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A Poliovirus 2A^{pro} Mutant Unable To Cleave 3CD Shows Inefficient Viral Protein Synthesis and Transactivation Defects

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Four poliovirus mutants with modifications of tyrosine 88 in 2A^{pro} were generated and introduced into the cloned poliovirus genome. Mutants Y88P and Y88L were nonviable, mutant Y88F showed a wild-type (WT) phenotype, and mutant Y88S showed a delayed cytopathic effect and formed small plaques in HeLa cells. Growth of Y88S in HeLa cells was restricted, giving rise to about 20% of the PFU production of the WT poliovirus. The 2A(Y88S) mutant synthesized significantly lower levels of viral proteins in HeLa cells than did the WT poliovirus, while the kinetics of p220 cleavage were identical for both viruses. Strikingly, the 2A(Y88S) mutant was unable to cleave 3CD, as shown by analysis of poliovirus proteins labeled with [^{35}S]methionine or immunoblotted with a specific anti-3C serum. The ability of the Y88S mutant to form infectious virus and cleave 3CD can be complemented by the WT poliovirus. Synthesis of viral RNA was diminished in the Y88S mutant but less than the inhibition of translation of viral RNA. Experiments in which guanidine was used to inhibit poliovirus RNA synthesis suggest that the primary defect of the Y88S mutant virus is at the level of poliovirus RNA translation, while viral genome replication is much less affected. Transfection of HeLa cells infected with the WT poliovirus with a luciferase mRNA containing the poliovirus 5′ untranslated sequence gives rise to a severalfold increase in luciferase activity. This enhanced translation of leader-luc mRNA was not observed when the transfected cells were infected with the 2A(Y88S) mutant. Moreover, cotransfection with mRNA encoding WT poliovirus 2A^{pro} enhanced translation of leader-luc mRNA. This enhancement was much lower upon transfection with mRNA encoding 2A(Y88S), 2A(Y88L), or 2A(Y88P). These findings support the view that 2A^{pro} itself, rather than the 3C′ and/or 3D′ products, is necessary for efficient translation of poliovirus RNA in HeLa cells.

A great number of RNA-containing animal viruses encode polyproteins that are proteolytically cleaved by host or viral proteases to produce the mature viral products (7, 27). This genetic strategy is exacerbated in picornaviruses, in which all of the proteins derive from a polyprotein precursor synthesized by a single initiation event (13, 27). Such a polyprotein is not found in infected cells because it is proteolytically degraded while the nascent polypeptide chain is still attached to the ribosome (7, 27). In poliovirus, proteases 2A^{pro} and 3C^{pro} (or 3CD^{pro}) accomplish all but one of the proteolytic cleavages of the polyprotein needed to produce mature viral proteins. The two proteinases belong to the family of serine-like proteinases (7). In poliovirus 2A^{pro}, the catalytic triad is formed by His-20, Asp-38, and Cys-109 (14, 42). This active-site nucleophilic cysteine residue replaces the serine found in serine proteases (7).

2A^{pro} is responsible for two cleavages of the poliovirus polyprotein (7). The first one, between P1 and 2A, releases from ribosomes all of P1, containing the four structural proteins (35). A second cleavage catalyzed by 2A^{pro} occurs on the 3CD precursor, between amino acids Y-148 and G-149 of 3D, to produce 3C′ and 3D′ (26), two products with unknown functions (38). In an alternative cleavage, 3CD is a substrate that allows 3C^{pro} to produce itself and poliovirus RNA polymerase 3D^{pol}. Apparently, the former cleavage event is not required for poliovirus growth in HeLa cells. A mutation (Thr-Ala) at position P2 produces a modified 3CD that is not a substrate for 2A^{pro} (18). Plaques are produced by this mutant in HeLa cells, leading to the conclusion that the 3CD cleavage by 2A^{pro} was not important for the poliovirus life cycle, at least in tissue culture cells (18). However, it is puzzling that this cleavage site is conserved in most virulent strains of poliovirus, whereas poliovirus type 1 Brumhilde, type 3 Sabin, and type 3 Leon appear not to produce 3C′ and 3D′ (18).

In addition to the two cleavages produced by 2A^{pro} on the poliovirus proteins, this protease may also degrade cellular proteins (36). The best-illustrated case of a cellular protein being degraded by a viral protease is the cleavage of p220 (the gamma subunit of eukaryotic initiation factor 4), a component of eukaryotic initiation factor 4F, which is internally cleaved once by rhinovirus 2A^{pro} (32, 33). It is still unknown if poliovirus 2A^{pro} cleaves p220 directly or by activating a cellular protease (39, 40). It is still a matter of debate to what extent cleavage of p220 is responsible for the shutoff of host translation induced by poliovirus (34). The suggestion that p220 cleavage would selectively impair the translation of capped mRNAs (8) has not been supported by more recent evidence indicating that the kinetics of p220 cleavage does not correlate with the shutoff of host translation by poliovirus (3, 6, 19, 28).

The function of 2A^{pro} may not be restricted to its role as a protease. Indications that the protein acts as a transactivator for the translation of its own mRNA (12) and that the protein is required for poliovirus RNA synthesis (23) have been reported recently. Although a number of poliovirus replicons lacking part of the entire P1 region replicate in cells, this ability is lost when 2A^{pro} is deleted (11, 25), strongly arguing in favor of the idea that the 2A^{pro} function is required for events other than P1-2A cleavage. Elegant experiments constructing viable poliovirus with the encephalomyocarditis virus IRES sequence placed between P1 and 2A reinforced this idea (23, 38). This hybrid poliovirus was unable to replicate the genome when the 2A^{pro} sequence was deleted. Mutations in Cys-109 of 2A^{pro} also abrogate the replication of minireplicons or hybrid poliovirus (23), arguing that the protease function of 2A is not

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restricted to cleavage of the P1-2A junction but is necessary for another, unidentified event(s) involved in viral genome replication (38). Therefore, the possibility is still open that other cleavage products of 2A^{pro} are required for poliovirus RNA synthesis. Alternatively, it might be that 2A^{pro} itself is endowed with other functions, for instance, the RNA-binding capacity necessary for genome replication (23).

A number of 2A^{pro} mutants have been generated, and their ability to cleave in *cis* and *trans* has been analyzed (38). Many mutations in the catalytic triad, particularly in Cys-109, render this protease totally inactive, whereas mutations in other residues of the protein generate a 2A^{pro} still able to cleave P1-2A in *cis* but devoid of p220 cleavage activity (14, 42). This has been well illustrated in tyrosine residue 88 mutants, suggesting that this residue participates in substrate recognition (14, 42). Since these studies were accomplished with 2A^{pro} synthesized in vitro and all of the assays were done with cell-free systems, we considered it of interest to reconstruct polioviruses with tyrosine residue 88 replaced by other amino acids. We now report that one such mutant, Y88S, efficiently cleaves p220 in HeLa cells, but cleavage of 3CD does not occur. This mutant poliovirus shows defects in viral protein synthesis, transactivation of the poliovirus leader sequence, and plaque size, confirming a role of 2A^{pro} in the stimulation of poliovirus mRNA translation during the virus replication cycle (12).

After completion of this study, we learned that Yu et al. (41) also reconstructed a poliovirus with the Y88S mutation in 2A^{pro}. Contrary to their previous report (42), they observed cleavage of p220 with the in vitro-translated proteins from the mutated poliovirus RNA. Although they found some replication of viral RNA in transfected cells, they were unable to grow and recover virus. We have no explanation for their failure to isolate the poliovirus mutant described in the present work.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum (GIBCO) and incubated at 37°C in a 5% CO_2 atmosphere. Wild-type (WT) and mutant polioviruses were isolated from single plaques grown in HeLa cells after transfection with in vitro-synthesized RNA from plasmid pT7XLD (37). To generate high-titer stocks, poliovirus was grown in Vero cells for two additional passages. In some experiments, poliovirus type 1, not derived from the infectious clone, was obtained and used as previously described (30). Vesicular stomatitis virus was grown in BHK-21 cells as previously described (16).

