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Attenuating Mutations in the Poliovirus 5′ Untranslated Region Alter Its Interaction with Polypyrimidine Tract-Binding Protein

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Mutations in the 5′ untranslated regions (5′-UTRs) of all three serotypes of the Sabin vaccine strains are known to be major determinants of the attenuation phenotype. To further understand the functional basis of the attenuation phenotype caused by mutations in the 5′-UTR, we studied their effects on viral replication, translation, and the interaction of the viral RNA with cell proteins. A mutation at base 472 (C472U), which attenuates neurovirulence in primates and mice, was previously found to reduce viral replication and translation in neuroblastoma cells but not in HeLa cells. This mutation reduced cross-linking of the poliovirus 5′-UTR to polypyrimidine tract-binding protein (pPTB) in neuroblastoma cells but not in HeLa cells. These defects were absent in a neurovirulent virus with C at nucleotide 472. When C472U and an additional mutation, G482A, were introduced into the 5′-UTR, the resulting virus was more attenuated, had a replication and translation defect in both HeLa cells and neuroblastoma cells, and cross-linked poorly to pPTB from both cell types. A neurovirulent revertant of this virus (carrying U472C, G482A, and C529U) no longer had a replication defect in HeLa and SH-SY5Y cell lines and cross-linked with pPTB to wild-type levels. The results suggest that the attenuating effects of the mutation C472U may result from an impaired interaction of the 5′-UTR with pPTB in neural cells, which reduces viral translation and replication. Introduction of a second mutation, G482A, into the 5′-UTR extends this defect to HeLa cells.

Each of the three serotypes of poliovirus causes an identical disease in humans, which may be controlled by use of the live, attenuated vaccine strains developed by A. B. Sabin. These attenuated strains were isolated by passage of viruses in animals and cultured cells until viruses with reduced neurovirulence in primates were identified (31). Understanding the molecular and functional bases of the attenuation phenotype has provided insight into the replication of poliovirus and has suggested how to improve the existing vaccines (reviewed in reference 23).

All three serotypes of the Sabin vaccine strains contain a determinant of attenuation in the 5′ untranslated region (5′-UTR) of the single-stranded RNA genome, at nucleotide (nt) 480, 481, or 472 in Sabin types 1, 2, and 3, respectively (16, 24, 40). These mutations reduce viral replication in the central nervous system (CNS) and in neuroblastoma cells but do not have a general negative effect on viral replication in cell culture and therefore may be considered host range mutations (17, 18). The precise mechanism by which these mutations reduce viral replication in the CNS is not known. The 5′-UTR contains an internal ribosome entry site (IRES) and harbors sequences that control viral RNA replication (2, 27, 28, 30, 33, 39). Attenuated strains with single base changes at nt 480, 481, or 472 are translated less efficiently in vitro than their virulent counterparts (36–38). An attractive hypothesis for the mechanism of action of attenuating mutations is that they alter the secondary structure of the 5′-UTR, thereby interfering with its ability to interact with *trans*-acting factors that are required for internal initiation and which may be limiting in neural tissues (20, 33, 34).

Many cell proteins that interact with the 5′-UTR of poliovirus have been described, including eIF-2 (3), pyrimidine tract-binding protein (pPTB) (12, 29), La (21), p100 (13), p48/50, p38/39, and p35/36 (4, 6, 11, 25), and p60 (11). The translation factor eIF-2, which interacts with nt 502 to 636 of the poliovirus 5′-UTR (3), is present in a fraction from HeLa cells that corrects the aberrant translation initiation of poliovirus RNA in reticulocyte lysates (37). The expression of this factor is subject to a variety of cellular controls (14, 19). La, a protein that plays a role in the termination of RNA polymerase III transcription (8, 9), appears to be essential for poliovirus translation, since its addition to La-deficient rabbit reticulocyte lysates restores initiation of translation at the authentic start codon (22). pPTB, which is involved in differential splicing of pre-mRNA (5, 7, 26), interacts with three sites in the poliovirus 5′-UTR (13). Depletion of pPTB from cell extracts inhibits translation of mRNAs containing the poliovirus or encephalomyocarditis virus IRES, and the addition of pPTB restores cap-independent translation (12, 15). p48, p38, and p35 have a higher affinity for RNA of the virulent P1/Mahoney strain than for that of P1/Sabin (4). Whether any of these proteins are involved in the cell type specificity of the attenuation phenotype has not been determined.

To further understand the functional basis of the attenuation phenotype caused by mutations in the 5′-UTR, we studied their effects on viral replication, translation, and the interaction of the viral RNA with cell proteins. A mutation at base 472 (C472U), which attenuates neurovirulence in mice, was previously found to reduce viral replication and translation in neuroblastoma cells but not in HeLa cells (17, 18). This mutation reduced cross-linking of the poliovirus 5′-UTR to pPTB in neuroblastoma cells but not in HeLa cells. These defects were

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absent in a neurovirulent virus with C at nt 472. When both C472U and G482A were introduced into the 5′-UTR, the resulting virus was more attenuated (33), had a replication and translation defect in both HeLa and neuroblastoma cell lines, and cross-linked poorly to pPTB from both cell types. A neurovirulent revertant of this virus (carrying U472C, G482A, and C529U) no longer had a replication defect in the HeLa and SH-SY5Y cell lines and cross-linked with pPTB to wild-type levels. These results suggest that the attenuating effects of the mutation C472U may result from an impaired interaction of the 5′-UTR with pPTB in neural cells, which reduces translation and replication. Introduction of a second mutation, G482A, into the 5′-UTR extends this defect to HeLa cells.

