The Deletion of 41 Proximal Nucleotides Reverts a Poliovirus Mutant Containing a Temperature-Sensitive Lesion in the 5' Noncoding Region of Genomic RNA

SANDRA L. DILDINE AND BERT L. SEMLER*

Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92717

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We generated a number of small deletions and insertions in the 5' noncoding region of an infectious cDNA copy of the poliovirus RNA genome. Transfection of these mutated cDNAs into COS-1 cells produced the following phenotypic categories: (i) wild-type mutations, (ii) lethal mutations, (iii) mutations exhibiting slow growth or low-titer properties, and (iv) temperature-sensitive (*ts*) mutations. The deletion of nucleotides 221 to 224 produced a *ts* virus, 220D1. Mutant 220D1 was found to have a dramatic reduction in growth, virus-specific protein and RNA synthesis, and the shutoff of host cell protein synthesis at 37 or 39°C compared with 33°C. Temperature shift experiments showed that the mutant viral RNA is not an effective template for protein or RNA synthesis at 39°C and suggested a decreased stability of the 220D1 RNA at 39°C. Selection for a non-*ts* revertant of 220D1 yielded the virus R2, which was no longer *ts* for growth or viral protein and RNA synthesis. Sequencing the 5' noncoding region of the genomic RNA from R2 revealed the deletion of 41 proximal nucleotides for an overall deletion of nucleotides 184 to 228. These data suggest that the deleted sequences are nonessential to the poliovirus life cycle during growth in HeLa cells. According to computer-predicted RNA secondary structures of the 5' noncoding region of poliovirus RNA, the R2 revertant virus has deleted an entire predicted stem-loop structure.

One of the unique properties of poliovirus and the picornavirus family in general is the presence of an unusually long 5' noncoding region (NCR) (from 600 to 1,200 nucleotides in length) preceding the single long open reading frame in viral RNAs (1, 4, 6, 11, 19, 24, 25, 28, 32, 39, 40, 42). Although the precise functions or functional domains have not been determined, ~740 nucleotides composing the 5' NCRs of RNAs from all the poliovirus serotypes share some interesting features and sequence similarities which support a role for the functional significance of this region in poliovirus replication. Greater than 90% nucleotide sequence homology occurs between nucleotides 1 and 650, followed by a highly variable region showing very little nucleotide sequence homology through the remainder of the 5' noncoding sequences (42). Another interesting feature of the 5' NCR of polioviruses is the presence of six to eight AUG codons upstream from the AUG codon that is used for initiation of translation of the viral polypeptide. The position of three of these upstream AUGs is conserved among the three poliovirus serotypes, although the lengths of their corresponding open reading frames vary. The upstream AUGs are not believed to be utilized for initiation of translation since no proteins have been identified from this region in vivo or in vitro (10) and they lack the preferred context consensus sequence displayed by the initiator AUG codon (20). The functional significance of these upstream AUGs is not known. The viral RNA must serve as a template for both protein and RNA synthesis as well as possibly encoding signals for uncoating virions and/or packaging of viral RNA. The high degree of sequence conservation of the 5' NCR and the multiple functions the viral RNA must serve suggest an important functional-regulatory role for this portion of the viral genome in the poliovirus life cycle.

The availability of infectious cDNA clones of poliovirus RNA has allowed for alteration of the genome by specific genetic manipulation (27, 31, 33, 37). A number of investigators have taken advantage of this technology to begin to probe the functions of the 5' NCR. It has been shown that the 5' NCR is not essential for translation in vitro (26), although it certainly is essential in vivo since deletion of the first 115 nucleotides of the 5' NCR or nucleotides 225 to 629 does not yield infectious virus upon transfection (18, 33). Pelletier et al. (30) have identified *cis*-acting regions (nucleotides 320 to 631) which are required for cap-independent translation in in vitro systems, and Bienkowska-Szewczyk and Ehrenfeld (2) have narrowed this region to nucleotides 567 to 627. Another approach has involved assaying the phenotypes of virus following the generation of short deletions and insertions in the 5' NCR. Racaniello and Meriam (34) have shown important structure-function relationships by isolation of a virus containing a single nucleotide deletion in a proposed 5'-terminal hairpin structure. This mutation was shown to have detrimental effects on viral biosynthetic functions (34). Probable restoration of the stem-loop structure by a second-site mutation was shown to partially restore wild-type functions. Kuge and Nomoto (21) have also detected second-site mutations in the 5' NCR of viral RNA from the Sabin 1 strain which have partially restored wild-type functions to a mutant virus.

Previous work from our laboratory described the generation of defined recombinants between poliovirus type 1 Mahoney [PV1(M)] and coxsackievirus B3 (CB3) in the 5' NCR (18, 38). These recombinant experiments led to the identification of a region around nucleotide 220 in poliovirus RNA that is important for viral functions. A temperature-sensitive (*ts*) phenotype was found in recombinant virus PCV110 (containing CB3 sequences from nucleotides 220 to 627) but not in the recombinants containing progressively

* Corresponding author.

more CB3 sequences toward the 5' end. These later recombinant viruses (PCV202 and PCV305) had growth properties similar to those of wild-type poliovirus. The above results suggested a possible disruption of important structures in nucleotide sequences proximal to nucleotide 220 in the viral RNA. In addition, a pseudo wild-type revertant of PCV110 was generated as a result of a second-site mutation downstream from the poliovirus/coxsackievirus junction at nucleotide 224, providing further evidence for putative secondary structures with important functional roles within the 5' NCR of viral RNAs. These findings led us to investigate the sequences around nucleotide 220 as well as other regions in the intact 5' NCR of the poliovirus genome.

The approach described here involved the introduction of small deletions and insertions into the 5' NCR of a cDNA copy of the entire poliovirus genome. These mutant cDNAs were then assayed for their ability to produce infectious virus upon transfection of COS-1 cells, and any resulting virus was analyzed for defects in various biological functions. The mutations fell into four categories: (i) lethal mutations, (ii) wild-type mutations, (iii) mutations leading to virus with slow growth or low-titer properties, and (iv) *ts* mutations. A *ts* mutant virus (220D1) was analyzed further to determine the primary defect. Temperature shift experiments suggested that increased instability of the viral RNA from mutant 220D1 is responsible for the *ts* phenotype. A non-*ts* revertant of mutant 220D1, R2, was selected by growth at 37°C. RNA sequencing of the 5' NCR of the revertant virus showed the deletion of 41 additional nucleotides, producing a virus missing nucleotides 184 to 228 in its genomic RNA. These results identify a substantial region of the 5' NCR, 184 to 228, which appears to be dispensable for a productive poliovirus infection. Finally, we discuss the significance of such a deletion with respect to computer-predicted RNA secondary structures (35) and the structure-function relationships implicit in our findings.

MATERIALS AND METHODS

Construction of mutant plasmids. The subgenomic plasmid pMV1.8 containing PV1(M) sequences from nucleotides 1 to 1809 in a pBR322 background was the vector for genetic manipulation of poliovirus sequences (38). In general, small deletions and insertions were constructed by digesting with the appropriate restriction enzyme and either chewing back or filling in the overhanging ends. A *Bam*HI restriction site was utilized to construct both the 220 and 670 mutants. Partial *Bam*HI digests of pMV1.8 were carried out to linearize the plasmid. Linearized plasmid was isolated by gel purification on a 1.4% agarose gel. Plasmid DNA was purified from agarose by electroelution. To insert four nucleotides, the linearized plasmid was treated with the Klenow fragment of DNA polymerase I, blunt ends were ligated with T4 DNA ligase, competent *Escherichia coli* cells were transformed, and the resulting transformants were then screened by restriction enzyme mapping for the loss of the *Bam*HI site at either position 220 or position 670. The deletion of four nucleotides was achieved by digestion of linearized plasmid with mung bean nuclease (New England BioLabs, Inc., Beverly, Mass.), which chews back 3' extensions. The newly generated blunt ends were ligated with T4 DNA ligase, competent *E. coli* cells were transformed, and the resulting transformants were screened by restriction enzyme mapping for the loss of the *Bam*HI site at either position 220 or position 670. The four-nucleotide insertion at position 66 was achieved by digestion of pMV1.8 with *Asp*

718 and isolation of linearized plasmid as described above. The resulting 5' overhang was filled in with the Klenow fragment of DNA polymerase I.

