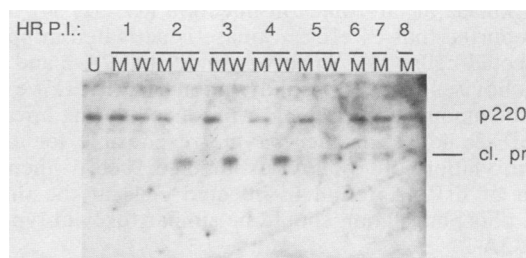


FIG. 4. p220 cleavage in virus-infected HeLa cells. At indicated times after infection, cytoplasmic extracts were prepared from HeLa cell monolayers and fractionated on a 12.5% SDS-polyacrylamide gel. After transfer to nitrocellulose, filters were probed with antiserum against p220. cl. pr., p220 cleavage products; U, uninfected cells; M, 2A-2 infected cells; W, wild-type infected cells; HR P.I., hour postinfection.

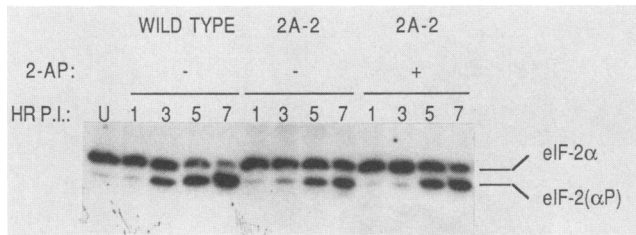


FIG. 5. Phosphorylation of eIF-2 α in wild-type- and mutant-infected cells. At indicated times after infection, whole-cell lysates were made from HeLa cell monolayers which were then separated on an isoelectric focusing gel. Proteins were transferred to nitrocellulose and probed with a mouse monoclonal antibody against eIF-2 α . 2-AP, 2-Aminopurine; eIF-2 α and eIF-2(α P), unphosphorylated and phosphorylated forms, respectively, of eIF-2 α , as identified previously (33); HR P.I., hour postinfection; U, uninfected cells.

eIF-2 α phosphorylation. Overall rates of protein synthesis in mammalian cells are reduced during a variety of physiological stresses. Phosphorylation of eIF-2 α inhibits initiation of protein synthesis (for reviews, see references 14 and 31) and occurs in a number of systems in which translation is inhibited, such as amino acid starvation (33), heat shock (8, 33), heme deficiency in reticulocyte lysates (12), and infection of cells by the adenovirus mutant *dI331* (29, 32, 34). To determine whether phosphorylation of eIF-2 α is the mechanism of inhibition of protein synthesis in cells infected with the poliovirus mutant 2A-2, total cell proteins were resolved at various times postinfection on an isoelectric focusing gel and immunoblotted with monoclonal antibody against eIF-2 α . Isoelectric focusing can resolve the phosphorylated and nonphosphorylated forms of eIF-2 α , with the phosphorylated form emerging as the more acidic species (33, 39). eIF-2 α was largely unphosphorylated in uninfected cells but progressively phosphorylated during the course of infection with 2A-2 (Fig. 5). However, wild-type poliovirus also induced phosphorylation of eIF-2 α . This result was surprising, since others have reported that eIF-2 α is not phosphorylated in poliovirus-infected cells (28). Pulse-labeling of infected-cell proteins showed that virus-specific translation peaks at 5 h postinfection and declines thereafter (Fig. 3). This decline of viral translation was probably due to the extensive phosphorylation of eIF-2 α .

RNA synthesis. A kinase that phosphorylates eIF-2 α , P1/eIF-2 α kinase, is an antiviral protein which is latent in the uninfected cell and is activated by dsRNA (for a review, see reference 14). The adenovirus mutant *dI331* activates P1/eIF-2 α kinase at late times in infection (29, 32, 34). It has been reported that P1/eIF-2 α kinase is activated in poliovirus-infected cells (28). Since we found that 2A-2 and wild-type poliovirus cause phosphorylation of eIF-2 α , we compared the amounts of viral RNA produced during infection. If P1/eIF-2 α kinase is the enzyme responsible for eIF-2 α phosphorylation in poliovirus-infected cells, then the amount of dsRNA found in infected cells at the time of eIF-2 α phosphorylation should be similar for wild-type and mutant 2A-2.