Bacterial strains, enzymes, and plasmid construction. All plasmids were isolated from *Escherichia coli* DH-5 and manipulated in accordance with standard procedures (31). Restriction enzymes were purchased from New England Biolabs. T7 RNA polymerase, RNasin, and T4 DNA ligase were obtained from Promega. Avian myeloblastosis virus reverse transcriptase was from Seikagaku America, Inc. The infectious clone of poliovirus type 1 (pT7XLD) was generously provided by E. Wimmer (Stony Book, N.Y.). Plasmid pT75′NCpolioLUC was obtained with primers 5′Luc (GAC CCG GGC GAA GACGCCAAAAAC ATAAAGAAAGGCC) and 3′Luc (CCG CA AT ATTTGGACTTTCCGCCC), which were designed for PCR amplification of the coding region of the firefly luciferase gene (sequences underlined) from plasmid pDO432, provided by S. H. Howell (University of California, La Jolla). The resulting 1.6-kpb DNA fragment was digested with *Sma*I and *Ssp*I and ligated to plasmid pT75′NCpolio containing the 5′ leader sequence of poliovirus type 1 (nucleotides 1 to 742) downstream of the T7 RNA polymerase promoter. The translation initiation codon of the luciferase gene starts at the same position as the poliovirus genome in the resulting construction. Plasmid KSLUC was obtained by subcloning the 1.8-kbp *Bam*HI fragment from plasmid pDO432 into pBluescript KS (Stratagene). This plasmid contains the 5′ leader sequence (80 nucleotides) of the luciferase gene. Plasmid pTM12C, containing the poliovirus 2C sequence under the control of the encephalomyocarditis virus 5′ untranslated region, was obtained as previously described (1). Plasmid pTM12A, containing the poliovirus 2A sequences or its variants, was made similarly.

Site-directed mutations of 2A^{pro}. The 2A^{pro} mutants were obtained by overlap extension with the PCR as previously described (15, 22). One synthetic oligonucleotide containing four possible site-specific substitutions in nucleotides 3645 to 3647 from pT7XLD were generated (primer A: GCTGGGTAAG/A G/AGTTA TTAGCG). The first PCR was carried out under standard conditions with plasmid pT7XLD as the template, primer A, and primer 5′2AE1A (GGC CGG CC CG GGATTCGGACACCAAAAC), complementary to nucleotides 3386 to 3403 from pT7XLD (underlined). The amplified DNA fragment was extracted from agarose gels and mixed at a molar ratio of 10:1 with a DNA fragment encompassing nucleotides 3417 to 3925 from pT7XLD, and the second PCR was performed by overlap extension. The resulting DNA fragment was digested with *Msc*I and subcloned into plasmid pBR322Polio2954-4600 containing the *Sna*BI-*Bam*HI fragment from pT7XLD. Mutant clones were screened by sequencing and introduced into full-length poliovirus cDNA with the enzyme *Bst*EII.

In vitro transcription and RNA transfection. pT7XLD and the luciferase-derived plasmids were linearized with *Eco*RI and *Ssp*I, respectively, and used as templates for in vitro transcription. The reaction mixtures contained 40 mM Tris-HCl (pH 7.9); 6 mM MgCl; 2 mM spermidine; 10 mM dithiothreitol; 1 mM each CTP, UTP, GTP, and ATP; 160 U of RNAsin RNase inhibitor (Promega); and 100 U of T7 RNA polymerase (Promega) and were incubated at 37°C for 120 min. Capped luciferase mRNAs were prepared in a similar fashion, except that 1 mM 7mG(5′)ppp(5′) (Biolabs) was also present. About 5 μg of in vitro-synthesized, full-length poliovirus RNA was used to transfect HeLa cells in 60-mm-diameter plates at 50 to 60% confluence with lipofectin reagent (GIBCO BRL), and the plates were incubated at 37°C for several days. WT poliovirus RNA produced a total cytopathic effect (CPE) at 72 h after transfection, whereas mutant Y88S showed only a weak effect at that time. Several single plaques from both viruses were picked and amplified in Vero cells by two additional passages to obtain high-titer virus stocks. To verify the presence of the mutated 2A, the sequence was amplified from the extracted viral RNA by reverse transcriptase PCR and sequenced by the dideoxy method.

Virus infection of cell monolayers. HeLa or Vero cells were infected with WT or mutant virus in Dulbecco modified Eagle medium at the multiplicity of infection (MOI) indicated elsewhere in this report for each experiment. After 1 h of adsorption at 37°C (time zero of infection), the monolayers were washed and fresh medium containing 2% calf serum was added. To analyze one-step virus growth, cells growing in 24-well plates were infected at an MOI of 10 PFU per cell. At various times after infection, cells were scraped from the dishes and lysed by three cycles of freezing and thawing. Lysates were clarified by centrifugation at 3,000 rpm for 25 min in an SS-34 rotor, and virus titers were obtained by plaque assay on HeLa cells.

Analysis of proteins by polyacrylamide gel electrophoresis (PAGE). Cells were infected with WT or mutant virus at an MOI of 25 PFU per cell. At the times indicated elsewhere in this report, cells were incubated for 1 h in methionine-free medium containing 25 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham) per ml. The monolayers were washed with phosphate-buffered saline (PBS) and solubilized in 100 μl of sample buffer (62 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 0.1 M dithiothreitol, 17% glycerol, 0.024% bromophenol blue). The samples were sonicated and heated at 90°C for 5 min. An aliquot was applied to an SDS–15% polyacrylamide gel and run overnight at 80 V. Fluorography was carried out in 1 M sodium salicylate. Finally, the gels were dried and exposed to X-ray film (Agfa) at −70°C.

Immunoblot analysis. Immunodetection of proteins with specific antisera was carried out with the ECL Western blotting (immunoblotting) detection kit (Amersham). Briefly, cellular extracts harvested in sample buffer were electrophoresed and transferred to a nitrocellulose membrane (Bio-Rad). After the nitrocellulose sheet was blocked with 5% nonfat milk in PBS for 1 h, the specific antiserum (anti-p220 or anti-3C) was added at a dilution of 1:1,000 in a PBS–1% nonfat milk solution and incubated for 2 h at room temperature. The blot was then washed three times with PBS containing 0.05% Tween 20 and incubated for 1 h with the second antibody (biotinylated anti-rabbit immunoglobulin antiserum). The blot was washed three times, and a streptavidin-peroxidase conjugate was added at a dilution of 1:20,000. After 1 h of incubation, the luminescence reaction was performed with 1 volume of solution A (100 mM Tris-HCl [pH 8], 5 mM H_2O_2) mixed with 1 volume of solution B (2.5 mM luminol, 78 mM luciferin). The blot was air dried and exposed to X-ray film (Agfa) for several seconds to several minutes.