The 456 deletions were generated by digestion of pMV1.8 with *Bsm* I. pMV1.8 contains *Bsm* I sites at positions 456 and 1513 of the poliovirus sequences. pMV1.8 was digested to completion, and the two resulting fragments of 1,057 and 2,845 base pairs were isolated by agarose gel electrophoresis and electroelution. Both fragments were treated with either the Klenow fragment of DNA polymerase I or T4 DNA polymerase to chew back the 3' overhanging ends under conditions which favor the 3'-5' exonuclease activity. The fragments were then religated, competent *E. coli* cells were transformed, and the resulting transformants were screened for the loss of the *Bsm* I sites.

In each of the above-described cases, CsCl₂-purified DNA was prepared from the pMV1.8 plasmid containing the introduced mutations. From this DNA, a 1,118-base-pair *Eco*RI-*Aat*II fragment was gel purified. This fragment contains the first 1,118 base pairs of poliovirus cDNA sequences and all the introduced mutations. The fragment was then ligated in each case, in a three-fragment ligation, to *Eco*RI-linearized pES131 (containing the simian virus 40 *ori* sequences) and a 6.4-kilobase *Eco*RI-*Aat*II fragment from pPV16 representing the remainder of the poliovirus genome (37). The correct three-fragment ligation resulted in full-length cDNA copies of the poliovirus genome containing the introduced mutations in a plasmid competent for DNA transfections (37). Each mutation was ultimately verified by sequencing the region containing the introduced mutation in the full-length cDNAs by a modification of the T7 DNA polymerase method (41) for sequencing double-stranded DNA (46).

Cells and virus. Virus for propagation and plaque assays was grown on HeLa cell monolayers. Transfections with plasmids containing full-length poliovirus cDNAs were done on COS-1 cells (13, 37). The wild-type poliovirus stock [designated PV1(M) or PV1_{tsf}] used for experiments reported here was derived from a single plaque isolate produced after transfection of 293 cells with a cDNA clone of PV1(M) RNA as described previously (37). The mutant virus isolates were designated Se1-5NC for Semler laboratory, type 1 (Mahoney), 5' NCR, and followed by a number denoting the genomic location of the mutation, the letter D (for deletion) or F (for insertion), and an additional number denoting the specific virus isolate.

Transfections. Subconfluent monolayers of COS-1 cells were transfected with 0.1 to 5 µg of CsCl₂-purified plasmid DNAs by the calcium phosphate precipitation technique (14) with a 3-min glycerol boost (12, 29). For 60-mm plates, 0.1 to 5.0 µg of mutant plasmid DNA was transfected with sufficient salmon sperm DNA carrier to total 10 µg of DNA per plate. After transfection (4 to 6 h), monolayers were overlaid with either semisolid medium consisting of Dulbecco modified Eagle medium, 6% fetal calf serum, and 0.45% agarose or liquid medium consisting of Dulbecco modified Eagle medium with 10% fetal calf serum.

Virus stocks and plaque assays. Mutant virus stocks were prepared by picking well-isolated plaques under semisolid agar from COS-1 monolayers on day 4 or 5 posttransfection. The plaques were then expanded by serial passages through HeLa cell monolayers. When no plaques were observed in the initial transfection, the transfection was repeated with a liquid rather than with a semisolid overlay. On day 4 or 5 posttransfection, the liquid supernatants were harvested and used to infect HeLa monolayers, which were then overlaid

with semisolid agar. Plaque assays of mutant virus stocks were done in 60-mm plates of HeLa cell monolayers under semisolid medium. Cells were incubated at 33 or 37°C for 2 to 4 days and stained with crystal violet (17).

Sequencing of mutant viral RNA. Mutant viral RNA was prepared by either the method of sucrose gradient purification (8, 16) or extraction of total cytoplasmic RNA from infected cells (5, 9). In the extraction method, confluent HeLa cell monolayers were infected at a multiplicity of infection (MOI) of 10 or 25 and incubated at 33°C for 8 to 12 h (8 h for wild-type-like mutants and 12 h for *ts* mutants). Infected cells were then lysed in TSM-0.2% Nonidet P-40 (0.15 M NaCl, 5 mM MgCl₂, 10 mM Tris hydrochloride [pH 7.6], 0.2% (vol/vol) Nonidet P-40). Cell debris and nuclei were pelleted by centrifugation. The supernatant was equilibrated with an equal volume of RNA extraction buffer (8 M urea, 2% sodium dodecyl sulfate [SDS], 20 mM EDTA, 0.3 M NaCl, 50 mM Tris, pH 8.0) and then extracted once with phenol-chloroform (1:1). The aqueous layer was ethanol precipitated. The RNA pellet was suspended in H₂O, extracted twice with phenol-chloroform, and ethanol precipitated. Approximately 30 µg of total cytoplasmic RNA was used in the sequencing reactions. Sequencing of viral RNA was performed by the annealing of deoxyoligonucleotide primers to viral RNA and then extension with [α -³²P]dATP, deoxynucleotides, dideoxynucleotides, and reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) (7, 15, 36). Deoxyoligonucleotide primers used to sequence viral RNA were designated DB5, DB6, L164, and L392 and are complementary to poliovirus nucleotides 272 to 291, 421 to 440, 633 to 652, and 760 to 779, respectively.

One-step growth curves. The kinetics of viral production were compared for Se1-5NC-220D1 and PV1_{tsf} at 33 and 37°C. HeLa cell monolayers were infected at an MOI of 10. Virus was allowed to adsorb at room temperature for 30 min, inoculum was removed, and monolayers were washed two times with phosphate-buffered saline and then overlaid with medium. Infections were incubated at 33 or 37°C. At various times after infection, medium and cells were harvested together. Samples were frozen and thawed four times to release virus from cells. Virus production was then measured by standard plaque assay at the temperature of the original infection.

Labeling of infected cells with [³⁵S]methionine. HeLa cell monolayers in 60-mm plates (~10⁶ cells) were infected with wild-type or mutant virus isolates at an MOI of 10 or 25 in methionine-free minimal essential medium-8% newborn calf serum. Infected cells were incubated at 33, 37, or 39°C. At specific times after infection (see Fig. 3), cells were pulse-labeled with [³⁵S]methionine (50 µCi per plate; 1 Ci = 37 GBq; Amersham Corp., Arlington Heights, Ill.) for 2 h. At the end of the labeling period, the medium was removed and the cells were washed twice with phosphate-buffered saline (8 mM sodium phosphate, 137 mM NaCl, pH 7.2). The washed cells were scraped from the plates with a rubber policeman and then suspended in Laemmli sample buffer (22). Samples were boiled and electrophoresed on 12.5% polyacrylamide gels containing SDS (22). Protein gels were fluorographed (3) and exposed to Kodak XAR film. Temperature shift experiments were performed as above, except that infections were shifted either up or down between 33 and 39°C at the indicated times (shown in Fig. 4).