Total viral RNA was examined by trichloroacetic acid precipitation of cytoplasmic RNA labeled with [5,6- 3 H]uridine in the presence of actinomycin D. The level of viral RNA in 2A-2-infected cells was about 50% of that in wild-type-virus-infected cells (Fig. 6A). The approximately two-fold-higher viral RNA level contrasts with the far smaller amounts of viral proteins found in mutant-infected cells.

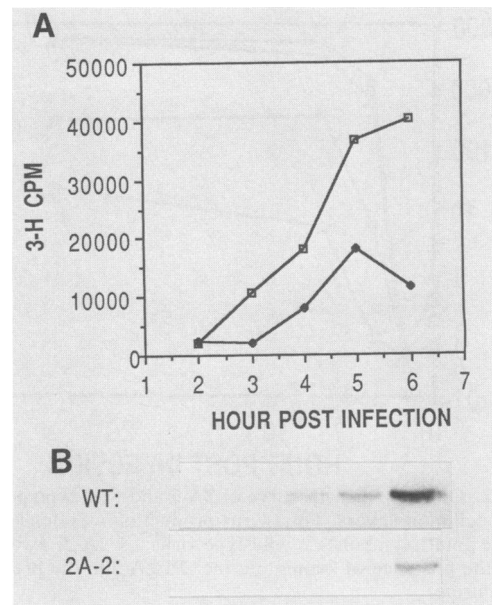


FIG. 6. Actinomycin D-resistant RNA synthesis in infected HeLa cells. (A) Incorporation of [5,6- 3 H]uridine into trichloroacetic acid-insoluble material in the presence of actinomycin D. Symbols: \square , wild-type virus; \blacklozenge , mutant 2A-2. (B) Electrophoresis of [5,6- 3 H]uridine-labeled poliovirus dsRNA from wild-type (WT)-virus- and 2A-2-infected cells. Cytoplasmic RNA purified from infected cells at 2, 3, 4, 5, and 6 h postinfection was fractionated on a nondenaturing agarose gel as described in Materials and Methods.

Others have demonstrated that very little viral protein is necessary for synthesis of large amounts of viral RNA in infected cells (27, 37).

Levels of viral dsRNA were examined by separation of [5,6- 3 H]uridine-labeled cytoplasmic RNA on a nondenaturing agarose gel followed by autoradiography (1). The dsRNA resolved as a tight band (Fig. 6B), while single-stranded RNA migrated faster, as determined by coelectrophoresis of a viral RNA marker (data not shown). dsRNA was detected in 2A-2-infected cells, although there was a 1- to 2-h delay compared with detection in wild-type-virus-infected cells. The slower accumulation of dsRNA in 2A-2-infected cells correlates with delayed eIF-2 α phosphorylation, as eIF-2 α phosphorylation lagged 1 to 2 h behind that seen in wild-type-virus-infected cells (Fig. 5). These observations suggest that dsRNA may be responsible for activation of P1/eIF-2 α kinase in wild-type- and mutant-virus-infected cells.

Rescue of 2A-2 by 2-aminopurine. 2-Aminopurine has been used to inhibit P1/eIF-2 α kinase in *in vitro* translation assays (6, 12, 20) and protein kinases *in vivo* (40). It was therefore of interest to determine whether viral yields of 2A-2 could be enhanced by treating cells with 2-aminopurine. In preliminary experiments, we found that HeLa cells remained viable when incubated in medium containing 10 mM 2-aminopurine for 36 to 48 h, a period longer than that required for one round of poliovirus replication. Therefore, a one-step growth analysis was performed in the presence and absence of 10 mM 2-aminopurine. The drug enhanced virus production in cells infected with 2A-2, whereas wild-type virus production was not altered by the presence of this compound (Fig. 2).

