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A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome

(poliovirus cDNA/site-directed mutagenesis/persistent viral infection/complementation)

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ABSTRACT **We have constructed an 8-base-pair insertion mutation in the 3′ noncoding region of an infectious poliovirus cDNA clone that gives rise to a temperature-sensitive RNA synthesis mutant upon transfection into mammalian cells. The mutated cDNA was used to establish a cell line that releases the mutant poliovirus in a temperature-dependent fashion, representing a unique persistent viral infection. A poliovirus mutant mapping in the noncapsid region of the viral genome can be complemented in this cell line, implying that the cell line expresses viral proteins at the nonpermissive temperature.**

Many events take place in a cell infected by poliovirus. The positive-strand RNA genome of the virus must be replicated, viral proteins made, cellular functions inhibited, and new virions produced. Although much has been learned about these and other aspects of the virus life cycle (1), clear understanding of the functions of the various viral proteins and their interaction with cellular constituents still lies ahead. This is due in part to the lack of well-defined poliovirus mutants.

The discovery that a cloned cDNA copy of the poliovirus RNA genome gives rise to infectious poliovirus upon transfection into mammalian cells (2, 3) opened up the possibility of constructing defined poliovirus mutants by mutagenizing the infectious cDNA. The opportunity to store the genetic information encoding a mutant poliovirus as a cDNA clone makes it possible to determine the nucleotide sequence of the mutated genome and to ensure that the mutation is responsible for the observed phenotype.

We report here the construction of several linker–insertion mutations in the 3′ noncoding region of the viral genome. One of these mutations, PTH7387, gave rise to a temperature-sensitive poliovirus mutant. The infectious cDNA bearing this mutation was used to establish stable persistently infected human HeLa cell lines that release infectious poliovirus at the permissive temperature. Such cell lines, at the nonpermissive temperature, can complement another poliovirus mutant, showing that the integrated viral genome is translated.

MATERIALS AND METHODS

Cells and Viruses. HeLa cells were maintained in suspension culture in Joklik's modification of minimal essential medium (MEM) supplemented with 7% horse serum (GIBCO). CV1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Hazleton). Wild-type poliovirus was isolated from a single plaque derived from HeLa cells transfected with a pSV2-polio plasmid as described (2). Protocols for preparation of virus stocks and viral infections have been described (4).

Bacterial Strains and Plasmids. *Escherichia coli* strain HB101 was the host for all plasmids used and was obtained from C. Cepko (Harvard Medical School). Plasmid pSV2-polio was originally obtained from V. R. Racaniello (Columbia University). Plasmid pUC9 was purchased from Bethesda Research Laboratories.

Enzymes. Restriction endonuclease enzymes were purchased from New England Biolabs. Klenow enzyme and T4 DNA ligase were obtained from Boehringer Mannheim.

Construction of Linker–Insertion Mutations. Plasmid pSV2-polio was digested to completion with *Eco*RI and *Hin*dIII. The DNA fragments were separated by low-melting agarose (Sigma) gel electrophoresis. The gel piece containing the 978-base-pair (bp) *Eco*RI/*Hin*dIII fragment (see Fig. 1) was excised with a razor blade and melted at 68°C for 15 min. After sequential extractions with phenol and chloroform/isoamylalcohol (24:1, vol/vol), the DNA fragment was precipitated with ethanol and stored at −20°C. The vector pUC9 was digested with *Eco*RI and *Hin*dIII, extracted with phenol, and precipitated with ethanol. Equimolar amounts of the digested vector pUC9 and the 978-bp fragment were ligated overnight at 15°C (5). The ligation mixture was transformed into HB101 and the recombinant pUC-polio was isolated using an alkaline minipreparation of bacterial cultures obtained from single bacterial colonies (6). The pUC-polio plasmid was then digested with *Taq* I in the presence of ethidium bromide in order to increase the number of molecules containing only one double-stranded break (7), and full-length linear molecules were isolated by low-melting agarose gel electrophoresis. Linear molecules (1 μg) were resuspended in 20 μl of 50 mM Tris·HCl pH 7.6/50 mM NaCl/1 mM dithiothreitol/2 mM dNTPs. The single-stranded 5′ termini were repaired by addition of 5 units of Klenow enzyme for 30 min at room temperature. The reaction was stopped by adding $NaDodSO_4$ to a final concentration of 0.1%, followed by heating at 68°C for 5 min, phenol extraction, and precipitation with ethanol. The DNA molecules were either religated alone, to create 2-bp insertions, or after the addition of phosphorylated *Hpa* I or *Xho* I-linker oligomers (Collaborative Research, Waltham, MA) (5). Ligated molecules were transformed into HB101 and screened for recombinants that had either lost the targeted *Taq* I restriction endonuclease site or had acquired a new *Hpa* I or *Xho* I site at that position. The *Eco*RI/*Hin*dIII fragments of the pUC-polio subclones were prepared as described above and ligated with the 9028-bp *Hin*dIII/*Eco*RI DNA fragment prepared from the wild-type pSV2-polio plasmid. In this way, plasmids were obtained bearing a 2-bp insertion (PTF7387),