MATERIALS AND METHODS

Cells. Suspension cultures of HeLa S3 cells were grown in Joklik minimal essential medium (MEM) containing 10% horse serum and 10 mg of gentamicin per ml. Monolayers of human neuroblastoma (SH-SY5Y) (35) and HeLa cells were maintained in Dulbecco MEM supplemented with 10% fetal calf serum and 10 mg of gentamicin per ml.

Viruses. PRV6.1 contains the 5′-UTR from P3/Leon and the coding region from P2/Lansing (17). PRV7.3 contains the 5′-UTR from P3/Sabin and the coding region from P2/Lansing (17). SFP8 is PRV7.3 with a mutation at base 482 (G to A) (33). V957 (previously called 8 $SC10^{-1}$) is a neurovirulent revertant of SFP8 selected in the CNSs of infected mice that contains a reversion at base 472 (U to C) and a mutation at base 529 (C to U) and maintains the SFP8 mutation at base 482 (G to A) (33). (See Fig. 1A for a summary of the mutations in the 5′-UTRs of these viruses.) Virus stocks were obtained after infection of HeLa cells at a multiplicity of infection (MOI) of 50.

One-step growth curve experiments. Monolayers of SH-SY5Y cells were dispersed by pipetting. SH-SY5Y cells and suspension cultures of HeLa cells were centrifuged and infected with viral strains at an MOI of 10 PFU/cell. After 45 min of adsorption at room temperature (RT), cells were diluted to 2×10^6 cells/ml in MEM supplemented with 5% fetal bovine serum. Infected HeLa cells were incubated at 32.5 or 38.5°C, and infected SH-SY5Y cells were incubated at 37°C for 7 h. At different times postinfection, cells and medium were frozen and thawed three times and clarified by centrifugation, and virus titers were determined by plaque assay on HeLa cell monolayers at 37°C (18).

Measurement of viral RNA synthesis. Infections were performed as described above, and at different times postinfection HeLa and SH-SY5Y cells were placed on ice, washed once with cold phosphate-buffered saline (PBS), and lysed in 10 mM Tris-HCl (pH 7.5)–10 mM NaCl–0.1% Nonidet P-40. Nuclei and debris were removed by centrifugation. RNA was obtained by phenol-chloroform extraction and ethanol precipitation.

Aliquots of RNA were denatured in 10× SSC (1.5 M NaCl, 150 mM sodium citrate)–17% formaldehyde and bound to nitrocellulose membranes by aspiration with a slot blot apparatus. Membranes were UV-cross-linked for 2 min in a Stratalinker apparatus and prehybridized and hybridized in 50% formamide–6× SSC–1% sodium dodecyl sulfate (SDS)–5× Denhardt's solution–100 mg of herring sperm DNA per ml at 42°C. A ^{32}P-labeled negative-strand RNA probe from the VP2-coding sequence was produced by T7 transcription. After overnight hybridization at 42°C, filters were washed in 2× SSC–0.1% SDS twice at room temperature and in 0.2× SSC–0.1% SDS twice at 65°C and exposed to X-ray film. Radioactivity on the nitrocellulose filters was determined by scintillation counting.

Plasmid construction. The cDNAs of the 5′-UTRs (nucleotides 1 to 686) from all strains were obtained after *Bam*HI digestion of the full-length cDNAs. The ends of the DNA fragments were made blunt with DNA polymerase I (Klenow fragment) and ligated to *Hin*dIII linkers. DNA fragments were cloned into the *Hin*dIII site of plasmid pS-2, which contains the simian virus 40 (SV40) promoter, the chloramphenicol acetyltransferase (CAT) gene, and a poly(A) signal from SV40 (the *Hin*dIII site is located between the promoter and the CAT gene). For cross-linking assays, nt 275 to 686 of the 5′-UTRs from wild-type and mutant cDNAs were obtained by cleavage with *Taq*I and *Bam*HI, followed by cloning into the *Acc*I and *Bam*HI sites of pGEM 4Z (Promega).

DNA transfection. Monolayers of HeLa and SH-SY5Y cells were cotransfected by the calcium phosphate method as described previously (32). Plasmids pRM-61, pRM-73, pRM-8, and pRM-D, containing the wild-type and mutant 5′-UTR and CAT gene (4 mg of DNA per 6-cm-diameter plate), and plasmid pGC-9, containing the β-galactosidase gene (1 mg of DNA per 6-cm-diameter plate), were used in transfection assays. At 48 h posttransfection, cells were washed with cold PBS, treated with 350 μl of 40 mM Tris-HCl (pH 7.4)–1 mM EDTA–150 mM NaCl for 5 min at RT, and harvested by centrifugation. The pellet was resuspended in 200 μl of 250 mM Tris-HCl (pH 8) and lysed by three freeze-thaw cycles. Nuclei and debris were eliminated by centrifugation, and the supernatants were assayed for β-galactosidase or CAT activity.

β-Galactosidase and CAT assays. Fifty microliters of cell extract was mixed with 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml in phosphate buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$]) and 1 μl of β-mercaptoethanol in phosphate buffer. The reaction mixtures were incubated for 1 h at 37°C, the reactions were stopped with 500 μl of 1 M sodium carbonate, and the optical density at 420 nm was determined. CAT assays were performed as described previously (32). The CAT activity obtained with a wild-type 5′-UTR was assigned a value of 1.

Preparation of RSW and S10. Ribosomal salt washes (RSW) from HeLa and SH-SY5Y cells were prepared as described previously (3). S10 was obtained by washing cells twice with cold PBS and resuspending them in 5 cell volumes of 10 mM HEPES (pH 7.9)–1.5 mM $MgCl_2$–10 mM KCl. Cells were centrifuged immediately and resuspended in 2 cell volumes of the same buffer. The cells were broken with 20 strokes in a Dounce homogenizer, and the homogenate was centrifuged at 10,000 rpm for 30 min in a Sorvall GSA rotor. The supernatant was divided into aliquots, and the amount of protein was determined with the Bradford assay.