Since 2A-2 was defective in inducing p220 cleavage and since it had been previously reported that 2-aminopurine inhibits P1/eIF-2 α kinase *in vitro*, we expected that in the presence of 2-aminopurine, translation of cellular mRNAs

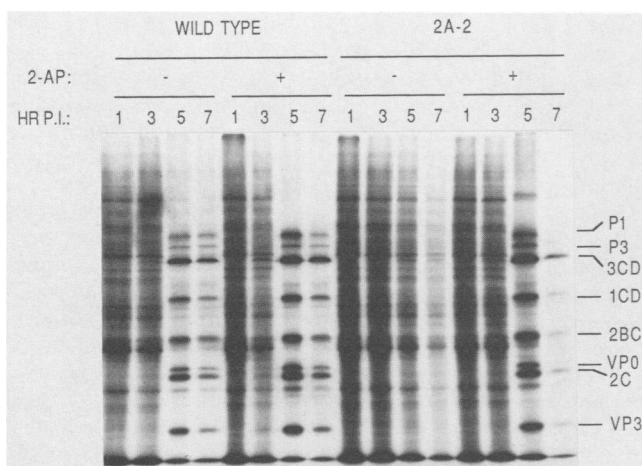


FIG. 7. Effect of 2-aminopurine on protein synthesis in virus-infected cells. Where indicated, 2-aminopurine (10 mM) was added to the medium 1 h before infection. Cells were pulse-labeled with [³⁵S]methionine at indicated times after infection. Extracts were fractionated on a 12.5% SDS-polyacrylamide gel and then autoradiographed. HR P.I., Hour postinfection; 2-AP, 2-aminopurine.

would not be as severely inhibited at late times after infection with 2A-2. Surprisingly, in the presence of 2-aminopurine, the pattern of protein synthesis in 2A-2-infected cells was similar to the pattern in wild-type-virus-infected cells (Fig. 7). Furthermore, p220 was cleaved in 2A-2-infected cells in the presence but not in the absence of 2-aminopurine (Fig. 8). These results show that 2-aminopurine rescues the viral function that induces p220 cleavage.

In the presence of 2-aminopurine, 2A-2 virus yields were still $10^{1.3}$ PFU per cell lower than yields of wild-type virus (Fig. 2). This difference may be related to our observation that, in the presence of 2-aminopurine, p220 cleavage occurs much later in mutant-virus-infected cells than in wild-type-virus-infected cells. Whereas wild-type virus induces complete cleavage by 3 h postinfection, there is still a small amount of uncleaved p220 in 2A-2-infected, 2-aminopurine-treated cells at 5 h postinfection (Fig. 8).

Although 2-aminopurine rescued mutant 2A-2, it had no effect on the phosphorylation of eIF-2 α in mutant- or wild-

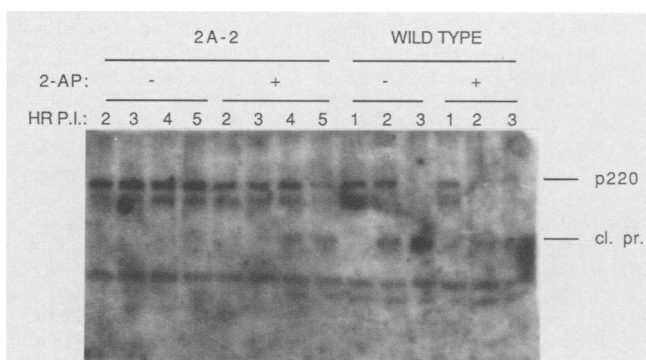


FIG. 8. Effect of 2-aminopurine on p220 cleavage in poliovirus-infected cells. Where indicated, 2-aminopurine (10 mM) was added to the medium 1 h before infection. At indicated times after infection, cytoplasmic extracts were prepared from HeLa cell monolayers and separated on a 12.5% SDS-polyacrylamide gel. After transfer to nitrocellulose, filters were probed with antiserum against p220. cl. pr., p220 cleavage products; 2-AP, 2-aminopurine; HR P.I., hour postinfection.

type-infected cells (Fig. 5). Perhaps not enough 2-aminopurine enters the cell to inhibit phosphorylation of eIF-2 α . Thus the ability of this compound to rescue 2A-2 is not based on blocking eIF-2 α phosphorylation.

The intensity of individual polypeptides labeled with [³⁵S]methionine in wild-type-virus-infected cells was increased in the presence of 2-aminopurine, indicating that translation was in some way potentiated (Fig. 7). It is possible that 2-aminopurine partially inhibits phosphorylation of eIF-2 α or inhibits another modulator of protein synthesis, enabling a greater rate of protein synthesis at late times in infection. However, the higher levels of viral protein observed in the presence of 2-aminopurine did not result in increased yields of wild-type virus (Fig. 2).