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Abbreviation: bp, base pair(s).

an 8-bp insertion (PTH7387), or a 10-bp insertion (PTX7387) at the *Taq* I restriction endonuclease site at nucleotide position 7387 of the viral genome. Predicted nucleotide sequence was verified by sequencing the 978-bp *Eco*RI/*Hin*dIII fragment (8).

Construction of Persistently Infected Cell Lines. Persistently infected HeLa cell lines were constructed by cotransfection of PTH7387 and pSV2-neo at a molar ratio of 20:1 using the calcium phosphate coprecipitation method (9). At 4 hr after transfection, cells were shifted to 39.5°C and maintained at this temperature. The drug G418 (GIBCO) was added to the medium 48 hr after transfection according to the protocol of Southern and Berg (10). At 18 days after transfection, individual G418-resistant colonies were cloned and grown in duplicate at 39.5°C and 32°C. Of 136 cloned cell lines, 12 released PTH7387 at 32°C within 3 days after the temperature shift. Representative persistently infected cell lines that were examined further were designated numbers 35 and 40. As a control cell line, we selected a G418-resistant line that does not contain poliovirus cDNA sequences, termed cell line 107.

RESULTS

Construction of Linker–Insertion Mutations in the Poliovirus cDNA 3′ Noncoding Region. As part of a more general program of linker-insertion mutagenesis of an infectious poliovirus cDNA clone, we chose to insert linkers in *Taq* I sites near the 3′ end of the viral genome (Fig. 1). The pSV2-polio plasmid, containing polio cDNA and a simian virus 40-derived promoter (11), has 17 *Taq* I sites, so we subcloned a 978-bp *Hin*dIII/*Eco*RI fragment into the unique *Hin*dIII and *Eco*RI sites of the plasmid pUC9 (12). The pUC-polio plasmid contained five *Taq* I sites, one within the ampicillin-resistance gene, one in the 3′ untranslated region of the cDNA, and three in coding sequences. The recombinant plasmid was partially digested with *Taq* I and full-length linear DNA molecules were isolated. These molecules, containing 2-bp 5′ overhangs resulting from the *Taq* I digestion, were filled in at both ends with the Klenow fragment of DNA polymerase (5) and recircularized by ligation with DNA ligase, either immediately or after addition of DNA oligomers containing either 6-nucleotide bps of an *Hpa* I site or 8-nucleotide bps including an *Xho* I site. The plasmids containing insertions at the *Taq* I site in the 3′ noncoding region (position 7387) were identified by restriction endonuclease digestions and their *Hin*dIII/*Eco*RI fragments were isolated and relegated to the largest fragment of a *Hin*dIII/*Eco*RI partial digest of the pSV2-polio plasmid. Recombinant full-length cDNA clones were obtained bearing 2-bp (PTF7387), 8-bp (PTH7387), or 10-bp (PTX7387) insertions in the 3′ untranslated region at nucleotide position 7387 (Fig. 2).

Phenotype of Poliovirus Mutants Mapping in the 3′ Noncoding Region. Wild-type and linker-insertion-mutagenized pSV2-polio plasmids were transfected into HeLa cells by the DEAE-dextran transfection procedure (16) and were scored for the formation of poliovirus plaques at 32°C and 39.5°C. The cDNAs containing a 2-bp or a 10-bp insertion at position 7387 gave rise to plaques of wild-type size, and the yields of viruses were identical to wild-type at both temperatures. In contrast, the 8-bp *Hpa* I linker-containing cDNA (PTH7387) produced plaques only at 32°C. Virus stocks were prepared from such plaques and characterized for growth at both temperatures (Table 1). PTH7387 was clearly extremely temperature sensitive for growth. It grew equally well in human HeLa cells and in African green monkey kidney cells (CV1) at 32°C, but at 39.5°C it produced <0.1% of the yield at permissive temperature. Furthermore, plaques picked at the restrictive temperature displayed wild-type phenotypes, suggesting that they were revertants.