UV-induced cross-linking assays. Labeled RNAs from nt 275 to 628 of the 5′-UTRs from wild-type and mutant strains were prepared by in vitro transcription of pGEM-derived plasmids, linearized with *Bal*I, in the presence of [α-^{32}P]UTP as described previously (3). Transcription reaction mixtures were treated with DNase RQ1 (Promega), and unincorporated nucleotides were removed by gel filtration. Cross-linking assays were performed as described previously (36). Samples were fractionated on SDS–12.5% polyacrylamide gels. The gels were fixed, dried, and autoradiographed. For competition experiments, unlabeled RNAs were first incubated with cell extract for 15 min at RT, and then the labeled RNA was added and incubation was continued for an additional 15 min prior to the cross-linking reaction.

Western blot assay. Cross-linking assays were performed as described above. SDS-polyacrylamide gels were equilibrated for 15 min at RT with transfer buffer (0.025 M Tris-HCl [pH 9.5], 0.019 M glycine, 20% [vol/vol] methanol) and transferred to nitrocellulose membranes by using a semidry Transblot apparatus at 20 V for 45 min. Membranes were blocked at RT for 1 h in PBS containing 3% (wt/vol) bovine serum albumin and then washed three times in 0.5% (wt/vol) Tween 20 in PBS. The first antibody was a monoclonal anti-pPTB antibody (provided by E. Wimmer, SUNY, Stony Brook, N.Y.) diluted 1:150 or a monoclonal anti-La antiserum (provided by N. Sonenberg, McGill University) diluted 1:100 in incubation buffer (1% bovine serum albumin in PBS) and incubated overnight at 4°C. The second antibody was anti-mouse immunoglobulin G conjugated to alkaline phosphatase, diluted 1:1,000 in incubation buffer, and incubated at RT for 2 h. Color was developed with BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) and nitroblue tetrazolium chloride, and this reaction was stopped after 30 min with water.

RESULTS

Phenotypic analysis of viral strains. Mutations in a stem-loop structure known as domain V in the poliovirus 5′-UTR were previously shown to reduce neurovirulence in a mouse model for poliomyelitis (17, 33). The effects of specific mutations on neurovirulence were determined by constructing recombinant viruses in which the 5′-UTR of the mouse-adapted P2/Lansing strain was replaced with corresponding sequences from different type 3 strains. Recombinant virus PRV6.1 contains the wild-type 5′-UTR from P3/Leon and is neurovirulent in mice (Fig. 1A). PRV7.3 contains the 5′-UTR from P3/Sabin, which contains a mutation at base 472 (C472U) that is dramatically attenuating (Fig. 1A). SFP8 contains C472U and the additional mutation G482A and is more attenuated than PRV7.3 (Fig. 1A). V957 is a neurovirulent revertant of SFP8 that has reverted at base 472 (U472C) but not at base 482 (G482A) and has a second mutation at base 529 (C529U) (Fig. 1A). The single mutation at base 482 (G482A) has been reported to be responsible for an attenuated phenotype in mice (33). The C472U mutation is host cell specific, as it reduces viral replication in neuroblastoma cells and in the CNS but not in HeLa cells (17, 18). Experiments were carried out to understand how mutations C472U and G482A influence viral neurovirulence.

To determine the effect of the mutations C472U and G482A in SFP8, one-step growth curve experiments were performed with HeLa cells and SH-SY5Y cells. In HeLa cells, infections were carried out at 38.5°C, a temperature at which the differences between viruses were emphasized compared to the case at lower temperatures (10). In HeLa cells, replication of SFP8 was slower than replication of PRV7.3, PRV6.1, and V957, and

A

VIRUS	220	472	482	529	LD50
PRV6.1	G	C	G	C	$<6 \times 10^2$
PRV7.3	U	U	G	C	$>2 \times 10^7$
SFP8	U	U	A	C	1.6×10^8
V957	U	C	A	U	$<5.5 \times 10^4$

B

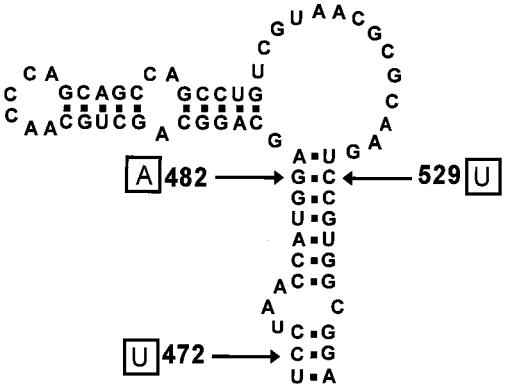

FIG. 1. Mutations in virus strains used in this study. (A) Mutations at nt 472, 482, and 529 and 50% lethal doses (LD50) reported previously (33). (B) Schematic representation of the secondary structure of domain V of the poliovirus 5′-UTR (33). Mutations at nt 472, 482, and 529 are boxed.

final yields were nearly 10 times lower (Fig. 2A). As previously reported, the growth curves of PRV7.3 and PRV6.1 in HeLa cells were similar (17). The revertant of SFP8, V957, replicated with kinetics similar to those of PRV6.1 and PRV7.3 (Fig. 2A). Growth curves were determined at 37°C for SH-SY5Y cells, which did not tolerate higher or lower temperatures. The replication of SFP8 and PRV7.3 in SH-SY5Y cells was reduced compared to that of PRV6.1 and V957 (18) (Fig. 2B). Consistent with these findings, SFP8 produced small plaques in both cell lines, while PRV7.3 produced small plaques only in SH-SY5Y cells. However, SFP8 is not temperature sensitive in HeLa cells, because equal numbers of plaques are observed at both temperatures (10). These results confirm previous findings (18) that the mutation C472U impairs poliovirus replication in human neuroblastoma cells but not in HeLa cells. The additional 5′-UTR mutation in SFP8 extends this growth defect to HeLa cells, while the mutations in the neurovirulent revertant V957 significantly improve its replication in both cell types.