DISCUSSION

The mutant 2A-2 reported here, which contains a 2-amino-acid insertion in 2A^{PRO}, displays a phenotype that includes small-plaque formation and reduced viral yield in HeLa cells, inability to cleave p220 and specifically inhibit host cell translation, and global inhibition of translation late in infection. During studies on the phenotype of 2A-2, we surprisingly found that eIF-2 α is extensively phosphorylated in wild-type-poliovirus-infected cells. Viral dsRNA is produced in both wild-type- and mutant-infected cells; these molecules may activate the P1/eIF-2 α kinase that is responsible for phosphorylation of eIF-2 α . It was previously demonstrated that P1/eIF-2 α kinase is activated in poliovirus-infected cell extracts (28). However, those authors also reported that eIF-2 α is not phosphorylated in poliovirus-infected cells, and they concluded that poliovirus encodes a product able to inhibit phosphorylation of eIF-2 α (28). It is not clear why extensive phosphorylation of eIF-2 α was not detected previously. The method for assaying eIF-2 α phosphorylation used in our studies is significantly different from the method of Ransone and Dasgupta (28). We examined phosphorylation of eIF-2 α by isoelectric focusing of whole-cell extracts followed by immunoblotting, whereas Ransone and Dasgupta examined the amount of ³²P-phosphorylated eIF-2 α in ribosomal salt washes from infected and uninfected cells by two-dimensional gel electrophoresis (28). It has been reported that the majority of eIF-2 α which pellets with ribosomes is not phosphorylated, whereas eIF-2 α which remains in the soluble fraction is phosphorylated (39). This may account for the previous failure to detect increased eIF-2 α phosphorylation in wild-type-poliovirus-infected cells.

eIF-2 α is the second known initiation factor that is modified in poliovirus-infected cells. We suggest that restriction of translation to viral messages in poliovirus-infected cells requires both cleavage of p220 and phosphorylation of eIF-2 α . In support of this hypothesis, it has been reported that infection of cells with poliovirus in the presence of inhibitors of poliovirus replication such as guanidine or 3-methylquercetin, under conditions in which p220 is completely cleaved, results in suppression of host protein synthesis by only 55 to 77%, suggesting that p220 cleavage is necessary but not sufficient for total suppression of host protein synthesis (3). Furthermore, the kinetics of p220 cleavage and host shutoff do not coincide. In our studies, cleavage of p220 always preceded host translational inhibition (e.g., compare p220 cleavage 2 and 3 h postinfection with wild-type virus in Fig. 3 and 4; see also reference 11). Finally, it was observed that preparations of eIF-2 α slightly stimulated the translation of vesicular stomatitis virus

mRNA in extracts from poliovirus-infected cells (30). Although it is possible that the eIF-2 α preparation was contaminated with the CBP complex, which is known to fully restore translation in poliovirus-infected extracts, we suggest that endogenous eIF-2 α was partially phosphorylated in the infected cell extract and that exogenous eIF-2 restored the limited ability of capped vesicular stomatitis virus mRNA to be translated in the absence of functional CBP complex.

On the basis of these considerations, we propose the following model for the shutoff of protein synthesis in wild-type-poliovirus-infected cells. Cleavage of p220 reduces the translation efficiency of cellular messages relative to viral RNA. Cellular mRNAs continue to be translated until (i) eIF-2 becomes limiting and (ii) enough viral RNA accumulates to compete with cellular mRNAs for the modified translation apparatus. Cells infected with viral mutants which fail to induce p220 cleavage (1; this work) do not selectively translate viral RNA even when these two criteria are met, perhaps because the translation efficiency of cellular messages is greater than that of viral RNA when the CBP complex is intact. In cells infected with 2A-2, cleavage of p220 does not occur and therefore host translation is not inhibited. However, eIF-2 α phosphorylation still occurs, perhaps because of the presence of viral dsRNA; as a result, overall translation slowly declines.