Measurement of viral RNA synthesis. Total poliovirus RNA synthesis was measured by determining the incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable material from dactinomycin-treated cells as described previously (30). To detect poliovirus positive-strand RNA, dot blot analysis was performed as follows. Total cytoplasmic RNA of poliovirus-infected cells was obtained by the Nonidet P-40 lysis method (9). One-microgram samples of RNA were denatured in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–7% (wt/vol) formaldehyde at 55°C for 1 h. The samples were bound to nitrocellulose filters by aspiration with a dot blot apparatus (Bio-Rad). Filters were dried at 80°C for 2 h and prehybridized at 55°C for 2 h in a solution containing 50% formamide, 5× SSC, 50 mM Na_2HPO_4 (pH 6.5), 0.1% SDS, 1 mM EDTA, 2.5× Denhardt's solution, and 200 μg of salmon sperm DNA per ml. Hybridization was continued overnight at 55°C in the same solution containing a 0.2-μg/ml concentration of a biotinylated RNA probe generated by in vitro transcription of a poliovirus fragment cDNA encompassing nucleotides 2099 to 4600 subcloned in a Bluescript KS vector (Stratagene). Synthesis of the biotinylated riboprobe was carried out as recommended by the manufacturer (Clontech). After hybridization, the filters were washed four times in 0.1× SSC–0.1% SDS at 65°C, air dried, and blocked in buffer B (PBS–0.05% Tween 20, 3% bovine serum albumin) at room temperature for 30 min. After incubation with a

streptavidin-peroxidase conjugate (diluted 1:10,000) for 30 min in buffer A (PBS–0.05% Tween 20), the filters were washed extensively with the same buffer and detection was carried out as described for immunoblot analysis. Quantitation of specific RNA was performed by densitometric analysis with a 300A computing densitometer (Molecular Dynamics).

Complementation assay. Two different assays were carried out to test the complementation of mutant 2A(Y88S) by WT poliovirus. To test the complementation for PFU formation, HeLa cells were simultaneously infected with 10 PFU of each virus per cell. After 6 or 8 h of incubation, the medium and intracellular virus were collected and plaque formation was assayed by the standard technique. To assay the effect of complementation on 3CD cleavage, cells were simultaneously infected with 10 PFU of each virus per cell. After 6 h of incubation at 37°C, the cell monolayer was dissolved in sample buffer and the proteins were separated by SDS-PAGE as described above. Immunoblot analysis with specific anti-3C serum was performed as described above.

Polysome analysis. HeLa cells (5×10^7) were infected with WT poliovirus or mutant 2A(Y88S) at 50 PFU per cell as described above. At 4 h 40 min postinfection (p.i.), the cells were treated with 100 μg of cycloheximide per ml; 5 min later, the cells were washed twice with PBS and lysed in 0.5 ml of polysome buffer (20 mM Tris-HCl [pH 7.6], 60 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 2 mM dithiothreitol, 0.5% Nonidet P-40) supplemented with 100 μg of cycloheximide per ml and 100 U of RNAsin. After 15 min of incubation on ice, the extracts were centrifuged at 12,000 rpm for 15 min in a microcentrifuge, and the resulting postmitochondrial supernatants were applied to a 7 to 47% sucrose gradient in polysome buffer. Centrifugation was done at 38,000 rpm for 110 min at 4°C in a Beckman TST 41.14 rotor. Gradients were fractionated with an ISCO UA-5 gradient fractionator, and the A_{254} was recorded. Eight fractions were collected from each gradient and adjusted to 1% SDS, and total RNA was purified by phenol extraction and ethanol precipitation. The distribution of poliovirus-specific RNA in the different fractions was analyzed by dot blot hybridization as described above.

Transfection of in vitro-synthesized luciferase RNA molecules into HeLa cells. HeLa cells grown in 35-mm-diameter plates at 70 to 80% confluence were transfected with 1 μg of uncapped 5′NCpolioLUC or capped LUC RNA transcripts derived from pT75′NCpolioLUC and KSLUC, respectively, made in vitro with 5 μg of lipofectin reagent (GIBCO BRL). After 3 h of incubation at 37°C, the monolayers were washed with cold PBS and lysed in 200 μl of buffer L (25 mM glycylglycine [pH 7.8], 1 mM dithiothreitol, 0.5% Triton X-100) for 10 min at 0°C. Nuclei and cell debris were pelleted by low-speed centrifugation, and a 20-μl aliquot of the resulting supernatant was used to measure luciferase activity (6a) in a Monolight 2010 apparatus (Analytical Luminescence Laboratory). Luciferase activity was corrected by the amount of protein present in each sample as estimated by the Bio-Rad protein assay. To determine the effect of WT or mutant poliovirus infection on luciferase expression, the cells were infected with these viruses at an MOI of 50 PFU per cell and transfected 2 h later with the luciferase RNAs. Three hours posttransfection (5 h p.i.), extracts were made and luciferase activity was measured.

RESULTS

Construction of poliovirus containing mutations at residue Y-88 of $2A^{pro}$. Our initial aim was to construct viable poliovirus $2A^{pro}$ mutants able to cleave the P1-2A substrate in *cis* but impaired in the ability to hydrolyze substrates in *trans*. We chose residue Y-88 of $2A^{pro}$, since some of the $2A^{pro}$ Y-88 mutants showed such behavior, at least in cell-free systems (42). Y-88 was mutated to F, S, P, and L, and the resulting $2A^{pro}$ was inserted into the infectious poliovirus genome (Fig. 1). RNA transcripts were generated by T7 RNA polymerase and transfected into HeLa cells, and single plaques were picked and amplified. The Y88F mutant behaved in the same way as WT poliovirus, in agreement with a recent report by Yu et al. (41). However, mutants Y88P and Y88L were nonviable. Interestingly, mutant 2A(Y88S) had a small-plaque phenotype (Fig. 2B), suggesting defects in $2A^{pro}$ function. In contrast to our findings, Yu et al. (41) found that the 2A(Y88S) mutant did not produce plaques in COS cells, even after prolonged incubation. Although we have no explanation for their findings, it is possible that the different systems used could account for these differences.

Growth of $2A^{pro}$ mutant Y88S. Initially, the growth characteristics of mutant 2A(Y88S) in HeLa cells were analyzed. The growth of Y88S in HeLa cells was restricted compared with that of WT poliovirus derived by transfection of RNA transcribed from plasmid pT7XLD (Fig. 2A). The differences in

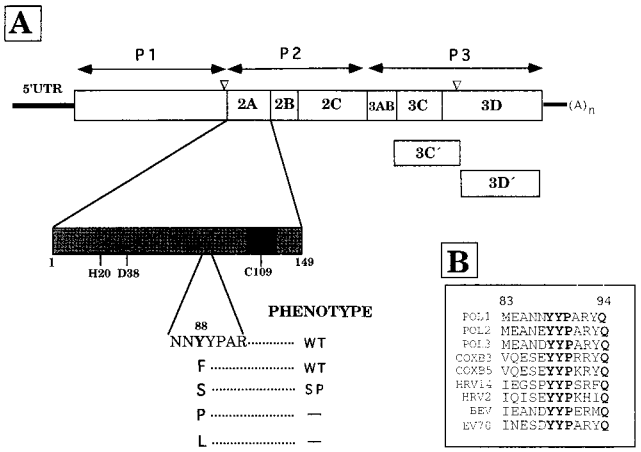

FIG. 1. Schematic representation of $2A^{pro}$ mutations in the poliovirus genome. (A) Poliovirus genome structure depicting the $2A^{pro}$-mediated cleavage sites (▽). The mutant sequences used in this study are located within the 2A molecule. The phenotype of each mutant virus was based on the plaque size obtained after several independent transfections as described in Materials and Methods: WT, large plaques; SP, small plaques; —, undetectable plaques. (B) Alignment of the amino acid sequences surrounding residue Y-88 of the 2A gene from several enteroviruses and rhinoviruses. The conserved residues are in boldface. Abbreviations: POL1, POL2, and POL3, poliovirus types 1, 2, and 3, respectively; COXB3 and COXB5, coxsackieviruses B3 and B5, respectively; HRV14 and HRV2, human rhinovirus types 14 and 2, respectively; BEV, bovine enterovirus; EV70, echovirus type 70; 5′ UTR, 5′ untranslated region.

virus growth were also apparent when the CPE was analyzed. Mutant 2A(Y88S) manifested a CPE in some HeLa cells of the culture only after prolonged incubation, while this effect was observed with WT poliovirus much earlier (Fig. 2A). Finally, the 2A(Y88S) mutant gave smaller plaques than did the WT in HeLa cells (Fig. 2B). No differences were found in this behavior when the plaques were formed at 32 or 39°C, suggesting that 2A(Y88S) is not a temperature-sensitive mutant. The plaques formed by mutant Y88S were homogeneous, and less than 1% reversion to the WT phenotype was apparent.