RNA extraction and slot-blot analyses. HeLa cell monolayers were infected at an MOI of 10 or 25. At various times after infection, total cytoplasmic RNA was extracted as described above in the sequencing section. Total cytoplas-

mic RNA (3 µg) from each sample was diluted in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and immobilized on nitrocellulose with a vacuum slot-blot apparatus. Nitrocellulose filters were baked at 80°C for 2 h. Filters were prehybridized for 4 h at 50°C in 5× Denhardt solution-6× NET (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris hydrochloride, pH 8.0)-0.1% SDS. Poliovirus-specific deoxyoligonucleotides were labeled with [γ -³²P]dATP with polynucleotide kinase and then purified from unincorporated label over a G-50 Sephadex column. The γ -³²P-labeled oligonucleotide was added to the prehybridization mix at a concentration of 10⁶ cpm/ml and hybridized at 50°C for 18 to 24 h. Filters were washed four times in 6× NET-0.5% SDS for 10 min at room temperature and once at 50°C for 5 min. Hybridized filters were exposed to XAR film.

cDNA synthesis from viral RNA. A cDNA copy of viral RNA from the revertant Se1-5NC-220D1-R2 was synthesized by a modification of the techniques used by Semler et al. (37). HeLa cell monolayers were infected at an MOI of 25 at 37°C. Total cytoplasmic RNA was extracted as described above. Total cytoplasmic RNA (500 µg) was the starting material for cDNA synthesis. The RNA was treated for 15 min at 37°C with 12 U of DNase (Worthington Diagnostics, Freehold, N.J.) to remove any contaminating DNA. After phenol-chloroform extraction and ethanol precipitation, the RNA was used as a template for the synthesis of the first strand of DNA synthesis by reverse transcriptase. Total cytoplasmic RNA (500 µg) and 36 pmol of the L392 20-mer deoxyoligonucleotide primer (complementary to nucleotides 760 to 779) were incubated in reverse transcription buffer (37), 0.5 mM of each deoxynucleoside triphosphate, 120 units of RNasin (Promega Biotec, Madison, Wis.), and 270 U of avian myeloblastosis virus reverse transcriptase (Life Sciences). Synthesis was allowed to proceed for 2 h at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. Residual RNA in the reaction mixture was degraded by incubation in 20 mM EDTA-0.1 N NaOH at 60°C for 1 h. The reaction was then adjusted to 50 mM Tris hydrochloride (pH 8) and neutralized by the addition of HCl. The remaining free nucleotides were removed from the reaction by two ethanol precipitations in the presence of ammonium acetate. Second-strand synthesis was then performed with the Klenow fragment of DNA polymerase I. The reaction was done in 0.1 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 6.9)-5 mM MgCl₂-5 mM dithiothreitol-0.2 mM each deoxynucleoside triphosphate-30 units of Klenow fragment (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Second-strand synthesis was allowed to proceed for 90 min at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. The resulting cDNA fragment, presumably from nucleotides 1 to 779, was then digested (separately) with an excess of *Kpn*I and *Bam*HI restriction enzymes. The *Kpn*I-*Bam*HI cDNA fragment was then ligated into a poliovirus cDNA subclone, pMV1.8, which had the *Kpn*I-to-*Bam*HI insert removed. Competent *E. coli* cells were then transformed, and the resulting transformants were initially screened for the loss of the *Bam*HI site at position 220 and the presence of a *Kpn*I site at position 66 and a *Bam*HI site at position 670. Rapid DNA from these transformants was then sequenced by a modification of the T7 DNA polymerase protocol (41, 46) to verify the presence of the 45-nucleotide deletion in the cDNA. Sequencing also confirmed the remainder of the cDNA fragment sequences from nucleotides 71 to 670 to be wild-type sequences (with the exception of the expected deletion). The cDNA-containing sequences

TABLE 1. Description of lesions generated in poliovirus cDNAs

cDNA mutation	Virus	<i>ts</i> at 37°C ^a	Plaque size	Sequence verified		P2 stock (PFU/ml) (33°C/37°C)
				DNA (nucleotides)	RNA (nucleotides)	
220D	220D1	+++	Very small ^b	-4	-221 to 224	$2.8 \times 10^8/1.0 \times 10^5$
	220D2	+	Small	-4		$6.6 \times 10^7/3.0 \times 10^6$
220F ^c	220F1	- ^d	Wild type	+4	220-GAUC-221	$3.0 \times 10^7/4.8 \times 10^7$
670D	670D2	-	Wild type	-4	-671 to 674	$3.0 \times 10^8/7.4 \times 10^8$
670F	670F1	-	Wild type	+4	670-GAUC-671	$4.0 \times 10^7/7.3 \times 10^7$
456D13	None			-13 (450 to 462) ^e		
456D2	None			-2 (461 to 462)		
66F	None			+4 (66-GTAC-67)		

^a +++, Titer 1,000-fold higher at 33°C than at 37°C; +, titer 1 log higher at 33°C than at 37°C.

^b Requires 4-day incubation.

^c P1 stock titer <10-fold higher at 33°C than at 37°C.

^d -, P2 stock, not *ts*.

^e Or -448 to 460 or -452 to 464. Exact 13-nucleotide deletion cannot be determined.

were cloned into a full-length poliovirus cDNA as described above in the construction of mutant plasmids.

RESULTS

Transfection of COS-1 cells with mutant cDNAs and recovery of virus. The lesions that we generated in poliovirus cDNAs by in vitro genetic manipulation are shown in Table 1. As described in Materials and Methods, the mutations consist of small deletions and insertions at specific restriction endonuclease cleavage sites within a cDNA corresponding to the 5' NCR of poliovirus RNA (Fig. 1). To test whether the mutant cDNAs would produce infectious virus, we carried out DNA transfections of COS-1 cells. Transfected cell cultures were incubated under either liquid medium or agar overlay for 5 to 6 days at either 33 or 37°C. If plaques were observed, these were picked individually and virus stocks were grown on HeLa cell monolayers at the temperature corresponding to the original transfection. If no plaques were observed, the cell culture medium was then harvested and analyzed for virus growth on HeLa cells by standard plaque assay. Any resulting plaques were individually picked, and virus stocks were grown on HeLa cells at the temperature corresponding to the original transfection. If no plaques resulted from assaying transfection culture medium, the mutation was defined as being lethal to the production of infectious virus.

The results of the transfection experiments with the mutant cDNAs are shown in Table 1. Transfection with 66F and 456D2 or 456D13 did not result in recovery of infectious virus even upon assay of transfection cell culture medium. It should be noted that a recent report presents data from RNA transfection experiments which demonstrate that the lesion in 66F can be recovered in mutant virus (43). Our inability to detect such a mutant virus may be due to the differences in sensitivity of DNA versus RNA transfection (45). Transfection with full-length cDNAs bearing either the 670D or the 670F mutation produced plaques at both 33 and 37°C. Individual plaques were isolated from the 33°C transfections

and used to generate virus stocks designated Se1-5NC-670D2 (670D2) and Se1-5NC-670F1 (670F1). First passage (P1) and second passage (P2) stocks were generated at 33°C. Plaque assays of 670D2 and 670F1 viral stocks revealed that both the P1 and P2 stocks grew to high titers at 33 and 37°C (Table 1). Further analysis of the P2 virus stocks showed that both the 670D2 and 670F1 mutant viruses were similar to PV1_{tsf} (a transfection-derived wild-type stock) in virus-specific protein and RNA synthesis and shutoff of host cell translation (data not shown).

Transfection with the 220F mutant cDNA produced viable virus at 33°C. A virus stock was generated from a single plaque, designated Se1-5NC-220F1 (220F1). The titer of the P1 stock of this virus was reduced by less than 10-fold when grown at 37°C compared with the titer obtained after growth at 33°C. Plaques appeared 2 days after infection with 220F1 (similar to the time of appearance for wild-type virus) and were heterogeneous in size. Upon passage to the P2 stock, 220F1 lost its slight *ts* phenotype, although it still exhibited a mixed plaque size phenotype. Further analysis of 220F1 revealed nearly wild-type properties for virus-specific protein and RNA synthesis and host cell shutoff (data not shown).

Mutant viruses with the most phenotypically distinct properties (compared with wild-type poliovirus) were obtained after transfection of COS cells with 220D cDNA. Since virus plaques could not be detected at 33 or 37°C following agar overlay of transfected cells, supernatants from similarly transfected cultures under liquid overlay were assayed for plaque production on fresh HeLa cell monolayers. Two individual plaque isolates (obtained after growth at 33°C) were obtained and used to generate virus stocks, designated Se1-5NC-220D1 (220D1) and Se1-5NC-220D2 (220D2). The P1 and P2 stocks of mutant 220D1 grew to titers that were 3 logs per milliliter lower at 37°C than at 33°C (Table 1). In addition, at both temperatures, the plaques were minute and required 3 to 4 days of incubation to appear compared with the normal 2-day incubation period for PV1. Upon further passage, the 220D1 mutant virus partially reverted in its growth properties to produce a P4 stock that was only slightly *ts* but still generated small plaques requiring 3 to 4 days of incubation to appear. The titers of virus generated from P1 and P2 stocks of the 220D2 isolate were reduced by 10-fold when assayed at 37°C compared with growth at 33°C. In addition, 220D2 produced plaques of heterogeneous size at both 33 and 37°C.