Others have reported the selective translation of viral messages when eIF-2 becomes limiting. Influenza virus mRNAs are selectively translated in cells coinfecting with adenovirus (16). In translation-competent extracts made from these cells, the translation of adenovirus mRNAs can be rescued by the addition of exogenous eIF-2. In wild-type-adenovirus-infected cells, eIF-2 α is phosphorylated to a limited extent (26). Adenovirus infection of a cell line which is unable to phosphorylate eIF-2 α results in incomplete inhibition of translation of host cell mRNAs (26), suggesting a possible role for eIF-2 phosphorylation in limiting translation to adenovirus messages in the infected cell. We suggest a similar role for eIF-2 α phosphorylation in the shutoff of host translation in wild-type-poliovirus-infected cells.

In wild-type-infected cells, as dsRNA accumulates and the extent of eIF-2 α phosphorylation increases, wild-type viral translation is also inhibited, as seen at late times postinfection. For example, the amount of viral polypeptides synthesized declines at 5 to 7 h postinfection in wild-type-poliovirus-infected cells (Fig. 7). This late decline in the synthesis of viral proteins is probably equivalent to the global inhibition of protein synthesis seen in 2A-2-infected cells. The correlation between dsRNA accumulation and the inhibition of viral protein synthesis was noted previously (5). At the same time, it was concluded that dsRNA was not the cause of the early, specific inhibition of host cell protein synthesis, since poliovirus dsRNA inhibited translation of both cellular and viral mRNAs *in vitro* (5).

The phenotype of 2A-2 is similar to that observed for a previously reported 2A^{pro} mutant, B2-2A-1 (1). However, in preliminary experiments, we found that 2-aminopurine does not increase the yield of B2-2A-1 in HeLa cells, nor does it lead to specific inhibition of translation by the mutant (data not shown). Therefore, protein kinases sensitive to 2-aminopurine do not play a role in the failure of B2-2A-1 to induce p220 cleavage. This difference is not surprising, since the inserted amino acids as well as the location of the insertion site differs in both mutants. In 2A-2, Leu-Thr was

inserted after amino acid 129, while in B2-2A-1, Leu was inserted after amino acid 102 (Fig. 1) (1).

We suggest two models to explain how the kinase inhibitor 2-aminopurine rescues 2A-2. Mutant 2A-2 contains an inserted threonine which is a potential phosphorylation site (Fig. 1). Phosphorylation of the mutant 2A^{pro} may lead to inhibition of its proteolytic activity, resulting in failure to cleave p220, reduced translation, and reduced virus yield. 2-Aminopurine might inhibit the cellular kinase that phosphorylates the mutant 2A^{pro}, thereby rescuing 2A^{pro} functions. Alternatively, failure of the mutant to induce p220 cleavage may be linked to phosphorylation of target polypeptides. For example, it has been suggested that 2A^{pro} activates a cell protein which then cleaves p220 (1, 22). Perhaps this putative cell protein is phosphorylated normally and cannot be activated by the mutant 2A^{pro}. In the presence of 2-aminopurine, the putative cell protein is not phosphorylated and can be activated by mutant 2A^{pro}. Experiments to determine whether phosphorylation of 2A^{pro} plays a role in the phenotype of 2A-2 are under way.

After this work was submitted for publication, it was reported that eIF-2 α is phosphorylated in cells infected with wild-type poliovirus, corroborating the results presented here (2).

ACKNOWLEDGMENTS

We thank L. Greenbaum for construction of mutant 2A-2, E. C. Henshaw for anti-eIF-2 α antibody, R. Panniers for advice on isoelectric focusing, E. Ehrenfeld for anti-p220 antiserum, H. Bernstein and D. Baltimore for anti-2A^{pro} antiserum, and R. Schneider for valuable discussion.

This work was supported by Public Health Service grant AI-20017 from the National Institute of Allergy and Infectious Diseases to V.R.R.