To ensure that the mutated cDNA contained the predicted alteration and that this alteration was responsible for the phenotype, we determined from the nucleotide sequence of a DNA fragment spanning the 3′ noncoding region that the 8-bp insertion was the only change present. This fragment was then cloned into an unmutagenized vector and shown to reproduce the mutant phenotype when transfected into mammalian cells. This "mix-and-match" experiment excluded the possibility that accidental second-site mutations acquired during the cloning procedures were responsible for the observed phenotype.

Establishment of a Cell Line Persistently Infected with a Mutant Poliovirus cDNA. PTH7387 makes <5% of the normal yield of viral RNA at the restrictive temperature (unpublished data). It might, therefore, not be able to spread through a cell population at all at 39.5°C. Should this be true, cells harboring the PTH7387 plasmid might be able to grow normally at 39.5°C but would yield virus when grown at 32°C. To examine this possibility, we cotransfected HeLa cells with the mutant plasmid along with a plasmid bearing the dominant selectable marker for G418 resistance (10) at a molar ratio of 20:1. Transfected cells were grown at 39.5°C and G418-resistant colonies were selected. At 18 days after transfection, the individual colonies were cloned and grown in duplicate at 39.5°C and 32°C. Of 136 cloned cell lines, 12 released poliovirus at 32°C during 3 days after the temperature shift. The supernatants of these producer cell lines were harvested and plaque assays were performed at both temperatures. The virus released by these cell lines had the same temperature sensitivity as the original mutant (data not shown). These results indicated that we had derived cell lines that harbored the intact mutant viral cDNA but allowed recovery of infectious poliovirus in a temperature-regulated fashion, representing a unique persistent viral infection.

To study the persistently infected cells further, we concentrated on two lines: a line designated cell line 40 that released virus on temperature downshift and had a single integrated copy of the PTH7387 plasmid (data not shown), and control line 107, which was G418 resistant but did not release virus after downshift and had no detectable integrated poliovirus sequences (data not shown). To examine the production of virus by individual cells after downshift, we overlaid dishes of confluent cells with agar and shifted some dishes to an incubation temperature of 32°C. After 3 days at 39.5°C or 5 days at 32°C, viable cells were stained with crystal violet. Neither line produced any plaques at 39.5°C (Fig. 3*A*) but cell line 40 produced many plaques of heterogeneous size at 32°C (Fig. 3*B*), while line 107 did not. When the lines were infected with wild-type virus at 39.5°C, they produced equal numbers of large, quite homogeneous plaques (Fig. 3*C*). Thus, both lines are permissive for growth of wild-type virus at 39.5°C but line 40 has the unique capability to yield plaques at 32°C. The inhomogeneity of plaque size produced by line 40 at 32°C suggests that initial virus release is asynchronous; an increasing number of plaques seen with increased time of incubation is consistent with this interpretation (data not shown). Only a few hundred cells of the $\approx 5 \times 10^6$ on the dish yielded a plaque, suggesting that virus activation is a relatively rare event.

Complementation of a Poliovirus Mutant in the Persistently Infected Cell Line 40. Recently it has been shown that several poliovirus mutants can effectively complement each other (ref. 17; H.D.B. and D.B., unpublished observations). Some of the complementable mutations map outside of the capsid region of the poliovirus genome, suggesting that some noncapsid poliovirus proteins can function in *trans* during the infectious cycle. This result encouraged us to attempt to complement a poliovirus mutant in persistently infected cell line 40 at the nonpermissive temperature. We chose a mutant that might be sensitive to small amounts of poliovirus protein