FIG. 1. Mutations in a cDNA corresponding to the 5' NCR of poliovirus RNA. Restriction sites are shown. nt, Nucleotides.

FIG. 2. One-step growth curve of mutant virus Se1-5NC-220D1 and PV1_{tsf} at 33 and 37°C. HeLa cell monolayers were infected at an MOI of 10 with either 220D1 or PV1_{tsf} virus stocks. Infections were then incubated at either 33 or 37°C. Infected cells and supernatants were harvested at 3, 4, 6, 8, and 24 h postinfection. Samples were frozen and thawed three times to liberate virus particles. Each sample was assayed for virus production by standard plaque assay at their respective temperatures. Symbols: □, PV1_{tsf} at 37°C; ■, PV1_{tsf} at 33°C; ○, 220D1 at 37°C; ●, 220D1 at 33°C.

Virus mutant 220D1 was subjected to further biochemical analysis because it displayed the most dramatic differences in growth properties compared with wild-type poliovirus. We first determined the kinetics and yield of virus infection by performing one-step growth experiments at 33 and 37°C (Fig. 2). At 33°C, 220D1 displayed delayed kinetics of viral production. At 33°C, PV1_{tsf} achieved peak levels of virus production by 8 h postinfection, whereas 220D1 virus production was significantly reduced (by ~4 logs per milliliter) at this time point. Mutant 220D1 eventually achieved wild-type levels of virus production by 24 h postinfection. At 37°C, 220D1 also displayed delayed kinetics of viral production but, in contrast, never achieved wild-type levels even by 24 h postinfection, when mutant virus production is 2 to 3 logs per milliliter lower than at 33°C and 3 to 4 logs per milliliter lower than PV1_{tsf} at 37°C. This delayed growth curve correlates well with the 3- to 4-day incubation required for plaque formation. The reduced virus production at 37°C is consistent with the *ts* phenotype of the 220D1 P2 virus stock.

Protein synthesis by Se1-5NC-220D1 at permissive and nonpermissive temperatures. To examine the effect (if any) of the 220D1 mutation on virus-specific protein synthesis and the shutoff of host cell protein synthesis (host cell shutoff), we labeled infected cells with [³⁵S]methionine at 33, 37, and 39°C. The source of viruses was a P2 stock of the 220D1 mutant and a transfection-derived wild-type stock, PV1(M). The results in Fig. 3A demonstrate that at 33°C, 220D1 showed delayed kinetics of viral protein synthesis with onset not occurring until 7 to 9 h postinfection [compared with the normal PV1(M) onset at 5 to 7 h postinfection]. Significant levels of viral proteins were eventually synthesized, and host cell protein synthesis was effectively shutoff. In contrast, at 37°C virtually no virus-specific proteins were detected above the background of host cell protein synthesis. Note that host cell protein synthesis was detectable as late as 15 h postin-

fection. At 39°C, there was also no detectable viral protein synthesis or significant host cell shutoff as late as 15 h postinfection. By comparison, in cells infected with PV1(M) at 37 and 39°C (Fig. 3B), viral proteins were synthesized and host cell shutoff occurred as early as 3 to 5 h postinfection. In summary, the deletion of four nucleotides, 221 to 224, yielded a virus, 220D1, which is slightly impaired in directing viral protein synthesis at the permissive temperature of 33°C and is severely impaired or unable to synthesize viral proteins at the nonpermissive temperatures of 37 or 39°C.

RNA synthesis in cells infected with Se1-5NC-220D1 at permissive and nonpermissive temperatures. We next determined the effects of the lesion in 220D1 on virus-specific RNA synthesis at 33, 37, and 39°C. Since the growth of mutant 220D1 was sensitive to actinomycin D (data not shown), RNA synthesis was measured by extraction of RNA from infected cells and slot-blot analysis as described above. Blots were hybridized with a ³²P-labeled oligonucleotide complementary to poliovirus sequences 6001 to 6020. At 33°C, virus-specific RNA synthesis in mutant 220D1-infected cells occurred with delayed kinetics and did not achieve peak levels until 9 h postinfection (Fig. 4). At 37°C, 220D1 also displayed delayed kinetics of RNA synthesis and did not produce detectable levels of viral RNA until 9 h postinfection. In addition, RNA synthesis levels produced by 220D1 at 37°C are less than 10% of the levels seen at 33°C (based on laser densitometer tracings of autoradiographs) (data not shown). At 39°C, significant levels of viral RNA synthesized by 220D1 were not detected by 12 h postinfection in the experiment shown in Fig. 4 and as late as 24 h postinfection in another experiment (data not shown). In contrast, RNA synthesis levels in cells infected with PV1(M) were significantly higher at 37 and 39°C than at 33°C. Thus, the four-nucleotide deletion at positions 221 to 224 also has a detrimental effect on RNA synthesis directed by mutant virus 220D1.

Effects of temperature shifts on protein synthesis in cells infected with Se1-5NC-220D1. The 220D mutation has deleterious effects on viral growth, viral protein synthesis, host cell shutoff, and viral RNA synthesis at the nonpermissive temperatures of 37 and 39°C. To determine the primary nature of the defect, we performed temperature shift experiments and analyzed the effects on viral protein synthesis. In the first set of experiments, infections were started by incubation of cells at the nonpermissive temperature of 39°C, followed by shifting to 33°C at various times after infection. The rationale for this experiment was to determine whether viral protein synthesis and host cell shutoff could be rescued at the permissive temperature or whether they were irreversibly inhibited by incubation at the nonpermissive temperature. Figure 5A shows the results of this shift-down experiment. Cultures of infected HeLa cells that were incubated at 39°C for 2 h and then shifted down to 33°C produced nearly normal kinetics of viral protein synthesis with onset occurring at 7 to 9 h postinfection (normal for 220D1 at a constant 33°C incubation). In contrast, infections started at 39°C and not shifted down until 3 h postinfection did not produce significant detectable levels of viral proteins or host cell shutoff by 11 h postinfection. Shifting down 4 h after infection by 220D1 also did not result in detectable levels of viral proteins or host cell shutoff by 11 h postinfection. The shift-down experiment was repeated (data not shown) and the time course was extended to 15 h postinfection. The same results were obtained as those seen in the 11-h time course, suggesting that a delay in kinetics of virus production is not the reason for the absence of viral protein

FIG. 3. [^{35}S]methionine labeling of HeLa cells infected with mutant virus Se1-5NC-220D1 and wild-type poliovirus. HeLa cell monolayers were infected at an MOI of 10 and were incubated at 33, 37, or 39°C. Infections were pulse-labeled with [^{35}S]methionine (50 μCi per plate) for the indicated 2-h periods from 3 to 5 h postinfection to 13 to 15 h postinfection. Lanes M, [^{35}S]methionine marker lysate from wild-type poliovirus-infected HeLa cells that had been labeled between 4 and 7 h after infection at 37°C. mock, Samples derived from mock-infected HeLa cells labeled from 5 to 7 h after mock infection at 33 or 39°C. (A) Cells infected with mutant virus Se1-5NC-220D1 at 33, 37, or 39°C. (B) Cells infected with wild-type transfection-derived poliovirus (PV1) at 33, 37, or 39°C. The figures display autoradiographs of SDS-12.5% polyacrylamide gels obtained after electrophoresis of extracts from samples. Viral proteins are shown on the left of each panel.

synthesis following shift down at 3 or 4 h postinfection. This experiment suggests that incubation of cells infected with 220D1 at 39°C for 2 h or less does not severely impair the ability of viral protein synthesis and host cell shutoff to be rescued upon further incubation at 33°C. In contrast, incu-

bation of infected cells at 39°C for 3 h or longer results in irreversible effects such that a productive infection (viral protein synthesis and host cell shutoff) cannot be rescued at the permissive temperature. Our data suggest that the input viral RNA may be unstable at 39°C for longer than 3 h and

FIG. 3.—Continued.

that an overall net degradation of viral RNA at 39°C is irreversibly affecting the ability of 220D1 to be rescued at 33°C.