Protein synthesis and cleavage of p220 and 3CD by the $2A^{pro}$ Y88S mutant. To characterize the defect of the 2A(Y88S) mutant at the molecular level during the virus replication cycle, we started by analyzing the kinetics of protein synthesis of the 2A(Y88S) mutant in HeLa cells. Figure 3 shows that the kinetics of protein synthesis by the WT poliovirus and mutant 2A(Y88S) were similar during the initial hours of infection of HeLa cells, but after 4 h p.i. the kinetics differed. Viral protein synthesis by WT poliovirus peaked at 6 h p.i., while viral translation by the mutant virus decreased after 4 h p.i. (Fig. 3A). Densitometric analysis of VP3 indicated that at 6 h p.i. the 2A(Y88S) mutant synthesized less than one-fifth of the VP3 of the WT poliovirus. It must be noted that viral protein synthesis by the WT poliovirus obtained after transfection of the RNA made by transcription of pT7XLD is slower than that of the adapted poliovirus routinely used in our laboratory. It is of interest that the 2A(Y88S) mutant synthesizes both P1 and 2A, indicating that $2A^{pro}$ is active in *cis* and catalyzes the hydrolysis between P1 and itself. Close inspection of the protein patterns of the WT and the Y88S mutant shows that protein migrating the same as 3C′ was absent in 2A(Y88S) mutant, suggesting a defect in 3CD cleavage by the mutated $2A^{pro}$ (Fig. 3A). Protein 3D′ migrated together with VP2 and was not distinguishable.

To assay the cleavage of p220 by the WT and the Y88S mutant, the proteins present at different times during infection

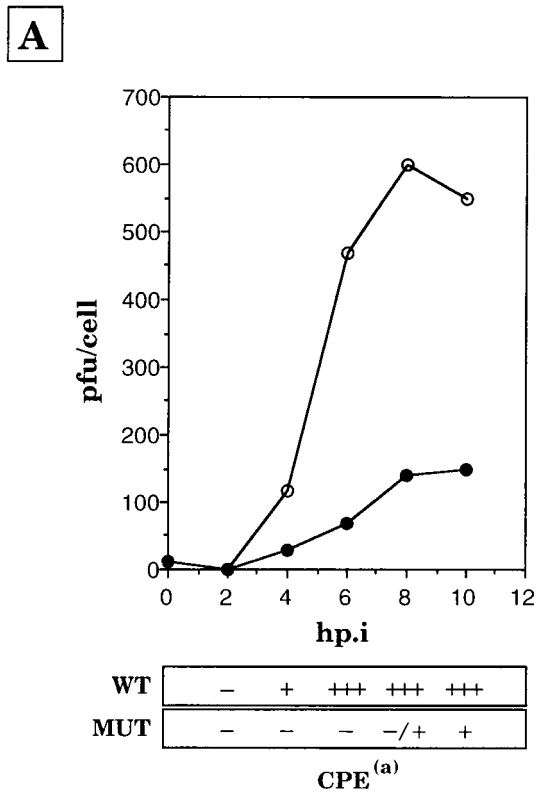

FIG. 2. Comparison of growth of WT poliovirus and the 2A(Y88S) mutant in HeLa cells. (A) Kinetics of WT (○) and 2A(Y88S) mutant (MUT) (●) poliovirus PFU in a single cycle of infection. HeLa cells were infected at an MOI of 10 PFU per cell, and at the times indicated, the virus titer was calculated by plaque assay. The time course of appearance of a CPE is shown below the graph. The CPE was scored as follows: −, undetectable; +, little rounding up visible in only ≤10% of cells; ++, 20 to 50% of cells rounded up; +++, more than 90% of cells rounded up and/or floating in suspension. (B) Plaque morphology of WT and 2A(Y88S) mutant virus stocks in HeLa cells. Plaques were visualized after staining with crystal violet 48 h after infection.

were transferred to a nitrocellulose membrane and immunoblotted with specific p220 antiserum (Fig. 3B). Both the Y88S mutant and WT poliovirus extensively hydrolyzed p220 with similar kinetics (Fig. 3B). Moreover, transient expression of the isolated 2A^{pro} gene with recombinant vaccinia virus that expresses the T7 RNA polymerase (24) also shows extensive cleavage of p220 upon expression of mutated 2A^{pro} (results not shown). These results contrast with those reported by Yu and Lloyd (42), who found that the isolated 2A^{pro} protein mutated in Y88S only partially cleaved p220 in cell-free systems. We believe that our results, obtained with intact cells and reconstituted polioviruses, are more definitive, indicating that 2A^{pro} with the Y88S mutation efficiently cleaves p220 in HeLa cells.

Apart from the cleavage at the P1-2A junction, the other poliovirus substrate for 2A^{pro} is 3CD. Cleavage at an internal

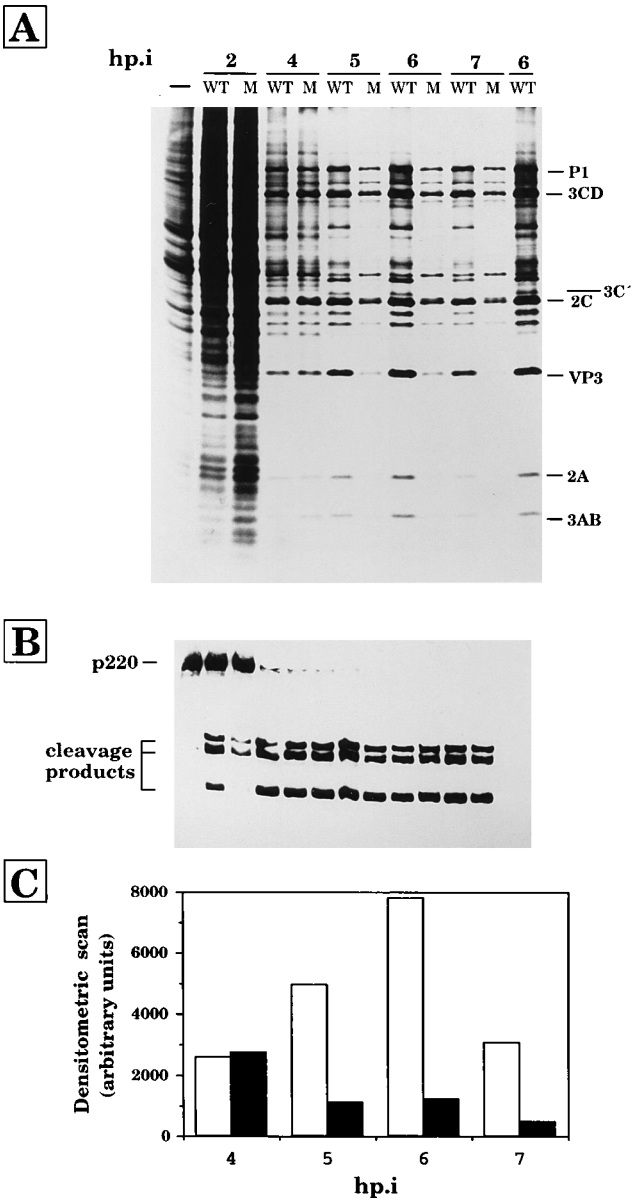

FIG. 3. Time course of protein synthesis and p220 cleavage in WT- and 2A(Y88S) mutant-infected HeLa cells. (A) Autoradiogram of proteins synthesized in infected cells during 1-h pulses with [^{35}S]methionine. At the times indicated, p.i. the cells were recovered in 100 μl of sample buffer. Aliquots of 25 μl were analyzed on SDS–15% polyacrylamide gels and processed as described in Materials and Methods. The pattern of proteins in mock-infected cells is shown in the leftmost lane. Lanes M contained 2A(Y88S) mutant virus. (B) Aliquots (50 μl) of the samples utilized for panel A were analyzed by SDS–7.5% PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-p220 serum. The order of the samples is the same as in panel A. Intact and proteolytic p220 products are shown. (C) Densitometric scan of viral VP3 band in WT (□)- and 2A(Y88S) mutant (■)-infected cells from the autoradiogram shown in panel A. Quantification is in arbitrary units.