To identify the viral function that is affected by the mutations in SFP8, viral RNA replication and translation were examined. To study viral RNA replication, total cytoplasmic RNA was extracted from cells at different times after infection, and viral RNA was quantitated by a slot blot assay with an antisense VP2 RNA probe. The results indicate that RNA synthesis in SFP8-infected HeLa cells was initially delayed but ultimately reached 70% of the levels observed with the wild-type strain, PRV6.1 (Fig. 3A). RNA synthesis of the neurovirulent revertant V957 was also delayed at early time points, although to a lesser extent than that of SFP8, but reached final levels that are similar to that of PRV6.1. As reported previously, the RNA syntheses of PRV7.3 and PRV6.1 in HeLa cells were similar (18) (Fig. 3A). In SH-SY5Y cells, RNA syntheses of both SFP8 and PRV7.3 were delayed and reached 50% of the final levels observed with PRV6.1. SFP8 RNA production resembled that of PRV7.3, while the pattern of RNA synthesis in the revertant V957 resembled that of PRV6.1 (Fig. 3B).

Translation of a reporter gene under the control of wild-type and mutant 5′-UTRs. Although the levels of PRV6.1 and SFP8 positive-strand RNAs were similar at 2 h postinfection (Fig. 4A), levels of PRV6.1 viral proteins in infected cells were much higher than those of SFP8 viral proteins (10). Furthermore, the results of shift-up experiments, in which HeLa cells were infected at 32°C and shifted to 38.5°C at different times postinfection, indicated that the defect in SFP8 occurs at approximately 2 h postinfection (10). These findings, together with the results of our studies on viral RNA synthesis, suggested that SFP8 contains a primary defect in translation that results in reduced RNA replication. To analyze the effect of attenuating mutations on translation in the absence of viral RNA replication, the expression of a reporter gene under the control of the different 5′-UTRs was determined. The 5′-UTRs of different viruses were placed upstream of the CAT gene, and HeLa and SH-SY5Y cells were cotransformed with the recombinant plasmids and with a plasmid containing β-galactosidase cDNA as a control for variation in transformation efficiency (Fig. 4A). At 48 h posttransfection, CAT and β-galactosidase activities were determined. In HeLa cells, the presence of a 5′-UTR from SFP8 reduced CAT activity by 70% compared with that of 5′-UTRs from PRV6.1 or PRV7.3 (Fig. 4B). DNAs containing the 5′-UTRs from PRV6.1 and PRV7.3 led to similar levels of CAT activity in HeLa cells, as expected, because the virus PRV7.3 has no translation defect in HeLa cells (18). In SH-SY5Y cells, the presence of the 5′-UTRs from PRV7.3 and SFP8 reduced CAT activity by 85 and 90%, respectively, compared with that for the 5′-UTR from PRV6.1 (Fig. 4C). A deletion in the poliovirus IRES (nt 489 to 630) completely abolished CAT activity in both cell lines (Fig. 4C). These results indicate that the mutations C472U and G482A present in the SFP8 5′-UTR are responsible for the reduced translation of the CAT gene in HeLa and SH-SY5Y cells.

UV-induced cross-linking of wild-type and mutant 5′-UTRs. The mutations in SFP8 are located in domain V, a hairpin structure that is believed to interact with cell proteins to facilitate internal initiation of translation (reviewed in reference 34). The translational defect associated with the 5′-UTR of SFP8 could therefore be caused, in part, by disruption of RNA-protein interactions. To address this possibility, UV-cross-linking assays were performed to examine the cell proteins that interact with nt 275 to 628 of wild-type and mutant 5′-UTRs. This region contains the major element that confers cap-independent translation to poliovirus mRNA (nt 320 to 620) (27) and to a reporter gene (27, 28).

To determine the effects of 5′-UTR mutations on RNA-protein interactions, UV-cross-linking assays were done with RNAs of nt 275 to 628 from PRV6.1, PRV7.3, SFP8, and V957 by using RSW from HeLa and SH-SY5Y cells. HeLa and SH-SY5Y cell proteins with molecular masses ranging from 28 to 115 kDa were cross-linked to PRV6.1 RNA (Fig. 5A and B, respectively). Competition studies revealed that binding of the 97-, 70-, 57-, 52-, and 42-kDa proteins was strongly reduced by the inclusion of a 50× molar excess of unlabeled homologous competitor but not by a 50× molar excess of heterologous