We then determined the effect of initiating the infections at the permissive temperature (33°C) and then shifting up to the nonpermissive temperature (39°C) at various times after infection. The rationale for this experiment is that starting the infections at 33°C allows for the synthesis of viral mRNA

template and viral proteins, and then shifting the cultures to 39°C will allow us to determine whether that template RNA is functional at 39°C. Shifting the infected cells up to 39°C at 5 or 7 h postinfection did not allow for the onset of viral protein synthesis within its normal time frame of 7 to 9 h postinfection or even up to 13 h postinfection (Fig. 5B). Infected cultures that were shifted up at 9 h postinfection (after viral proteins and RNA have accumulated to detect-

FIG. 4. RNA synthesis of HeLa cells infected with Se1-5NC-220D1 and wild-type poliovirus. HeLa cell monolayers were infected at an MOI of 10 and incubated at 33, 37, or 39°C. At the indicated times (hours) postinfection, total cytoplasmic RNA was prepared from the infected cells and from mock-infected (mock) cells. A 3- μ g portion of each RNA sample was immobilized on nitrocellulose filters with a vacuum slot-blot apparatus. The blot was hybridized to a γ - 32 P-labeled oligonucleotide probe, LV6, complementary to nucleotides 6001 to 6020 of the poliovirus genomic RNA. PV1 denotes a wild-type transfection-derived poliovirus stock.

FIG. 5. Temperature shift effects on protein synthesis of Se1-5NC-220D1-infected HeLa cells. The infections and labeling conditions were as described in the legend to Fig. 3 except for the changes in temperature of the infections. (A) Shift-down experiment in which cells were infected at the nonpermissive temperature (39°C) and at 2, 3, or 4 h postinfection were shifted down to the permissive temperature (33°C). (B) Shift-up experiment in which cells were infected at the permissive temperature (33°C) and then at 5, 7, or 9 h postinfection were shifted up to the nonpermissive temperature (39°C). (C) Extended shift-up experiment in which cells were infected at 33°C and then at 9 or 11 h postinfection were shifted up to 39°C. Lanes 5-7 through 13-15 under the 33°C heading are cells infected with 220D1 and incubated at a constant 33°C. Lanes M, Marker lysates from wild-type poliovirus-infected HeLa cells as described in the legend to Fig. 3. Mock lane sample is derived from mock-infected HeLa cells labeled from 5 to 7 h after mock infection at 39°C. The figures display autoradiographs of SDS-12.5% polyacrylamide gels obtained after electrophoresis of extracts from the samples. Viral proteins are shown to the left of each panel.

able levels) appear to cease the continued synthesis of viral proteins in the remaining time periods through 13 h postinfection. This experiment was repeated, and the production of virus proteins was assayed through 25 h postinfection (Fig. 5C). In the 9-h shift-up samples, no viral proteins were detected in a 15- to 25-h labeling period, suggesting that the cessation of viral protein synthesis at 39°C is not a transient effect. There were readily detectable levels of viral proteins and virtually complete host cell shutoff at the time of the 11-h shift up (Fig. 5C). Upon shift up to 39°C, only cellular proteins were detected in the 15- to 25-h labeling period. The above experiment suggests that although sufficient templates for protein synthesis exist at a given time (9 or 11 h postinfection), the mutant template mRNA is defective in directing the synthesis of viral proteins at 39°C.

Effects of temperature shifts on RNA synthesis of HeLa cells infected with Se1-5NC-220D1. The above temperature shift data suggested that the primary defect in *ts* mutant 220D1 is in the structure or stability of the viral RNA. It was of interest to then analyze the effect (if any) of temperature shift experiments on the accumulation of viral RNA in 220D1-infected cells. In the first set of experiments, 220D1-infected cells were incubated at the nonpermissive temperature of 39°C and then shifted down to the permissive temperature of 33°C at either 2 or 4 h postinfection. Total

cytoplasmic RNA was then extracted at 12 h postinfection in each case, and RNA synthesis was measured by slot-blot analysis as described above. Shifting the incubation temperature to 33°C at 2 h postinfection allowed for detectable levels of viral RNA by 12 h postinfection (Fig. 6). However, these RNA levels were significantly lower than levels seen at the 9- or 12-h time points in a 220D1 infection maintained only at 33°C. RNA synthesis levels detected for the infected cells shifted at 2 h postinfection were nearly five times lower than those seen at 12 h in a constant 33°C infection. In addition, 220D1-infected cells that were incubated only at 33°C for 9 h prior to RNA extraction produced three times as much viral RNA as infected cells that were incubated at 39°C for 2 h and then shifted to 33°C for 10 h prior to RNA extraction (based on laser densitometer tracings of autoradiographs) (data not shown). Such a result suggests a net degradation of input viral RNA at 39°C and not simply a time delay owing to no synthesis occurring until infections are shifted down to 33°C. Shifting the 220D1-infected cells from 39 to 33°C at 4 h postinfection did not allow for detectable levels of viral RNA synthesis by 12 h postinfection. This suggests that the input viral RNA is effectively being degraded or structurally altered at 39°C such that it can no longer serve as template for viral RNA synthesis upon shift down to 33°C.

Equivalent controls were somewhat difficult to carry out for the shift-down experiment because the time course of infection is very different for mutant 220D1 and PV1(M). Cells infected with PV1(M) for more than 6 h are already beginning to lyse and release viral particles into the culture medium. The experiment shown in Fig. 6 only detects viral RNA in intact cells. Therefore, the RNA levels detected for later time points in PV1(M)-infected cells probably do not reflect the total viral RNA synthesized by this time in infection. This explanation probably accounts for only slightly higher levels of viral RNA seen in the 12-h shift samples compared with the samples obtained after a 6-h constant 39°C incubation. In any case, the PV1(M) controls show no difference between infections shifted down at 2 or 4 h postinfection, with equivalent amounts of RNA detected at 12 h postinfection in each experiment (Fig. 6). The data from the RNA synthesis shift-down experiment agree with the protein synthesis shift-down data. Shifting the temperature of incubation of 220D1-infected cells down early on in infection (2 h postinfection) allows for the rescue of a productive viral infection (viral protein and RNA synthesis and host cell shutoff) upon continued incubation at the permissive temperature. However, shifting down to the permissive temperature at 3 or 4 h postinfection does not allow for rescue of a productive infection.

The effects of incubating poliovirus mutant 220D1-infected cells at the permissive temperature and then shifting up to the nonpermissive temperature at various times are also shown in Fig. 6. Mutant 220D1-infected cells were shifted to the nonpermissive temperature at 3 h postinfection and RNA was extracted at 6 h postinfection, or they were shifted up at 9 h postinfection and RNA was extracted at 12 h postinfection. The 3- to 6-h shift-up experiment yielded levels of viral RNA slightly lower than those in the infected culture maintained at 33°C for 6 h. These data suggest that continued viral RNA synthesis is impaired upon shift up to 39°C. In contrast, the PV1(M) control 3- to 6-h shift up showed increased levels of RNA accumulation compared with those observed for wild-type-infected cells maintained for 6 h at 33°C.

The data derived from the 9- to 12-h shift-up samples showed detectable levels of mutant viral RNA. However,

FIG. 5.—Continued.

FIG. 6. Temperature shift effects on RNA synthesis of Se1-5NC-220D1- and wild-type poliovirus-infected HeLa cells. The infections, RNA extractions, and hybridization conditions were as described in the legend to Fig. 4 except for the temperature shifts. Shift-up slots represent viral RNA synthesis levels from cells infected at the permissive temperature (33°C) and then shifted up to the nonpermissive temperature (39°C). 3-6 denotes infections shifted up at 3 h postinfection and then RNA harvested at 6 h postinfection. 9-12 denotes infections shifted up at 9 h postinfection and then RNA harvested at 12 h postinfection. Shift-down slots represent viral RNA synthesis levels from cells infected at the nonpermissive temperature (39°C) and then shifted down to the permissive temperature (33°C). 2-12 denotes infections shifted down at 2 h postinfection and then RNA harvested at 12 h postinfection. 4-12 denotes infections shifted down at 4 h postinfection and then RNA extracted at 12 h postinfection. a, Time points from constant 33°C infections. b, Time points from constant 39°C infections. mock, RNA samples from mock-infected HeLa cells harvested at 6 h postinfection. The blot was hybridized to a γ -³²P-labeled oligonucleotide probe, L391, complementary to nucleotides 253 to 272 of the poliovirus genomic RNA.

the levels were significantly lower than those seen in a constant 33°C infection when cultures were harvested at 9 or 12 h postinfection (Fig. 6). RNA synthesis levels seen in the 9- to 12-h shift-up samples were only ~35 and 54% of the levels seen in the constant 33°C infections harvested at 12 and 9 h postinfection, respectively (based on laser densitometer tracings of autoradiographs) (data not shown). Lower mutant viral RNA levels in the 9- to 12-h shift-up samples than in the 12-h constant 33°C samples suggest that continued net synthesis of viral RNA does not occur (at least at the same rate) upon shift up to 39°C. Lower mutant RNA levels in the 9- to 12-hr shift-up samples than in the 9-h constant 33°C time point suggest a net degradation of viral RNA.