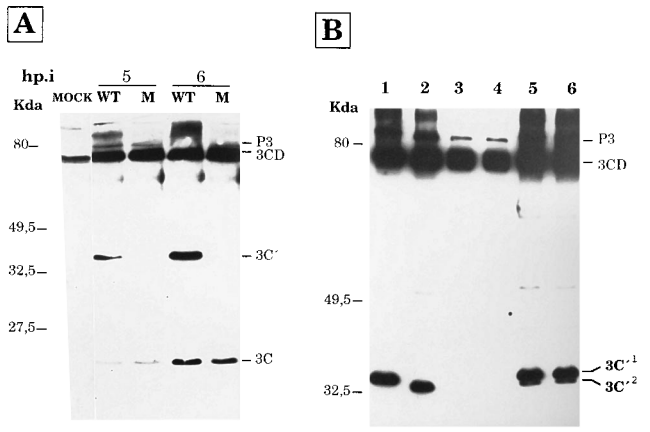

FIG. 4. Cleavage of 3CD to produce 3C′. (A) Failure of mutant 2A(Y88S) to mediate alternative cleavage of the 3CD polypeptide. Extracts from WT- or 2A(Y88S) mutant (M)-infected cells were analyzed by Western blotting with anti-3C serum as described in Materials and Methods. 2A(Y88S) mutant lanes contained twofold more protein than the WT to compensate for the difference in viral protein synthesis. Immunoreactive products and molecular size markers are indicated. (B) Complementation of mutant 2A(Y88S) by WT poliovirus for alternative cleavage of the 3CD polypeptide. HeLa cells were infected with 2A(Y88S) mutant virus alone or with PV1(M) to assay the effect of WT $2A^{pro}$ on proteolytic processing of the 2A(Y88S) 3CD polypeptide. At 6 h p.i., extracts were made and immunoblot analysis with anti-3C serum was performed. Lanes: 1, PV1(M) at an MOI of 10 PFU per cell; 2, pT7XLD WT at an MOI of 25 PFU per cell; 3, pT7XLD 2A(Y88S) at an MOI of 25 PFU per cell; 4, pT7XLD 2A(Y88S) at an MOI of 40 PFU per cell; 5, PVI(M) at an MOI of 10 PFU per cell plus pT7XLD2A(Y88S) at an MOI of 25 PFU per cell; 6, PVI(M) at an MOI of 10 PFU per cell plus pT7XLD 2A(Y88S) at an MOI of 40 PFU per cell. The gel was run for 40 h to separate the specific PVI(M) 3C′ protein ($3C'^1$) from pT7XLD-derived virus 3C′ protein ($3C'^2$).

sequence in 3D produces 3C′ and 3D′ (7). To analyze this cleavage by the mutant 2A(Y88S), the proteins present in the infected cells were transferred to a nitrocellulose membrane and immunoblotted with a specific anti-3C serum. No 3C′ protein was detected in the 2A(Y88S) mutant (Fig. 4), in agreement with the results obtained by analysis of the [^{35}S]methionine-labeled proteins (Fig. 3A). These results are striking because they illustrate and reinforce the idea that the specificity of $2A^{pro}$ for substrates in *trans* can be altered in a selective way (42) and open the possibility of finding $2A^{pro}$ mutants able to cleave P1-2A and 3CD but not p220.

Complementation of the $2A^{pro}$ Y88S mutant by WT poliovirus. Since the plaques produced by WT poliovirus and the $2A^{pro}$ mutant are distinguishable by size, HeLa cells were infected with each virus individually or together and the plaques formed were quantitated. WT poliovirus complements the $2A^{pro}$ mutant and enables it to grow in HeLa cells, suggesting that defective $2A^{pro}$ can be complemented in *trans* (results not shown). To assess if the 3CD polypeptide from the mutant virus can be cleaved by WT poliovirus $2A^{pro}$, we took advantage of the fact that the poliovirus strain routinely used in our laboratory has a 3C′ with electrophoretic mobility different ($3C'^1$) from that of the 3C′ derived from poliovirus pT7XLD ($3C'^2$) (Fig. 4B). Coinfection with both mutant 2A(Y88S) and our WT poliovirus gave rise to two bands, one of them corresponding to the 3C′ from mutant 2A(Y88S) ($3C'^2$) (Fig. 4B). These results indicate that there is not an intrinsic modification in the 3CD from the mutant that makes this protein refractory to cleavage by WT $2A^{pro}$.

Synthesis of viral RNA by the $2A^{pro}$ Y88S mutant. Activity of $2A^{pro}$ is necessary for poliovirus genome replication (23, 41), but it is still uncertain if $2A^{pro}$ directly participates in this process or if this requirement is due to the proteolytic generation of viral or host protein products that participate in that process. Therefore, viral RNA synthesis by the 2A(Y88S) mutant was tested by measuring [^{3}H]uridine incorporation and by dot blot analyses. In cells infected with the 2A(Y88S) mutant, incorporation of [^{3}H]uridine at 3 to 4 and 4 to 5 h p.i. was half of that seen in cells infected with the WT virus. However, almost no differences were found during the initial 2 to 3 h p.i. or at later times (Fig. 5A). Measurement of total viral RNA by dot blot analyses with a specific poliovirus riboprobe showed that HeLa cells infected with 2A(Y88S) contained about 50 to 70% of the positive-strand RNA of WT poliovirus (Fig. 5B). Thus, the Y88S mutant makes less viral RNA than does the WT poliovirus in HeLa cells. These results may reflect a defective $2A^{pro}$ activity of mutant 2A(Y88S) during RNA replication or a lower amount of other viral proteins made by the mutant virus that are needed for genome replication. It must be kept in mind that poliovirus RNA synthesis requires continuous viral protein synthesis (4). Therefore, a decrease in viral protein synthesis would have repercussions in viral RNA synthesis.