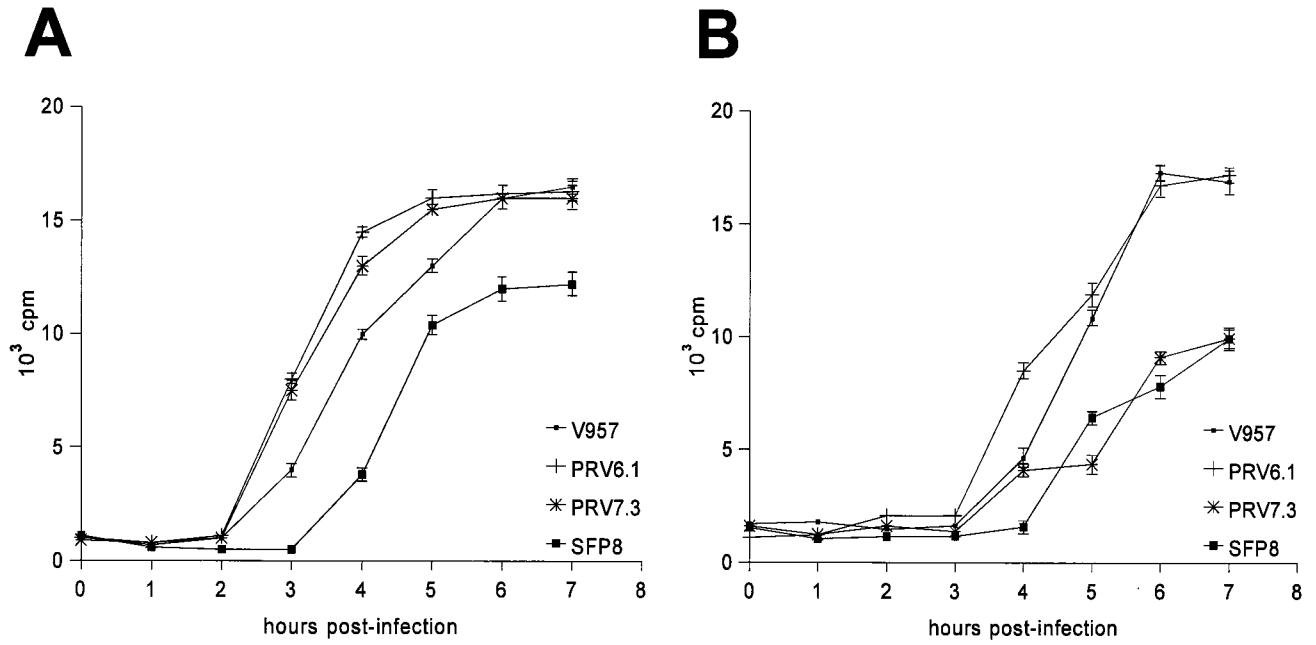

FIG. 2. One-step growth curve analysis of poliovirus strains in HeLa (A) and SH-SY5Y (B) cells. Cells were infected at an MOI of 10 and incubated at 38.5°C when HeLa cells were used and at 37°C when SH-SY5Y cells were used. The total virus titer was determined by plaque assay on monolayers of HeLa cells. Each point represents the mean (± standard deviation) of virus titers in three different experiments.

unlabeled competitor (Bluescript nt 643 to 977), suggesting that the interaction of these proteins with PRV6.1 RNA is specific (10).

A reduction in the cross-linking of the 57-kDa protein from HeLa RSW to SFP8 RNA was observed (Fig. 5A, lane 3) compared with that to PRV6.1, PRV7.3, and V957 RNAs (Fig. 5A, lanes 1, 2, and 4, respectively). When SH-SY5Y RSW was used, reduced cross-linking of a 57-kDa protein to both

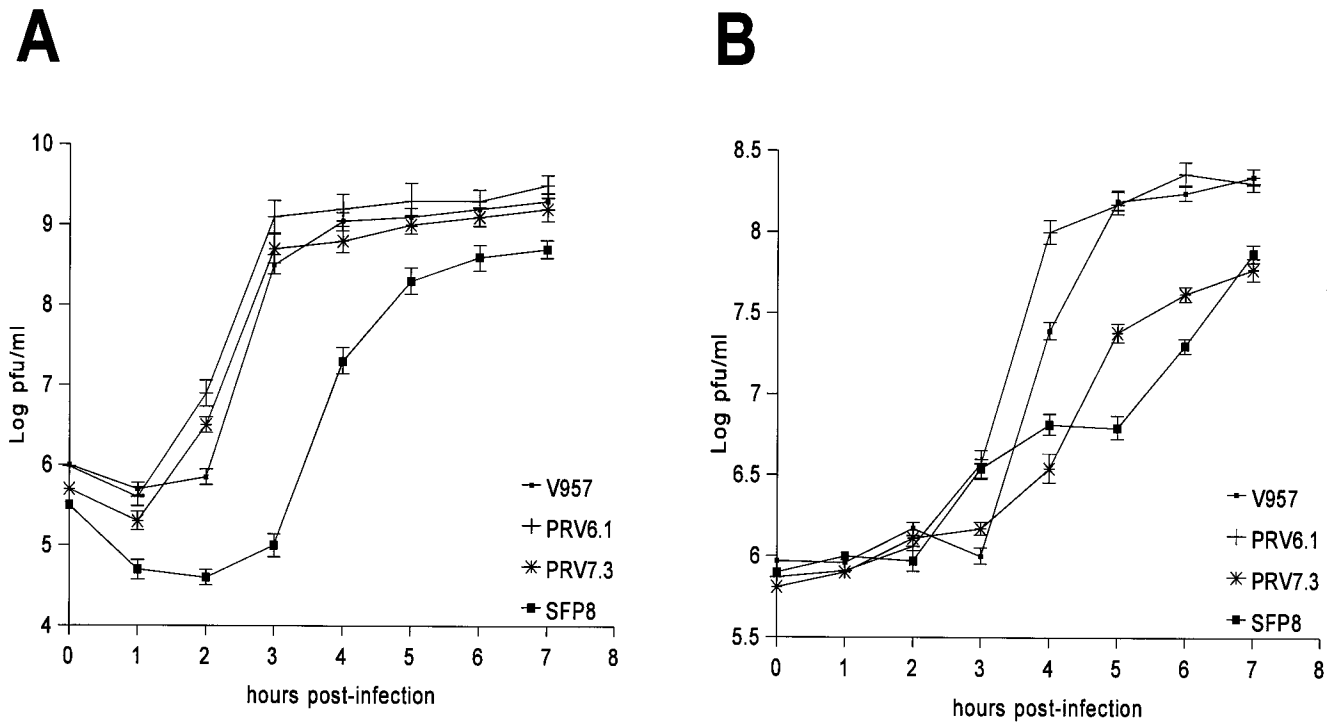

FIG. 3. Positive-strand viral RNA synthesis. HeLa (A) and SH-SY5Y (B) cells were infected with poliovirus strains at an MOI of 10 and incubated at 38.5°C when HeLa cells were used and at 37°C when SH-SY5Y cells were used. RNA levels were determined by slot blot analysis of total infected-cell RNA with a ^{32}P-labeled negative-strand VP2 RNA probe. Each point represents the mean (± standard deviation) of the amounts of positive-strand viral RNA in triplicate experiments.