LITERATURE CITED

- 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.
- Black, T. L., B. Safer, A. Hovanessian, and M. G. Katze. 1989. The cellular 68,000- M_r protein kinase is highly autophosphorylated and activated yet significantly degraded during poliovirus infection: implications for translational regulation. *J. Virol.* 63:2244-2251.
- Bonneau, A.-M., and N. Sonenberg. 1987. Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. *J. Virol.* 61:986-991.
- Buckley, B., and E. Ehrenfeld. 1987. The cap-binding protein complex in uninfected and poliovirus-infected HeLa cells. *J. Biol. Chem.* 262:13599-13606.
- Celma, M. L., and E. Ehrenfeld. 1974. Effect of poliovirus double-stranded RNA on viral and host-cell protein synthesis. *Proc. Natl. Acad. Sci. USA* 71:2440-2444.
- De Benedetti, A., and C. Baglioni. 1984. Inhibition of mRNA binding to ribosomes by localized activation of dsRNA-dependent protein kinase. *Nature (London)* 313:79-81.
- Dolph, P. J., V. Racaniello, A. Villamarin, F. Palladino, and R. J. Schneider. 1988. The adenovirus tripartite leader may eliminate the requirement for cap-binding protein complex during translation initiation. *J. Virol.* 62:2059-2066.
- Duncan, R., and J. W. B. Hershey. 1984. Heat shock-induced translational alterations in HeLa cells. *J. Biol. Chem.* 259:11882-11889.
- Ehrenfeld, E. 1984. Picornavirus inhibition of host cell protein synthesis, p. 177-222. *In* H. Fraenkel-Conrat and R. W. Wagner (ed.), *Viral cytopathology*. Plenum Publishing Corp., New York.
- Etchison, D., J. Hansen, E. Ehrenfeld, I. Edery, N. Sonenberg, S.