Apart from the analysis of total poliovirus RNA, the distribution of viral RNA after polysome fractionation was determined (Fig. 6). Lysates from cells infected with WT poliovirus or the 2A(Y88S) mutant were fractionated by sucrose gradient centrifugation. Both the optical density at 254 nm and the viral RNA present in each fraction were estimated. The amounts of ribosomal subunits (fractions 2 to 4) in the two lysates were similar, while the polysomal fraction (fractions 5 to 8) was more abundant in WT poliovirus-infected cells than in 2A(Y88S) mutant-infected cells (Fig. 6A). In agreement with the data obtained on the polysomal profile, the amount of viral RNA from WT poliovirus was more abundant in all fractions of the gradient and particularly in the heavy fractions, which represent polysomes, than in the fractions from the 2A(Y88S) mutant (Fig. 6B and C). The highest amount of viral RNA in the 2A(Y88S) mutant was located in fractions 4 and 5, which probably represent monosomes and virion RNA. These results reinforce the idea that the viral RNA from the mutant virus

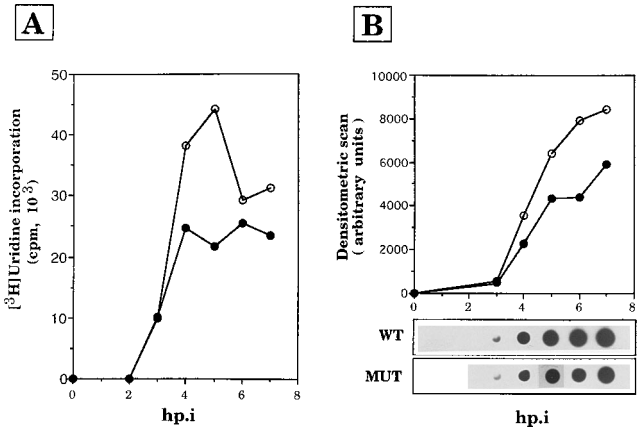

FIG. 5. Analysis of WT and 2A(Y88S) mutant poliovirus RNA synthesis in infected HeLa cells. (A) Total viral RNA synthesis estimated by incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable material at different times after infection in dactinomycin-treated cells infected with WT (○) and 2A(Y88S) mutant (●) viruses. (B) Estimation of poliovirus positive-strand RNA. Total RNA was extracted from WT (○)- or 2A(Y88S) mutant (MUT) (●)-infected cells, and dot blot analysis was performed with a biotinylated riboprobe to detect viral RNA as described in Materials and Methods. Only one of the eight serial dilutions in the linear range of the signal is shown below the graph, in which the densitometric quantification from the blot is plotted.

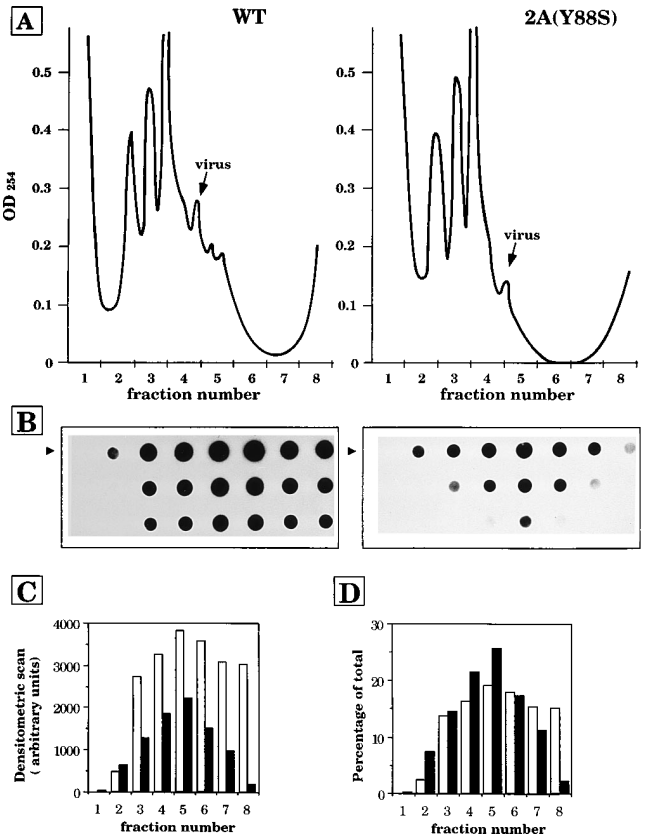

FIG. 6. Distribution of viral RNA in polysomes. (A) Absorbance profiles of cellular extracts from HeLa cells infected with WT or 2A(Y88S) mutant poliovirus analyzed by sucrose gradient centrifugation. Cell lysates were obtained 4.75 h p.i. and analyzed as described in Materials and Methods. The direction of sedimentation is left to right. The position of the viral particles is indicated. OD_{254}, optical density at 254 nm. (B) Distribution of poliovirus-specific RNA in the different fractions. Equivalent amounts of total RNA from each fraction were analyzed by dot blot hybridization as described in Materials and Methods. Three serial dilutions of the samples are shown. The arrowheads indicate the dilutions used for the densitometric analysis. (C) Densitometric quantitation of poliovirus RNA from the dot blot shown in panel B. The total hybridization signals were 19,947 (arbitrary units) for WT poliovirus (open bars) and 8,661 for the 2A(488S) mutant (full bars). (D) Data corresponding to panel C represented as percentages of the total fractions. The bars are the same as in panel C.

participates to a lesser extent in translation than does WT poliovirus mRNA.

Effects of guanidine on RNA and protein synthesis. To determine whether the primary defect of the 2A(Y88S) mutant is at the level of viral RNA replication or protein synthesis, the poliovirus RNA synthesis inhibitor guanidine was used. Addition of guanidine from about 2.5 h p.i. does not prevent the appearance of poliovirus proteins, while RNA synthesis is immediately halted (4, 17). Thus, we reasoned that addition of guanidine at 3.5 h p.i. would still allow the synthesis of viral proteins under conditions in which no differences in viral RNA content are observed between the 2A(Y88S) mutant and WT poliovirus. Figure 7 shows that addition of guanidine at 3.5 h p.i. arrested the synthesis of viral RNA (Fig. 7C) and the infected cells continued with the same amount of viral RNA until 6.5 h p.i. (Fig. 7C). Almost no difference in the total amount of poliovirus RNA was found between the WT and the mutant virus, and only a slight difference in the stability of viral RNA was observed (Fig. 7B).

Analysis of the proteins synthesized by WT poliovirus in the presence of guanidine showed efficient synthesis of virus proteins at 3.5 to 4.5 h p.i. and a decline of viral translation in the subsequent 2 h (Fig. 7A). This behavior may be due to sequestration of the already translated viral RNA in nonfunctional replication complexes or to the use of viral RNA for assembly of new virions. The most striking finding is that the 2A(Y88S) mutant synthesized 10-fold less protein than did the WT poliovirus, under conditions in which the total amount of viral RNA was similar to that of WT poliovirus (Fig. 7A). These results suggest that the viral RNA is not actively translated in HeLa cells infected by the 2A(Y88S) mutant.

Failure of the 2A^{pro} Y88S mutant to transactivate poliovirus leader-luc mRNA. One of the activities ascribed to poliovirus 2A^{pro} is the ability to transactivate the translation of mRNAs bearing the poliovirus 5′ untranslated sequence (12). Since the major defect found in the 2A(Y88S) mutant was at the translational level, we sought to analyze the transactivation capacity of both WT poliovirus and the 2A(Y88S) mutant. Two different mRNAs encoding luciferase were assayed, one containing the luciferase gene under the control of the poliovirus leader