Isolation and characterization of non-*ts* pseudorevertants of Se1-5NC-220D1. To identify possible second-site mutations in the 220D1 viral RNA, we selected revertants of the *ts* phenotype by growth of virus at 37 and 39°C. A P1 stock of mutant 220D1 was used to infect HeLa cell monolayers at an MOI of 1 and then incubated at 33, 37, and 39°C. The resulting virus stocks were assayed for viral production by standard plaque assay at their respective temperatures. Plaques were seen at 33 and 37°C but not at 39°C. Individual plaques were isolated from cultures incubated at 33 and 37°C and plaque purified one additional time at their respective temperatures. P1 and P2 revertant viral stocks were grown up from these two-times-purified plaque isolates. Standard plaque assays of these P1 and P2 stocks revealed that the revertant control stocks (grown at 33°C, called RC1 and RC3) grew to titers that were 2 to 3 logs per milliliter lower at 37°C compared with those obtained at 33°C (Table 2). The

revertant stocks, R2 and R4 (grown at 37°C), were no longer *ts* for growth at 37°C, and both grew to high titers (10^8 to 10^9 PFU/ml) at both 33 and 37°C (Table 2). Plaque sizes of R2 and R4 were considerably larger than those of RC1 and RC3. R2 and R4 plaques were larger at 37°C than at 33°C [as observed for PV1(M)], whereas RC1 and RC3 plaques were small at both temperatures but slightly larger at 33°C than at 37°C (data not shown).

The 37°C revertant, R2, was analyzed for its viral protein synthesis properties by [³⁵S]methionine pulse labeling as described above. HeLa cell monolayers were infected at an MOI of 25 with P2 stocks of the revertant R2, the revertant control RC1, and PV1 at 33 and 37°C (Fig. 7). At 33°C, R2 directed viral protein synthesis with kinetics similar to those of RC1, producing significant levels that were detectable by 7 to 9 h postinfection. Onset of virus-specific protein synthesis in cells infected with R2 at 33°C was slightly delayed compared with that seen in PV1-infected cells, which accumulated significant levels of viral proteins by 5 to 7 h postinfection. At 37°C, R2 synthesized virus-specific polypeptides with kinetics different from those observed for RC1. Low levels of viral proteins were detected in R2-infected cells as early as 3 to 5 h postinfection, with significant levels detectable by 5 to 7 h postinfection (concurrent with effective host cell shutoff). RC1-infected cells at 37°C did synthesize low levels of viral proteins during the 5- to 7-h and 7- to 9-h labeling periods but not to the same levels observed for R2 at 37°C or RC1 at 33°C. In addition, RC1 did not effectively shut off host protein synthesis by 9 h postinfection. Note that the RC1 P2 stock does not have protein

TABLE 2. Characteristics of mutant and revertant viruses

Virus	Origin	<i>ts</i> at 37°C	Plaque size	Sequence verified		P2 stock (PFU/ml) (33°C/37°C)
				DNA (nucleotides)	RNA (nucleotides)	
220D1	Transfection of mutant cDNA	Yes	Very small	—4	—4 (—221 to 224)	$2.8 \times 10^8/1.0 \times 10^5$
RC1	Selected from 220D1 at 33°C	Yes	Very small	—4	—4 (—221 to 224)	$1.5 \times 10^9/5.3 \times 10^6$
R2	Selected from 220D1 at 37°C	No	Wild type	—45	—45 (—184 to 228) ^a	$3.4 \times 10^8/1.1 \times 10^9$
R2 cDNA	Transfection of cDNA synthesized from R2 viral RNA	No	Wild type	—45	—45 (—184 to 228) ^a	$1.2 \times 10^8/8.0 \times 10^8$

^a Or —183 to 227; exact location of 45-nucleotide deletion cannot be determined.

FIG. 7. [^{35}S]methionine labeling of HeLa cells infected with the 37°C revertant virus R2, the revertant control virus RC1, and the wild-type poliovirus PV1. The infections and labeling conditions were as described in the legend to Fig. 3, except that infections were done at an MOI of 25. R2 denotes the 37°C-selected Se1-5NC-220D1-R2 virus stock, RC1 denotes the 33°C-selected Se1-5NC-220D1-RC1 virus stock, and PV1 denotes a transfection-derived wild-type poliovirus stock. Lane M, Marker lysate from wild-type poliovirus-infected HeLa cells as described in the legend to Fig. 3. mock, Samples derived from mock-infected HeLa cells labeled from 5 to 7 h after mock infection at 33 and 37°C. The figure displays an autoradiograph of SDS-12.5% polyacrylamide gels obtained after electrophoresis of extracts from the samples. Viral proteins are shown on the right side of the gel.

synthesis characteristics that are identical to those of the 220D1 P2 stock analyzed above. Such a result is not unexpected since RC1 represents a further passage of the 220D1 P2 stock at 33°C and may have accumulated some genomic RNA changes as it is passaged at 33°C. Differences related to

protein synthesis properties between the RC1 and 220D1 P2 stocks could also be due to the MOI used in the infections, since the RC1 infections were done at an MOI of 25 and the 220D1 infections were done at an MOI of 10. RC1 does differ significantly from revertant R2 in directing viral protein

FIG. 8. RNA synthesis levels of the 37°C revertant virus R2, the revertant control virus RC1, and the wild-type poliovirus PV1(M). The infections, RNA extractions, and hybridization conditions were as described in the legend to Fig. 4. R2 denotes the 37°C-selected Se1-5NC-220D1-R2 virus stock, RC1 denotes the 33°C-selected Se1-5NC-220D1-RC1 virus stock, and PV1(M) denotes a transfection-derived wild-type poliovirus stock. The blot was hybridized to a γ - 32 P-labeled oligonucleotide probe, LV6, complementary to nucleotides 6001 to 6020 of the poliovirus genomic RNA.

synthesis at 37°C. RC1 is still *ts* for protein synthesis at 37°C, but revertant R2, while not exhibiting a completely wild-type protein synthesis phenotype, is certainly no longer *ts* for protein synthesis or host cell shutoff at 37°C.

We then measured the accumulation of RNA in cells infected by revertant R2 and the 33°C revertant control RC1. HeLa cell monolayers were infected at an MOI of 25 with R2, RC1, and PV1 and incubated at 33 or 37°C. Total cytoplasmic RNA was isolated at various times after infection, and RNA synthesis levels were measured by slot-blot analysis as described above. Figure 8 shows that the results we obtained are consistent with results obtained in the protein-labeling experiments. At 33°C, the levels of R2 viral RNA synthesis were similar to those of RC1, and RNA synthesis for both mutants was slightly delayed compared with that of PV1. At 37°C, the levels of viral RNA synthesis in cells infected with revertant R2 were similar (but not identical) to those of wild-type-infected cells, with significant levels detectable at 6 h postinfection.