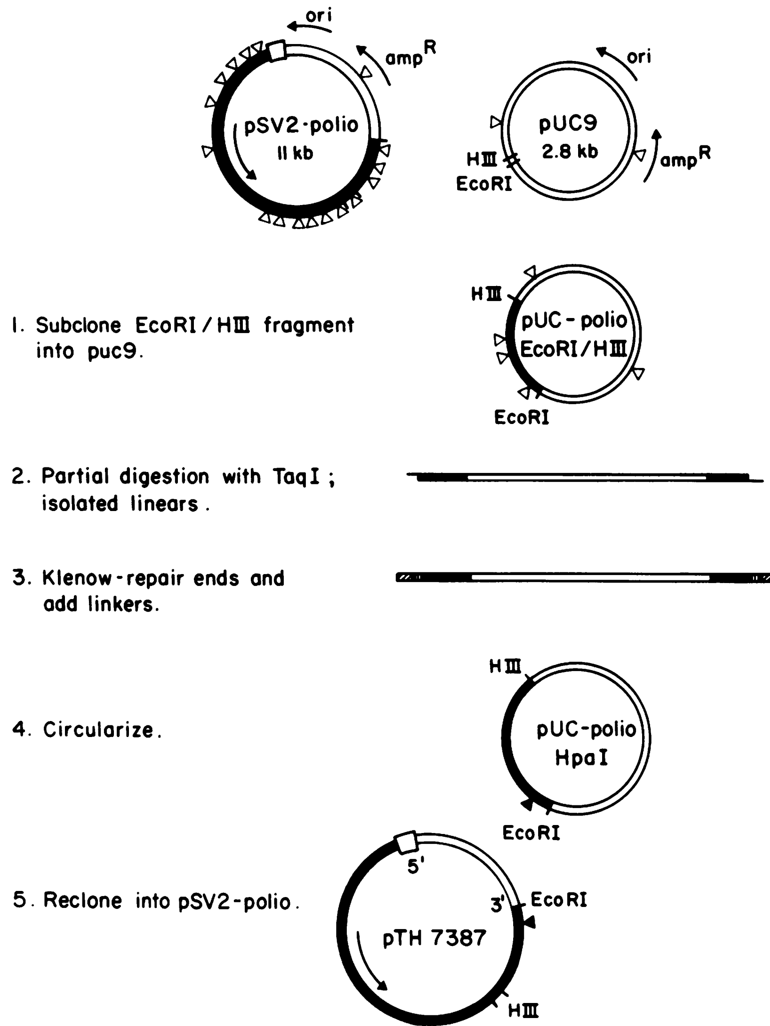

FIG. 1. Strategy for the construction of linker-insertion mutants in the poliovirus cDNA. The pSV2-polio plasmid contains pBR322 sequences (open bar), the simian virus 40 promoter and enhancer elements (box), and polio-cDNA (solid bar). The open triangles point to the *Taq* I restriction endonuclease site present in the plasmid. The solid triangle represents a mutated *Taq* I site. Hatched bars show the added linker oligomers. Construction of the recombinants is described in *Materials and Methods*. amp^R, Ampicillin resistance; kb, kilobases; HIII, *Hin*dIII.

and, therefore, would represent a sensitive assay of complementation. To this end, we chose to complement a small plaque mutant, termed HF121, which maps in the P2-2A region of the poliovirus genome and is deficient in the selective inhibition of host protein synthesis, a function that can be provided by small amounts of protein made very early in infection (4).

The complementation experiment was done by infecting either nonproducer cell line 107 (Fig. 4*A*), or producer cell line 40 (Fig. 4 *B* and *C*), with the mutant HF121 or wild-type poliovirus. The infected cell lines were overlaid with agar and incubated at 39.5°C for various lengths of time. The culture dishes were then stained with crystal violet and scored for plaques.

The HF121 mutant produced only very tiny plaques on cell line 107 (Fig. 4*A*), as it does on uncloned HeLa cells. However, when plated on cell line 40, HF121 formed significantly larger plaques (Fig. 4*B*), although still not wild-type

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MAHONEY  TAG TAA CCC TAC CTC AG|T CGA|ATT GGA TTG GGT CAT ACT GTT GTA GGG GTA AAT TTT TCT TTA ATT CGG AGA-polyA
SABIN 1  TAG TAA CCC TAC CTC AG|T CGA|ATT GGA TTG GGT CAT ACT GCT GTA GGG GTA AAT TTT TCT TTA ATT CGG AGG-polyA
SABIN 2  TAG TAA CCC TAC CTC AG|T CGA|ATT GGA TTG GGT CAT GCT GTT GTA GGG GTA AAT TTT TCT TTA ATT CGG -GA-polyA
SABIN 3  TAG TAA CCC TAC CTC AG|T CGA|ATT GGA TTG GGT CAT GCT GTT GTA GGG GTA AAT TTT TCT TTA ATT CGG AGG-polyA
                               TAQI
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PLASMID	CONSTRUCTION	INSERTION	STRUCTURE	PHENOTYPE
pSV2•polio	None	—	TCGA	W.T.
pTF 7387	Klenow	2 bp	TCGCGA	W.T.
pTH 7387	HpaI-Linker	8 bp	TCGGTTAACCGA	T.S.
pTX 7387	XhoI-Linker	10 bp	TCGCCTCGAGGCGA	W.T.