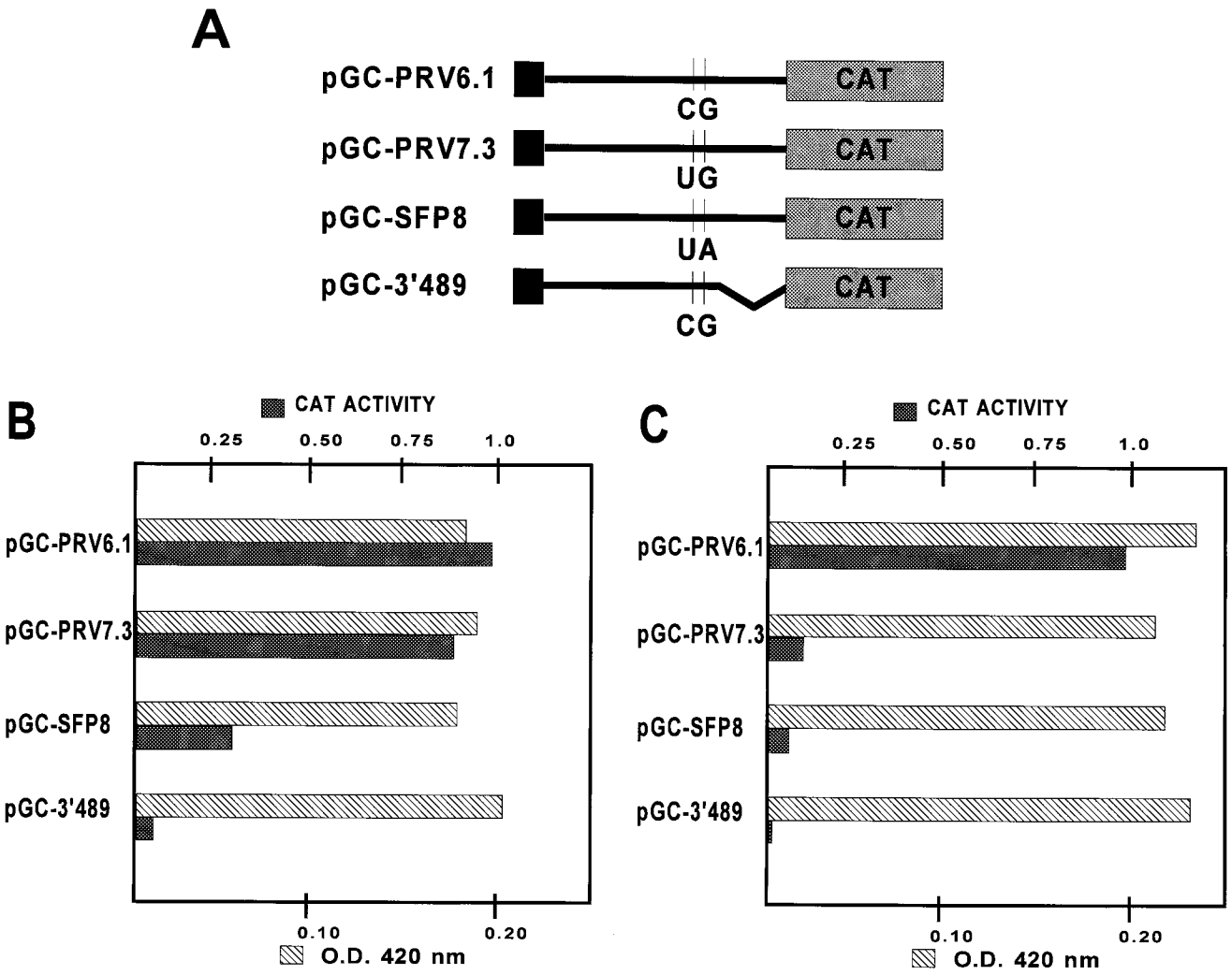

FIG. 4. CAT activity in cells transfected with 5′-UTR-CAT cDNAs. The 5′-UTRs of PRV6.1, PRV7.3, SFP8, and a deletion mutant of PRV6.1 lacking nt 489 to 629 were placed upstream of the CAT gene, under control of the SV40 promoter. (A) Schematic representation of the DNA inserts. Black box, SV40 early promoter; line, 5′-UTR with the bases at nt 472 and 482 indicated; stippled box, CAT gene. (B and C) HeLa cells (B) or SH-SY5Y cells (C) were cotransfected with recombinant plasmids and a β-galactosidase control plasmid. Forty-eight hours after transfection, cells were collected and β-galactosidase levels (optical density [O.D.] at 420 nm) and CAT activities in the cytoplasmic extract were determined. β-Galactosidase activity was used as a control for transformation efficiency, which did not vary significantly within and among cell lines.

PRV7.3 and SFP8 RNAs was observed (Fig. 5B, lanes 2 and 3, respectively) compared with PRV6.1 and V957 RNAs (Fig. 5B, lanes 1 and 4, respectively). Similar results were obtained when S10 extracts from HeLa and SH-SY5Y cells were used in the cross-linking reactions (10). The levels of the 52-kDa protein cross-linked to each RNA probe were similar when RSW (Fig. 5A) or S10 extracts (10) were used, although the amount of p52 in S10 was significantly higher than that in RSW, as previously reported (21).