The original purpose of selecting for *ts* revertants was to identify second-site mutations present in the altered viral RNA which may be responsible for the revertant phenotype and which may tell us something about the functional significance of sequences or structures. Viral RNA was isolated by the RNA extraction protocol described above. The viral RNA was sequenced from both 37°C revertants R2 and R4 and both 33°C revertant controls RC1 and RC3 by dideoxynucleotide sequencing with reverse transcriptase. RC1 and RC3 viral RNAs were both found to contain the introduced four-nucleotide deletion of sequences 221 to 224, and no other changes were detected in the 5' NCR. Both R2 and R4 viral RNAs also contained the introduced four-nucleotide deletions but had also accumulated deletions of 41 additional nucleotides in this same region. Overall, R2 and R4 viral RNAs contained a 45-nucleotide deletion of sequences 184 to 228.

Synthesis of an R2 cDNA and characterization of resulting virus. To rule out the presence of any other second-site mutations (other than the identified 45-nucleotide deletion) elsewhere in the genome contributing to the reversion of the 220D1 phenotype seen in the R2 virus, a cDNA copy of nucleotides 71 to 670 from R2 genomic RNA was synthesized and cloned into a full-length wild-type cDNA copy of PV1(M) (as described in Materials and Methods). The region from positions 71 to 670 of the cDNA clone was sequenced and found to contain only the 45-nucleotide deletion that was

identified in the R2 revertant virus. The R2 cDNA clone [containing only the 45-nucleotide deletion in a wild-type PV1(M) background] was used to transfect COS-1 cells. Transfection supernatants were assayed at 37°C, single plaques were isolated, and from these, virus stocks were grown. P1 and P2 stocks grew to high titers at 37°C and were not *ts* at 37°C (Table 2), similar to the phenotype of the R2 virus.

To compare the biochemical properties of the R2 cDNA virus with those of the original R2 revertant virus selected at 37°C, we performed [35 S]methionine protein-labeling experiments. HeLa cell monolayers were infected at an MOI of 25 with the R2 cDNA virus, the R2 revertant virus, RC1 (33°C R2 control), and a transfection-derived wild-type stock, PV1_{tsf}. Figure 9 shows the results of the R2 cDNA and R2 revertant at 33 and 37°C. In both cases, the presence of significant levels of viral proteins and detectable host cell shutoff at 33°C was not detected until the 7- to 9-h time point. This is slightly delayed from the 5- to 7-h onset in the PV1_{tsf} infection (data not shown). At 37°C, both virus stocks accumulated detectable levels of viral proteins and begin host cell shutoff in the 3- to 5-h time point, similar to PV1_{tsf}. This experiment shows that the original R2 revertant virus and its derivative R2 cDNA virus have virtually identical kinetics of viral protein synthesis and host cell shutoff.

DISCUSSION

The data presented here describe the effects of a number of small deletions and insertions in the 5' NCR of PV1 RNA. The effects of these mutations were initially assayed by the ability of a full-length cDNA copy containing the introduced mutations to produce infectious virus upon transfection of COS-1 cells. The lethal mutations, by definition, did not produce infectious virus upon transfection of COS-1 cells and have therefore not been studied in vivo. These mutations can certainly be studied in vitro to look at their effects on translational efficiency of the mutated RNA. The 670F and 670D mutations resulted in wild-type viruses. These mutations lie within the highly variable region from nucleotide 650 to the beginning of the polyprotein-coding region at nucleotide 743. Kuge and Nomoto (21) have shown that deletion of nucleotides 600 to 726 or insertion of up to 72 nucleotides in this region has no effect on wild-type properties of the Sabin 1 strain. The four-nucleotide insertion,

FIG. 9. [^{35}S]methionine labeling of HeLa cells infected with the 37°C revertant virus R2 and the R2 cDNA virus derived from it. The infections and labeling conditions were the same as described in the legend to Fig. 7. R2 denotes the 37°C-selected Se1-5NC-220D1-R2 virus stock, and R2 cDNA denotes the virus stock derived from transfection of a cDNA containing the R2 45-nucleotide deletion in a wild-type PV1(M) background. Lane M, Marker lysate from wild-type poliovirus-infected HeLa cells as described in the legend to Fig. 3. The figure displays an autoradiograph of SDS-12.5% polyacrylamide gels obtained after electrophoresis of extracts from samples. Viral proteins are shown on the left side of the gel.

220F, resulted in P1 and P2 virus stocks exhibiting slightly slower growth properties than wild-type poliovirus. The P2 stock of 220F1 displayed nearly wild-type properties for viral protein and RNA synthesis. The insertion of four nucleotides in this region does not appear to have gross detrimental effects on viral functions. It should be noted that Kuge and Nomoto (21) have also generated a mutant like 220F1 in the Sabin 1 strain of poliovirus that produces small plaques and yields second-site revertants upon further passage. In addition, Trono et al. (43) have also generated a mutant like 220F1 that appears to be slightly *ts* for virus growth and RNA synthesis.

The most interesting mutation in the 5' NCR of PV1 RNA

proved to be the four-nucleotide deletion of 221 to 224. Two virus stocks were generated from independent plaque isolates, 220D1 and 220D2. Mutant 220D2 grows to 10-fold lower titer at 37°C than at 33°C, while growth of 220D1 is reduced by 3 logs per milliliter at 37°C. Sequencing the 5' NCR of viral RNAs from these two virus stocks revealed only the introduced deletion of nucleotides 221 to 224. The reasons for the phenotypic differences between these two virus stocks have not yet been determined genetically. Further studies on 220D1 revealed numerous detrimental effects on the biological functions of the virus. At 33°C, 220D1 is delayed (compared to PV1_{tsf}) in viral protein synthesis, host cell shutoff, and viral RNA synthesis. At 37

or 39°C, these viral functions are further debilitated or nonexistent in the mutant virus.

To determine the primary defect in the 220D1 mutant virus, we performed temperature shift experiments and assayed separately for the effects on viral protein and viral RNA synthesis. The results of the shift-down experiments suggested that the primary defect is in the stability of the input viral RNA. In these experiments, similar results were obtained when analyzing for the effects on viral protein synthesis or viral RNA synthesis. Infections shifted down by 2 h postinfection could be rescued upon further incubation at 33°C, but infections allowed to proceed for 3 or 4 h at 39°C and then shifted down could not be rescued by further incubation at 33°C. The above results suggest that irreversible effects occurred at 39°C such that a productive infection (viral protein synthesis and viral RNA synthesis) could not be rescued upon further incubation at 33°C. We propose that these irreversible effects are secondary to the net degradation of the input viral RNA at the nonpermissive temperature of 39°C. An additional shift-down experiment was performed in which the effect of incubation at 39°C on virus production was analyzed (data not shown). A one-step growth curve was performed in which all infections were begun at 39°C and then at various times postinfection were shifted down to 33°C, and then cells and medium were harvested together at 12 h postinfection. Measuring virus yield by standard plaque assay revealed a progressive drop in virus yield upon increased incubation at 39°C. An approximate 4 logs per milliliter reduction in virus production occurred as a result of an initial 8-h incubation at 39°C followed by 4 h of incubation at 33°C compared with a 12-h constant incubation at 33°C.

The shift-up experiments are consistent with the shift-down experiments and also suggest a decreased stability of the mutant template RNA. In these experiments, if infections were shifted up before detectable levels of viral proteins or RNA accumulated, the synthesis of viral proteins or RNA was routinely not detected at later time points. If infections were shifted up after detectable levels of viral proteins and RNA were present, the continued synthesis of both viral proteins and RNA was reduced or completely inhibited following shift up. The lack of viral RNA synthesis in cells infected at 39°C is not simply due to the absence of viral proteins at 39°C. The shift-up experiment showed that although sufficient functional template viral RNA and viral proteins were present upon shift up to 39°C, this template RNA was no longer functional since it could not support continued viral protein or RNA synthesis.

The inability of the 220D1 mutant viral RNA to function as a template for viral protein or RNA synthesis at 39°C could be due either to the inability of the mutant RNAs to form the correct secondary structures for ribosome recognition or replication initiation or both or to an increased instability (and subsequent degradation) of the mutant viral RNA. The RNA shift-up experiments suggest the latter possibility as the primary defect, since the 9- to 12-h shift-up sample (Fig. 6) shows that levels of mutant viral RNA are lower than the levels present at the 9-h time point (before the shift). These results suggest an overall net degradation of the mutant viral RNA at 39°C. The net degradation could be due to either an increased rate of degradation at 39°C or a decreased rate of initiation of RNA synthesis with the rate of degradation remaining the same. The observation that there is a greater turnover of the mutant viral RNA (or a reduced synthesis rate) at 39°C at later times (9 to 12 h postinfection) than at earlier times (3 to 6 h postinfection) suggests that different cytoplasmic environments (free polysomes or membranous

replication complexes) affect the overall levels of the altered mutant viral RNA.