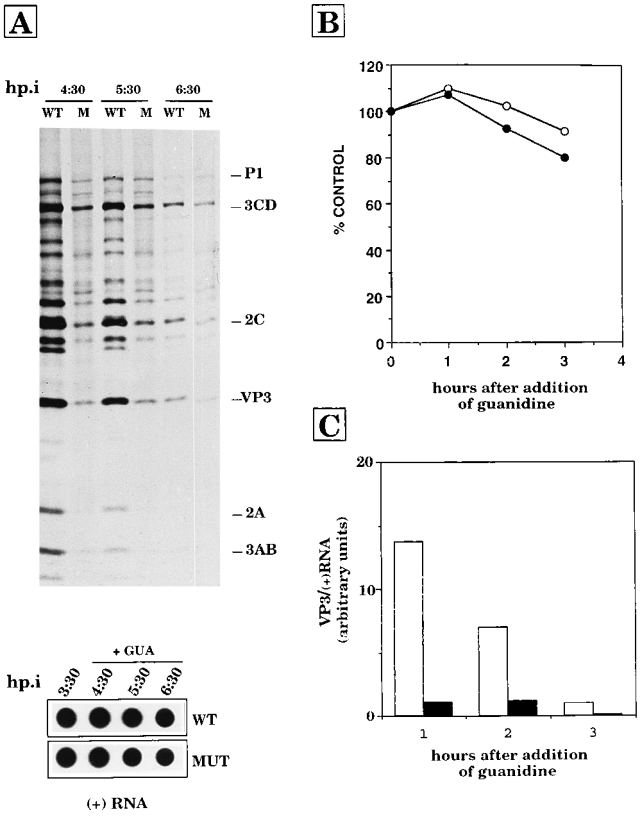

FIG. 7. Effect of guanidine on viral RNA synthesis and translation by WT poliovirus and 2A(Y88S) mutant. (A) SDS-PAGE analysis of proteins synthesized after addition of 3 mM guanidine at 3.5 h p.i. Infected cells were pulse-labeled with [^{35}S]methionine for 1 h and recovered in PBS at 4.5, 5.5, and 6.5 h p.i. (1, 2, and 3 h after guanidine treatment, respectively), as indicated. One-tenth of each sample was used to detect viral protein synthesis (top), and the remaining volume was used to extract total RNA. Dot blot analysis was performed to quantitate the amounts of viral positive-strand RNA present at the different times (bottom). Lanes: WT, WT virus; M, 2A(Y88S) mutant virus. (B) Stability of viral RNA after guanidine treatment. The dot blot from panel A was quantitated by densitometry. The amount of RNA at 3.5 h p.i. (time zero after guanidine addition) was taken as 100%. Symbols: ○, WT RNA; ●, 2A(Y88S) mutant RNA. (C) Relative translational capacities of WT (□) and 2A(Y88S) mutant (■) viruses. VP3 bands from the autoradiogram shown in panel A were quantitated by densitometry, and the resulting data were corrected by the amounts of specific viral RNA.

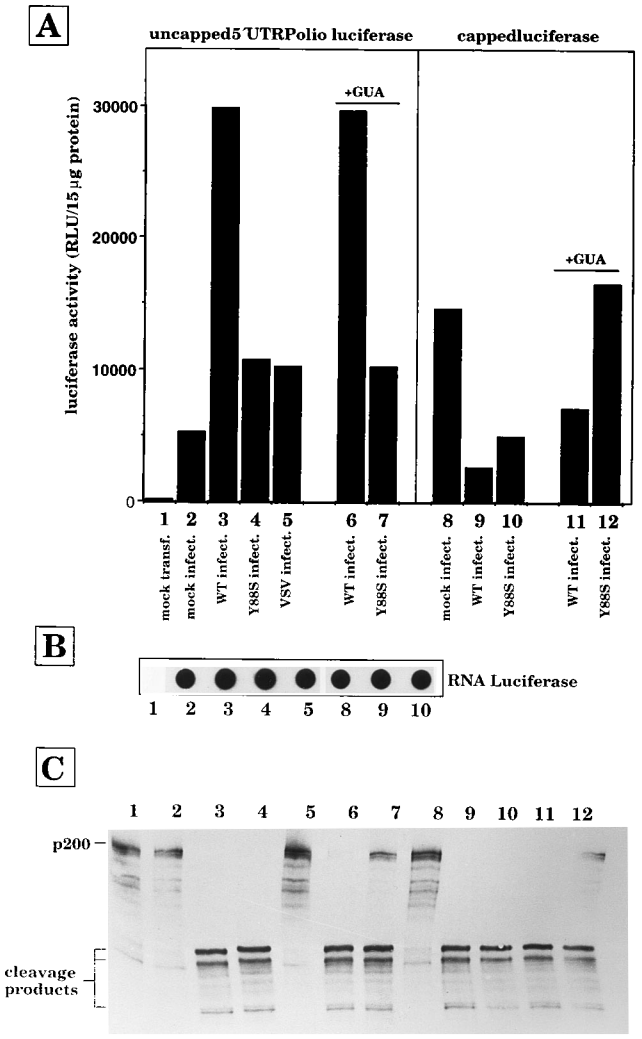

FIG. 8. Effect of WT and 2A(Y88S) mutant poliovirus infection (infect.) on translation of uncapped 5′ untranslated region (UTR) luc and capped luc mRNA. (A) HeLa cells were infected with the viruses indicated at an MOI of 50 PFU per cell, and 2 h later they were transfected (transf.) with 2 μg of the in vitro-synthesized RNA indicated, as described in Materials and Methods. Incubation was continued for 3 h (5 h p.i.), and then the cells were recovered in lysis buffer and the extracts were split into four portions. One-tenth of each sample was used to measure luciferase activity. Guanidine (GUA; 3 mM) was added at 1 h p.i. as indicated. All of the transfections were done in triplicate, and the data shown are means of the values obtained, normalized to the protein content in each sample. VSV, vesicular stomatitis virus. (B) Dot blot analysis of luciferase RNA transfected into the cells. One-half of the extracts described in panel A were treated with 5 U of RQ1 DNase for 15 min at 37°C and adjusted to 0.5% SDS, and the total RNA was extracted with phenol and precipitated with ethanol. Detection was carried out with a biotinylated luciferase riboprobe as described in Materials and Methods. The blot numbers correspond to the bars in panel A. (C) Immunoblot analysis of p220. The lane numbers correspond to the bars in panel A. The positions of intact p220 and poliovirus-induced p220 cleavage products are indicated.

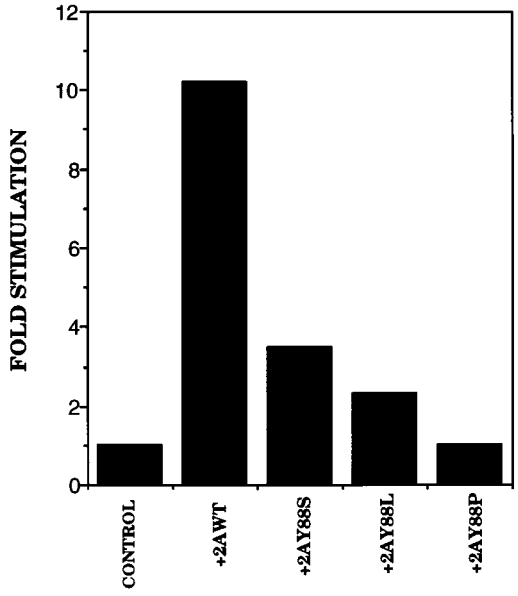

FIG. 9. Transactivation capacities of WT and mutant 2A proteins. HeLa cells were cotransfected with 1 μg of pT75′NCLUC RNA and 5 μg of pTM12C RNA (control) or with 5 μg of pTM1 2A WT, pTM1 2A(Y88S), pTM1 2A(Y88L), or pTM1 2A(Y88P) RNA as indicated. After 5 h of incubation, the cells were washed and incubated in Dulbecco modified Eagle medium–5% fetal calf serum for an additional hour. Extracts were then obtained, and luciferase activity was measured. The data were corrected for the protein content of each sample and for the specific luciferase RNA transfected in each sample as estimated by dot blot analysis.