FIG. 2. Poliovirus mutants mapping in the 3′ noncoding region. The nucleotide sequences of the Mahoney and Sabin strains are shown (13–15), with individual nonconserved nucleotides marked. The mutagenized *Taq* I restriction endonuclease site is indicated by a large box. Designation of wild-type (W.T.) phenotype means that the yield of the particular virus in a single-cycle infection is the same as wild-type poliovirus. Temperature-sensitive (T.S.) phenotype denotes that the virus yield of PTH7387 in a single-cycle infection at the nonpermissive temperature is 0.1% of the wild-type yield.

Table 1. Titer of wild-type poliovirus and temperature-sensitive PTH7387 virus stocks

	Wild type			PTH7387		
Cell line	32°C	39.5°C	39.5 °C/ 32°C	32°C	39.5°C	39.5°C/ 32°C
HeLa	2.5×10^9	3×10^9	1.2	5×10^9	2×10^6	4×10^{-4}
CV1	4×10^8	6×10^8	1.5	9×10^8	2×10^5	2×10^{-4}

Plaque assays were performed at the temperatures indicated and the virus titers are displayed as plaque-forming units of virus stock per ml.

size plaques (Fig. 4*C*). We suggest that this increase in plaque size represents complementation of the HF121 mutant in cell line 40. In another line that produces poliovirus after temperature downshift, cell line 35, HF121 also produced larger plaques.

Another possible explanation for the faster growth of HF121 in cell line 40 could be the formation of viral recombinants that overgrew the small plaque mutant viruses. To examine this possibility, we picked 20 plaques from the HF121-infected cell line 40 and assayed virus from each plaque on HeLa cells at 32°C and 39.5°C. Virus from all 20 plaques displayed the mutant small-plaque phenotype at all temperatures (not shown). In addition, the titers at both temperatures were the same indicating that little or no endogenous viral RNA derived from pSVPTH7387 had been rescued.

In a different kind of complementation experiment, we tested the virus yield during single-cycle infections of cell lines 107 and 40. The yield and growth curve of wild-type virus was the same in both cell lines. Mutant HF121 grew more slowly in both cell lines, but its yield was 10 times higher from line 40 than from line 107 (Fig. 5). Thus, cell line 40 apparently produces sufficient poliovirus products to complement at least one poliovirus mutant.

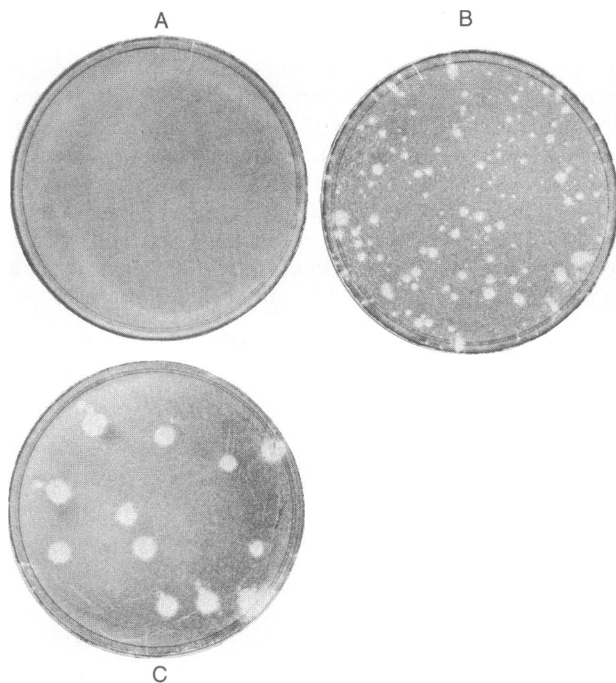

FIG. 3. Temperature-dependent spread of PTH7387 poliovirus in persistently infected cell line 40. Cell line 40 was overlaid with agar and (*A*) incubated at 39.5°C or (*B*) shifted to an incubation temperature of 32°C. After 3 days at 39.5°C or 5 days at 32°C, the dishes were stained with crystal violet and scored for plaques. (*C*) In a control experiment, cell line 40 was infected with wild-type virus, overlaid with agar, and incubated for 3 days at 39.5°C.