The molecular masses of the 52- and 57-kDa proteins suggested that they might be identical to La and pPTB, respectively, which have been shown to interact with the poliovirus 5′-UTR (12, 20). To identify these polypeptides, a Western blot analysis of UV-cross-linked complexes from S10 extracts was performed, using monoclonal antibodies against La and pPTB (Fig. 6). Monoclonal anti-La antibodies detected a 52-kDa protein (Fig. 6, lanes 1) which comigrates with the 52-kDa protein cross-linked to PRV6.1 RNA (Fig. 6, lanes 2 and 3) in HeLa cells (Fig. 6A) and SH-SY5Y cells (Fig. 6B). Monoclonal anti-pPTB antibodies react with a doublet of 57 to 60 kDa (Fig. 6, lanes 4) which comigrates with the 57- to 60-kDa protein cross-linked to PRV6.1 RNA (Fig. 6, lanes 2 and 3) in HeLa cells (Fig. 6A) and SH-SY5Y cells (Fig. 6B). When UV-cross-linking was followed by immunoprecipitation with anti-La antibodies, a labeled 52-kDa protein was detected in HeLa and SH-SY5Y cells (10). The monoclonal anti-pPTB antibodies used were unable to immunoprecipitate any protein from cell extracts.

DISCUSSION

The attenuation phenotype of the poliovirus vaccines isolated by Albert Sabin is a result of mutations in the coding and noncoding regions of the viral RNA (reviewed in reference 23). Major attenuation determinants in the 5′-UTR are located at bases 480, 481, and 472 for poliovirus types 1, 2, and 3, respectively (16, 24, 40), within a hairpin structure known as domain V. These mutations are believed to influence the trans-

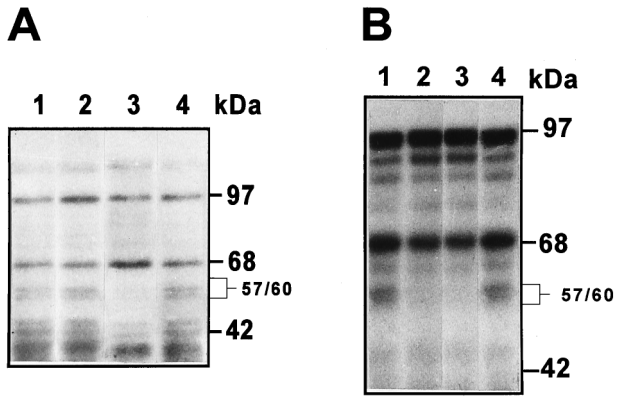

FIG. 5. UV-induced cross-linking of HeLa and SH-SY5Y RSW to ^{32}P-labeled RNA from the 5′-UTRs of mutant strains. ^{32}P-labeled RNAs consisting of nt 275 to 628 from PRV6.1 (lanes 1), PRV7.3 (lanes 2), SFP8 (lanes 3), and V957 (lanes 4) were UV-cross-linked with 30 μg of HeLa (A) and SH-SY5Y (B) RSW. Proteins were separated by SDS–12.5% polyacrylamide gel electrophoresis and detected by autoradiography. Positions of molecular mass markers are shown. The doublet of the 57/60-kDa protein is indicated.

lational efficiency of viral RNA, perhaps by disrupting the RNA secondary structure in this region and reducing the interaction with one or more *trans*-acting factors which facilitate ribosome binding (reviewed in reference 41).

It was previously shown that the mutation C472U in P3/Sabin, which attenuates viral neurovirulence in primates (40) and in mice, reduces viral replication in the CNS of mice (17) and appears to affect viral translation in neuroblastoma cells but not in HeLa cells (18). C472U is therefore a host range mutation. Introduction of both C472U and G482A into the 5′-UTR in SFP8 further attenuates neurovirulence in mice, perhaps by further disrupting domain V (33). A neurovirulent revertant of SFP8, selected in the spinal cord of an infected mouse, contains a reversion at base 472 (U472C), is unchanged at base 482 (G482A), and has a second mutation at base 529 (C529A) that restores base pairing disrupted by the original mutation at base 482 (33). Here we show that the double mutant has a replication defect in both SH-SY5Y and HeLa cells, in contrast to the single mutant, carrying C472U, which replicates poorly only in neuroblastoma cells (18). The neurovirulent revertant V957 has replication kinetics similar to those of PRV6.1 in both cell types. The analyses of SFP8 RNA and protein synthesis in infected HeLa cells are consistent with a defect in translation. When the 5′-UTRs of these viruses were linked to a reporter gene and expressed in cells, the results showed that the 5′-UTR of SFP8 reduced translation both in HeLa cells and in neuroblastoma cells, while the 5′-UTR of PRV7.3 reduced translation only in neuroblastoma cells, as expected. Thus, the single mutation C472U impairs translation only in neuroblastoma cells, while C472U plus G482A impairs translation in neuroblastoma cells and in HeLa cells.