- Milburn, and J. W. B. Hershey. 1984. Demonstration in vitro that eucaryotic initiation factor 3 is active but that a cap-binding protein complex is inactive in poliovirus-infected HeLa cells. *J. Virol.* **51**:832-837.
11. Etchison, D., S. C. Milburn, I. Edery, N. Sonenberg, and J. W. B. Hershey. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. *J. Biol. Chem.* **257**:14806-14810.
 12. Farrell, P. J., K. Baklow, T. Hunt, R. J. Jackson, and H. Trachsel. 1977. Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. *Cell* **11**:187-200.
 13. Hewlett, M. J., J. K. Rose, and D. Baltimore. 1976. 5'-terminal structure of poliovirus polyribosomal RNA is pUp. *Proc. Natl. Acad. Sci. USA* **73**:327-330.
 14. Jagus, R., W. F. Anderson, and B. Safer. 1981. The regulation of initiation of mammalian protein synthesis. *Prog. Nucleic Acid Res. Mol. Biol.* **25**:127-185.
 15. Kaplan, G., and V. R. Racaniello. 1988. Construction and characterization of poliovirus subgenomic replicons. *J. Virol.* **62**:1687-1696.
 16. Katze, M. G., B. M. Detjen, B. Safer, and R. M. Krug. 1986. Translational control by influenza virus: suppression of the kinase that phosphorylates the alpha subunit of initiation factor eIF-2 and selective translation of influenza viral mRNAs. *Mol. Cell. Biol.* **6**:1741-1750.
 17. Kaufmann, Y., E. Goldstein, and S. Penman. 1976. Poliovirus-induced inhibition of polypeptide initiation *in vitro* on native polyribosomes. *Proc. Natl. Acad. Sci. USA* **73**:1834-1838.
 18. Krausslich, H. G., M. J. H. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer. 1987. Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220. *J. Virol.* **61**:2711-2718.
 19. Lee, K. A. W., I. Edery, and N. Sonenberg. 1985. Isolation and structural characterization of cap-binding proteins from poliovirus-infected HeLa cells. *J. Virol.* **54**:515-524.
 20. Legon, S., A. Brayley, T. Hunt, and R. J. Jackson. 1974. The effect of cyclic AMP and related compounds on the control of protein synthesis in reticulocyte lysates. *Biochem. Biophys. Res. Commun.* **56**:745-752.
 21. Leibowitz, R., and S. Penman. 1971. Regulation of protein synthesis in HeLa cells. III. Inhibition during poliovirus infection. *J. Virol.* **8**:661-668.
 22. Lloyd, R. E., H. Toyoda, D. Etchison, E. Wimmer, and E. Ehrenfeld. 1986. Cleavage of the cap binding protein complex polypeptide p220 is not effected by the second poliovirus protease 2A. *Virology* **150**:299-303.
 23. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499-560.
 24. Moss, E. G., R. E. O'Neill, and V. R. Racaniello. 1989. Mapping of attenuating sequences of an avirulent poliovirus type 2 strain. *J. Virol.* **63**:1884-1890.
 25. Nomoto, A., Y. F. Lee, and E. Wimmer. 1976. The 5' end of poliovirus mRNA is not capped with m⁷G(5')ppp(5')np. *Proc. Natl. Acad. Sci. USA* **73**:375-380.
 26. O'Malley, R. P., R. F. Duncan, J. W. B. Hershey, and M. B. Mathews. 1989. Modification of protein synthesis initiation factors and the shut-off of host protein synthesis in adenovirus-infected cells. *Virology* **168**:112-118.
 27. Pelletier, J., M. E. Flynn, G. Kaplan, V. Racaniello, and N. Sonenberg. 1988. Mutational analysis of upstream AUG codons of poliovirus RNA. *J. Virol.* **62**:4486-4492.
 28. Ransone, L. J., and A. Dasgupta. 1987. Activation of double-stranded RNA-activated protein kinase in HeLa cells after poliovirus infection does not result in increased phosphorylation of eucaryotic initiation factor-2. *J. Virol.* **61**:1781-1787.
 29. Reichel, P. A., W. C. Merrick, J. Siekierka, and M. B. Mathews. 1985. Regulation of a protein synthesis initiation factor by adenovirus virus-associated RNAI. *Nature (London)* **313**:196-200.
 30. Rose, J. K., H. Trachsel, K. Leong, and D. Baltimore. 1978. Inhibition of translation by poliovirus: inactivation of a specific initiation factor. *Proc. Natl. Acad. Sci. USA* **75**:2732-2736.
 31. Safer, B. 1983. 2B or not 2B: regulation of the catalytic utilization of eIF-2. *Cell* **33**:7-8.
 32. Schneider, R. J., B. Safer, S. Munemitsu, C. E. Samuel, and T. Shenk. 1985. Adenovirus VAI RNA prevents phosphorylation of the eucaryotic initiation factor 2 alpha subunit subsequent to infection. *Proc. Natl. Acad. Sci. USA* **82**:4321-4325.
 33. Scorsone, K. A., R. Panniers, A. G. Rowlands, and E. C. Henshaw. 1987. Phosphorylation of eucaryotic initiation factor 2 during physiological stresses which affect protein synthesis. *J. Biol. Chem.* **262**:14538-14543.
 34. Siekierka, J., T. M. Mariano, P. A. Reichel, and M. B. Mathews. 1985. Translational control by adenovirus: lack of virus-associated RNAI during adenovirus infection results in phosphorylation of initiation factor eIF-2 and inhibition of protein synthesis. *Proc. Natl. Acad. Sci. USA* **82**:1959-1963.
 35. Sonenberg, N. 1987. Regulation of translation by poliovirus. *Adv. Virus Res.* **33**:175-204.
 36. Tahara, S. M., M. A. Morgan, and A. J. Shatkin. 1981. Two forms of purified m⁷G-cap binding protein with different effects on capped mRNA translation in extracts of uninfected and poliovirus-infected HeLa cells. *J. Biol. Chem.* **256**:7691-7694.
 37. Trono, D., R. Andino, and D. Baltimore. 1988. An RNA sequence of hundreds of nucleotides at the 5' end of poliovirus RNA is involved in allowing viral protein synthesis. *J. Virol.* **62**:2291-2299.
 38. Vogelstein, B., and D. Gillespie. 1979. Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci. USA* **76**:615-619.
 39. Wong, S. T., W. Mastropalo, and E. C. Henshaw. 1982. Differential phosphorylation of soluble versus ribosome-bound eucaryotic initiation factor 2 in the Ehrlich Ascites tumor cell. *J. Biol. Chem.* **257**:5231-5238.
 40. Zinn, K., A. Keller, L. A. Whittemore, and T. Maniatis. 1988. 2-Aminopurine selectively inhibits the induction of β -interferon, *c-fos*, and *c-myc* gene expression. *Science* **240**:210-213.