sequence and the other consisting of a capped mRNA containing the 5′ leader sequence of the luciferase gene (Fig. 8). Different transfection protocols were used to assay the transactivation effect; good transactivation was found when cells were first infected with poliovirus and then, after 2 h of poliovirus replication, transfected with mRNA by the lipofectin method. Cells were harvested 3 h after transfection, and extracts were prepared to assay luciferase activity. Notably, infection with WT poliovirus caused an about sixfold increase in luciferase activity when the leader-luc mRNA was analyzed, while no increase was found with the 2A(Y88S) mutant (Fig. 8A). This stimulation was observed even when the infected cells were treated with guanidine, suggesting that the amount of $2A^{pro}$ generated by translation of the input RNA is sufficient to transactivate the leader-luc mRNA. As a control, the amount of mRNA transfected into HeLa cells was quantitated. Figure 8B shows that the same leader-luc mRNA was transfected in mock-infected cells, WT poliovirus-infected cells, and 2A(Y88S) mutant-infected cells. These results suggest that the poliovirus $2A^{pro}$ mutant has a defect in the ability to transactivate the poliovirus leader sequence. Finally, infection by both WT poliovirus and the 2A(Y88S) mutant inhibited luciferase production from the capped luc mRNA, suggesting that the capacity to inhibit translation of capped mRNA is retained in the 2A(Y88S) mutant (Fig. 8). To assess the action of $2A^{pro}$ on p220 cleavage during these experiments, the extracts obtained to measure luciferase activity were immunoreacted with p220 antibodies. p220 was cleaved after infection with both WT poliovirus and mutant 2A(Y88S) in the absence or presence of guanidine (Fig. 8C).

Transactivation of leader-luc mRNA upon transfection with mRNA encoding $2A^{pro}$. Once it was determined that infection with mutant 2A(Y88S) did not transactivate translation of leader-luc mRNA, it was of interest to assay transactivation by the isolated $2A^{pro}$ gene. The sequences corresponding to WT $2A^{pro}$ were placed under control of the encephalomyocarditis virus 5′ untranslated region, and the corresponding mRNA was cotransfected with the leader-luc mRNA. Figure 9 shows a 10-fold enhancement of luciferase activity upon transfection with mRNA encoding WT poliovirus $2A^{pro}$. Notably, cotransfection with mRNA encoding 2A(Y88S) gave rise to diminished enhancement of leader-luc mRNA translation; even less

leader-luc transactivation was observed with the 2A(Y88L) mutant, and none was seen with the 2A(Y88P) mutant (Fig. 9).

These findings indicate that transactivation of the poliovirus 5′ leader sequence by 2A^{pro} can be achieved by cotransfection of the two mRNAs, i.e., leader-luc mRNA and mRNA encoding 2A^{pro}. Moreover, our results point to the crucial role of residue 88 of 2A^{pro} in the transactivation effect. Finally, the possibility that 2A(Y88S) is viable because it still possesses some transactivation activity while 2A(488P) has a null phenotype because it is unable to transactivate is attractive.

DISCUSSION

The availability of the cloned poliovirus genome allows the generation of an increasing number of mutants with changes in virtually all regions of the poliovirus genome (38). This research is providing a more detailed picture of the mechanisms by which the viral RNA is translated and replicated. However, many fundamental aspects of poliovirus biology remain unclear. It is hoped that manipulation of the cloned poliovirus genome will allow further insight into the poliovirus replication cycle (38). Analysis of the poliovirus 2A^{pro} mutant described in this work offers several new findings. One important result that emerged from this study is that the effect of the mutation of 2A^{pro} on the capacity to cleave p220 is much better analyzed when the protease is produced in virus-infected cells or in cells transfected with the 2A^{pro} gene than in cell-free systems. Our present results show that p220 is degraded in cells expressing mutant 2A^{pro} to the same extent as during WT poliovirus infection, a conclusion not reached when cell-free systems were used (42). Another aspect of this study is that 2A^{pro} can be selectively modified in such a way that the protease is still able to cleave in *trans* some substrates, such as p220, but not others, such as 3CD. This finding raises the possibility of finding 2A^{pro} mutants with the converse phenotype, i.e., poliovirus mutants selectively unable to cleave p220 that still retain the ability to hydrolyze 3CD. The region of 2A^{pro} located around residue 88 seems to be important in the recognition of substrates in *trans*, as already indicated by others (42).

Expression of 2A^{pro} enhances production of luciferase from a transfected reporter plasmid encoding luciferase under control of the poliovirus leader sequence (12). The mechanism by which 2A^{pro} induces this activation is unknown, but a more detailed analysis of the mutant described here will provide new clues to this activation. We believe that one of the most important characteristics of the 2A(Y88S) mutant described in the present work is that it shows defects in viral protein synthesis, perhaps as a consequence of its failure to transactivate the poliovirus leader sequence (12). If this is so, our findings demonstrate that the enhancing effect of 2A^{pro} on poliovirus protein synthesis is required for efficient virus growth. There are several possible explanations for the present results. (i) 2A^{pro} directly enhances the translation of poliovirus RNA; this enhancement could be achieved by its potential capacity to bind the viral RNA (10, 20). (ii) Y88S mutant 2A^{pro} is not only unable to cleave the 3CD substrate but is also unable to cleave an unidentified cellular protein necessary for transactivation of poliovirus RNA translation. (iii) The impairment of translation observed in the Y88S mutant is due to the lack of 3C′ and/or 3D′. The 3CD precursor, unlike 3C^{pro} or 3D^{pol}, has the ability to bind to a specific region at the 3′ end of poliovirus RNA (2). Thus, it would not be surprising if 3C′ or 3D′ showed RNA binding specificity different from that of 3C^{pro}, 3D^{pol}, or 3CD and if this RNA binding potentiated translation in HeLa cells. As already pointed out, the fact that the 3CD cleavage that gives rise to 3C′ and 3D′ remains after repeated passage of poliovirus suggests that the formation of these products is important in some step(s) of the poliovirus life cycle (18). If this is so, this finding means that the alternative cleavage of 3CD has a physiological meaning, providing an extreme example of the compactness of the information in the poliovirus 3CD region. However, we believe, that the third possibility is unlikely, since a poliovirus mutant with an alteration in 3CD which is no longer cleaved by 2A^{pro} shows no growth defects (18). Moreover, as shown in the present work, transactivation occurs with the transfection of mRNA encoding 2A^{pro} alone, and the 3C′ and 3D′ products have not been implicated in this process (12).

The finding that 2A^{pro} is necessary to stimulate the synthesis of poliovirus proteins (12) provides another example of a virus-encoded protein that selectively enhances the translation of some mRNAs from the same virus. Proteins such as rev or NS1 enhance the translation of some human immunodeficiency virus mRNAs or influenza virus mRNAs, respectively (5, 21, 29). The mechanism of this selective activation lies in the selective binding of these proteins to their corresponding mRNAs (5, 21, 29). Once the proteins are bound to these mRNAs, the initiation of translation on these messengers is more efficient, perhaps because of the recruitment of initiation complexes on these mRNAs. There are indications that picornavirus 2A possesses the capacity to interact with the leader sequence of the picornavirus mRNA (20). Since poliovirus 2A^{pro} enhances the translation of its own mRNA, it could be that binding of 2A^{pro} activates the initiation of the picornavirus mRNA. This is also suggested by the fact that 2A^{pro} needs to interact with p220 to cleave this factor. Moreover, we have found that the C terminus of p220 becomes associated with polysomes in poliovirus-infected cells (unpublished observations). The possibility that cleaved p220, or at least one of its fragments, participates in poliovirus mRNA translation is still open. The interaction of 2A^{pro} with the C-terminal p220 fragment and with the 5′ leader region of poliovirus RNA would provide a mechanism to account for the selective transactivation of poliovirus translation by 2A^{pro}. Experiments are in progress in our laboratory to assay this model.

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