We have also performed experiments in which we attempted to rescue the 220D1 mutant with a pseudo wild-type virus, PCV305 (18; data not shown). Recombinant PCV305 has been shown previously to be able to rescue *in trans* RNA synthesis of a mutant virus defective in the synthesis of the 3D viral RNA polymerase (9). Our attempts to rescue 220D1 viral RNA synthesis at 37 or 39°C were unsuccessful (levels of rescue of less than 1% were seen). The inability of the mutant viral RNA to be rescued *in trans* by PCV305 is consistent with instability of the mutant viral RNA, since an addition of functional viral replication proteins should not rescue a mutant virus with a defective template.

The origin of the proposed instability of the 220D1 viral RNA is not known. Does the four-nucleotide deletion lend some general inherent instability to the viral RNA or is it an inability to associate with the translational machinery or replication complexes which then leaves the RNA naked and more susceptible to nucleases present in the cytoplasm? There are a number of ways to address this question. We have initiated experiments to analyze the translational efficiency of this mutant RNA *in vitro*. In addition, a comparison of the ability of 220D1 and PV1 to bind ribosomes or other cellular factors *in vitro* by a band-shift assay may reveal important interactions that are affected by the lesion in 220D1. The question of instability of the mutant viral RNA at the nonpermissive temperature will be more directly addressed by performing RNA pulse-labeling experiments followed by hybridization to filters containing bound poliovirus cDNA and comparing the half-lives of mutant viral RNA at 33 and 39°C. Finally, it will be of interest to probe for the presence of negative-strand RNAs to determine if there are differential effects of the 220D1 lesion on positive-versus negative-strand synthesis and stability.

The results from the poliovirus/coxsackievirus recombinant experiments originally identified the region around nucleotide 220 as having an important functional role in the poliovirus life cycle (18, 38). These experiments showed that a recombinant virus, PCV305, which contained coxsackievirus sequences from 1 to 625 had wild-type properties. The fact that CB3 sequences, which are 30% divergent from the poliovirus sequences they have replaced, could substitute in this region for poliovirus sequences and seemingly provide the functions necessary for viral replication suggests an important functional conservation in the 5' NCR between these viruses. Such a functional conservation may be represented by secondary structures and/or interactions between nonadjacent nucleotides in this region that serve as important regulatory signals and not necessarily by specific nucleotide sequences that provide those signals. However, the poliovirus/coxsackievirus recombinant PCV110, which contains CB3 sequences from 220 to 627, is *ts* for viral functions. That the *ts* phenotype was only found in PCV110 and not in the recombinants containing progressively more CB3 sequences suggests a possible disruption of important structures in the nucleotide 220 junction region. The isolation of a second-site, pseudo wild-type revertant which had deleted nucleotides 231 to 234 was further evidence for structures (or sequences) in this region with important functional roles.

We selected for non-*ts* revertants of 220D1 to identify second-site mutations in the 220D1 viral RNA to gain insight into important structure-function relationships in the 5' NCR of the intact poliovirus genome. The 37°C revertant R2, although it did not exhibit completely wild-type characteristics, was certainly no longer *ts* for growth or viral protein

and RNA synthesis at 37°C. Most interestingly, the viral RNA of R2 had deleted 41 additional nucleotides for an overall deletion of positions 184 to 228. It was surprising to find that such a substantial deletion had occurred in a viable and nearly wild-type virus, since small deletions, even two nucleotides, elsewhere in the 5' NCR produced lethal mutations. The above reversion was also detected in the RNA of another revertant virus stock, R4, grown up from an independent plaque isolate. An independent transfection with the 220D cDNA yielded a virus, 220D3, which, although not as well characterized as 220D1, is similarly *ts* for growth at 37°C based on a standard plaque assay (data not shown). When selecting for 37°C revertants of this virus stock, we identified three slightly different deletions. Three independent plaque isolates were grown at 37°C, and RNA extracted from infections with P2 stocks was sequenced in the region of the expected deletion. One virus stock yielded the same 45-nucleotide deletion of positions 184 to 228 as the R2 revertant, another yielded a 45-nucleotide deletion of 185 to 229, and the third virus stock yielded a 44-nucleotide deletion of 184 to 227 (three independent 33°C control virus stocks had only the original 4-nucleotide deletion in this region). These results suggest some flexibility in this region as far as which sequences are required or dispensable for a successful poliovirus infection. A cDNA clone of the R2 revertant which contained only the 45-nucleotide deletion in a PV1(M) background produced a virus, R2 cDNA, with virtually identical biological properties to the original R2 revertant virus. These results provide further support for our conclusion that the reversion of the 220D1 *ts* phenotype found in R2 is due solely to the additional deletion of the 41 nucleotides proximal to the original 4-nucleotide deletion in 220D1.

It was of interest that we generated a 4-nucleotide deletion in the 5' NCR that is extremely detrimental to viral functions, and yet a 45-nucleotide deletion (inclusive of the original four deleted nucleotides) restores nearly wild-type functions to a revertant virus. The location of the 45-nucleotide deletion (184 to 228) in R2 is within a region that has recently been defined as essential for translation of poliovirus RNA (43, 44). The boundaries of this region (130 to 600) may require reexamination in light of the results we presented in this report. As we have pointed out previously, replicase recognition-translation initiation signals may be encoded in structures within the 5' NCRs of picornavirus genomes that are not strictly sequence specific (18).

An examination of a computer-predicted secondary structure of the 5' NCR of poliovirus RNA by Rivera et al. (35) provides a speculative basis for explaining our results. The four-nucleotide deletion in mutant 220D1 of nucleotides 221 to 224 occurs directly downstream of the base of a predicted stem-loop structure. The deletion in revertant R2 of nucleotides 184 to 228 would effectively delete this entire stem-loop structure as well as two nucleotides upstream and eight nucleotides downstream. It is possible that the original four-nucleotide deletion in mutant 220D1 destabilizes the proposed stem-loop structure and is detrimental to replication of the virus. The deletion of the stem-loop structure in R2 reverses such deleterious effects, perhaps by allowing surrounding sequences to fold as if the stable stem-loop were present. Since nucleotides 184 to 228 are not essential for virus growth in HeLa cells, the actual function of such sequences remains to be determined. Interestingly, one of the upstream AUG codons is present at the 5' nucleotide of the predicted stem-loop structure. This AUG is deleted along with the stem-loop structure in the genomic RNA of

the R2 revertant. Perhaps these sequences (and the putative stem-loop structure they generate) are intended to reduce accessibility and prevent aberrant ribosome binding at this upstream AUG codon.

There is little experimental evidence for the biological significance of computer-predicted secondary structures in the 5' NCR of poliovirus RNA. As one might expect from the nucleotide sequence homology, the predicted secondary structures of the 5' NCRs of genomic RNAs from all three poliovirus serotypes, Sabin and wild-type strains, are nearly identical (35). The predicted secondary structures for CB3 and human rhinovirus (types 2 and 14) genomic RNA (sharing ~70 and ~60% nucleotide sequence homology with poliovirus, respectively) share many structural features with poliovirus genomic RNAs despite their differences in nucleotide sequence (35). There is also enzymatic data for the existence of the 5'-terminal hairpin structure in poliovirus RNA that is predicted by computer analysis to occur in practically all picornavirus RNA sequences thus far (23, 35). In addition, data based on mutations in the 5' NCR of poliovirus RNA provide suggestive evidence for the existence of secondary structures. Deletion of nucleotide 10 at the base of the 5'-terminal hairpin resulted in a virus with defects in various biological functions (34). The mutant virus was then partially restored to wild type by a compensatory mutation at position 34, presumably restoring the hairpin structure. However, a much more extensive genetic analysis will be needed to define more precisely the functional domains in the 5' NCR and to verify any predicted RNA secondary structures and their important functional significance in the poliovirus life cycle.

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