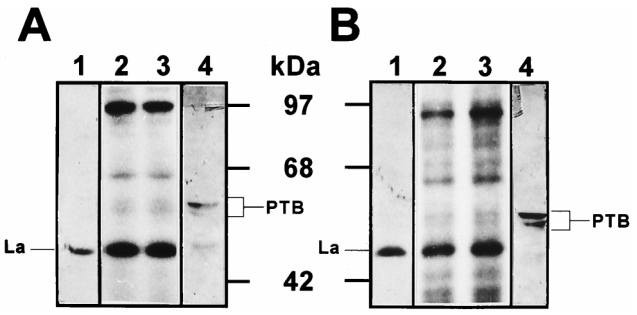

FIG. 6. Western blot analysis of UV-cross-linked proteins. ^{32}P-labeled RNAs consisting of nt 275 to 628 from PRV6.1 were UV-cross-linked with 60 μg of S10 from HeLa (A) and SH-SY5Y (B) cells, transferred to a nitrocellulose membrane, and incubated with anti-La (lanes 1) or anti-pPTB (lanes 4) antiserum. Lanes 2 and 3 show the results of UV-cross-linking only, so that the positions of cross-linked proteins can be compared with those of the proteins identified in the Western blot analysis. Positions of molecular mass markers are shown. The cross-linked doublet of 57/60 kDa which comigrates with pPTB and the 52-kDa cross-linked protein which comigrates with the La protein are indicated.

Cross-linking assays were performed to determine whether the mutations at nt 472 and 482 modify the interaction of the 5′-UTR with cellular proteins. In extracts of neuroblastoma cells, RNA probes containing C472U cross-linked poorly to pPTB, while in HeLa cells, the presence of C or U at this position did not influence cross-linking to pPTB. SFP8 RNA cross-linked poorly to pPTB in both HeLa and neuroblastoma cells, while RNAs of PRV6.1 and the neurovirulent revertant V957 cross-linked to pPTB at similar levels. No differences were observed in the cross-linking of La from either cell type with RNAs from the different mutants. The poor translational efficiency of the SFP8 5′-UTR in both cell types and of the PRV7.3 5′-UTR in neuroblastoma cells therefore correlates with reduced pPTB binding.

How might mutations at nt 472 and 482 interfere with binding of pPTB? It has been reported that pPTB directly binds to three sites in the poliovirus 5′-UTR, including nt 443 to 539 (12, 13). Nucleotides 472 and 482 are located in putatively base-paired regions of this pPTB binding site (Fig. 1). By disrupting base pairing, these mutations might change the overall structure of the hairpin and therefore influence its ability to bind pPTB. This hypothesis is consistent with the notion that pPTB recognizes RNA structural elements rather than specific sequences (42). In support of this hypothesis, revertant V957, which contains mutations at nt 529 and 482 that restore the base pairing disrupted by the original mutations, is no longer defective in binding pPTB in HeLa or neuroblastoma cells.

In a previous study, no differences in the cross-linking of nt 443 to 567 to pPTB were found when sequences from P1/Mahoney and P1/Sabin were compared (13). Possible explanations for the differences with our findings are that we used a longer RNA probe (nt 275 to 628), which might more closely reflect the substrate for pPTB binding present in viral RNA, and that we used 5′-UTRs from type 3 poliovirus strains.

The results presented here suggest that reduced pPTB cross-linking might be in part responsible for the reduced translational efficiency of the SFP8 5′-UTR. Although the role of pPTB in poliovirus translation is not known, it is clearly not a universal internal initiation factor that is indispensable for IRES function (15). While it appears to be essential for the function of the poliovirus and EMCV IRESs (12, 15), it is not needed for the function of the IRES of Theiler's murine encephalomyelitis virus or hepatitis C virus (15). It has been suggested that pPTB is likely to play a role in maintaining the correct folding of the IRES, and this requirement may vary among different IRESs.

In a recent study, it was suggested that the interaction of pPTB with domains V and VI of the poliovirus 5′-UTR is not required for IRES function and viral replication in cell culture, based on studies of a virus containing several base changes in the polypyrimidine stretch between domains V and VI (11). Although the mutant virus replicates normally in HeLa cells, an RNA probe consisting of domains V and VI from this mutant did not interact with purified pPTB. It is possible that domains V and VI from the mutant virus can bind pPTB only

when it is part of a larger and perhaps functional complex (1). Alternatively, in light of the proposed role of pPTB in folding of the IRES, it is possible that the mutations in the 5′-UTR permit it to fold correctly in the absence of pPTB. However, those authors (11) were not able to cross-link pPTB to either mutant or wild-type RNA in extracts prepared from HeLa cells.

It is not clear how the single mutation C472U selectively impairs pPTB binding in neuroblastoma cells but not in HeLa cells. The results of Western blot assays indicate that levels of pPTB in the two cell lines are similar. pPTB in neuroblastoma cells may have a lower intrinsic affinity for the 5′-UTR than pPTB in HeLa cells, and therefore its interaction with the 5′-UTR might be more sensitive to the mutation at nt 472. The lower affinity of pPTB might be due to tissue-specific differences in amino acid sequence and/or posttranslational modifications. Alternative splicing of pPTB mRNA produces three related proteins which may differ in their affinities for the 5′-UTR (7). The affinity of pPTB may also be regulated by other proteins which bind directly to the RNA or which participate in the interaction of pPTB with the 5′-UTR. Levels of these proteins may differ in HeLa and neuroblastoma cells. Two mutations at nt 472 and 482 reduce the interaction of the 5′-UTR with pPTB in both HeLa and neuroblastoma cells. Presumably, the defect imposed by two mutations cannot be overcome by cell-type-specific differences in pPTB or in other required factors.

The results presented here suggest that the attenuation determinant at nt 472 might disrupt the interaction of the poliovirus 5′-UTR with pPTB in the CNS, reducing viral translation, replication, and neurovirulence. Additional work is required to test this hypothesis. For example, it will be necessary to determine the effect of exogenous pPTB on the translation of RNAs carrying the C472U mutation in extracts prepared from neuroblastoma cells. It is also possible that the 5′-UTR mutations studied here impair the interactions of other proteins with the viral RNA, which were not detected by the UV cross-linking. A complete understanding of the attenuation caused by mutations in the 5′-UTR will require analysis of all *trans*-acting factors involved in internal initiation. These studies may also provide information about the basis of the restricted tissue tropism of poliovirus.

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