DISCUSSION

We report here the construction of a poliovirus mutant mapping in the 3′ noncoding region of the viral genome. The mutant was obtained by site-directed mutagenesis of an infectious cDNA clone using linker insertion at a known restriction endonuclease site. Having a cDNA copy of the mutation enabled us to perform "mix-and-match" experiments with DNA fragments bearing the desired mutation to unambiguously map the introduced mutation and the mutant phenotype to a single locus in the viral genome. Although we have not formally proven that the introduced mutation is present in the viral RNA genome, it is unlikely that other changes occur in the viral RNA molecule eliciting the mutant phenotype because every plaque isolate derived from the transfected mutated cDNA gives rise to the same temperature-sensitive mutant phenotype.

We chose the 65-nucleotide-long 3′ noncoding region as a target for mutagenesis because it contains highly conserved sequences that might, for instance, be involved in the initiation of minus-strand RNA synthesis. Despite the fact that only three nucleotide changes (at positions 7405 and 7409) are found in this region among the three poliovirus strains (Fig. 2) (13–15), nucleotide insertions at the *Taq* I site at position 7387 are tolerated. Mutants with 2- (PTF7387) or 10- (PTX7387) nucleotide insertions produced virus yields similar to wild-type virus, indicating that sequences 5′ and 3′ of this insertion need not be contiguous to provide their function. That an 8-nucleotide insertion at the same site gave rise to a conditional lethal mutant (PTH7387) is remarkable. Perhaps the RNA genome of PTH7387 folds properly at the permissive temperature but may contain a different secondary structure at the restrictive temperature and be unable to function. Temperature-dependent mutants of RNA molecules that affect the biological function of the nucleic acid have been described in tRNA (18, 19) and in mitochondrial rRNA (20, 21).

The very strong temperature sensitivity of PTH7387 allowed us to produce a unique type of persistently infected cell. Persistently infected cells with poliovirus have been described (22–24) but were presumably based on slow replication of viral RNA rather than on storage of the genome as a DNA copy (25). A previous claim of persistence of

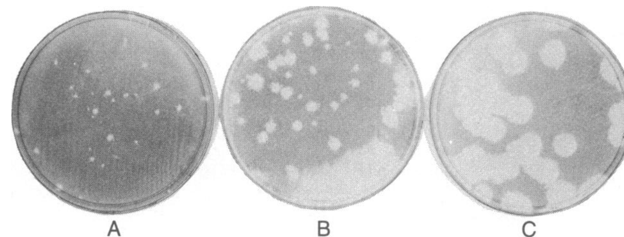

FIG. 4. Complementation of poliovirus mutant HF121 in persistently infected cell line 40. (*A*) Nonproducer cell line 107 and (*B*) persistently infected cell line 40 were infected with HF121, overlaid with agar, and incubated for 4 days at 39.5°C. (*C*) In addition, cell line 40 was infected with wild-type virus and incubated at 39.5°C for the same length of time. The dishes were stained with crystal violet.

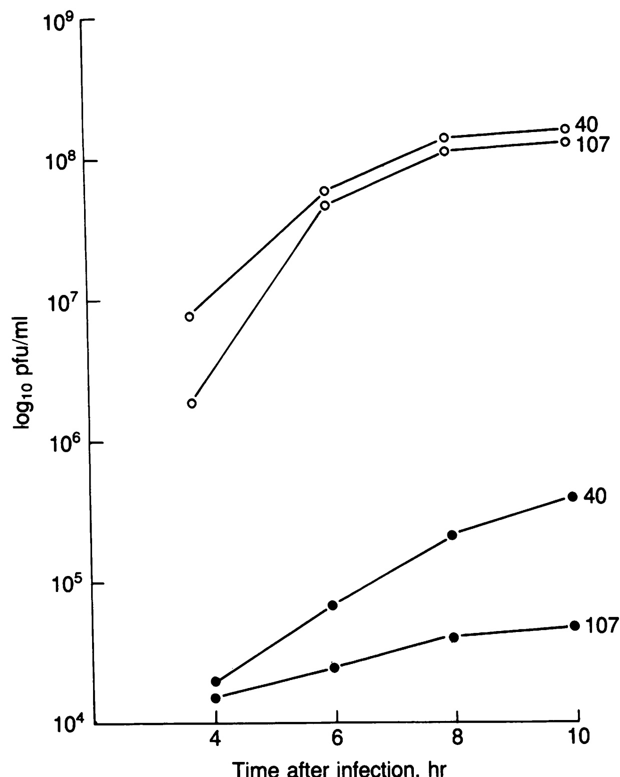

FIG. 5. Growth curves of wild-type and mutant-HF121 poliovirus in persistently infected cell line 40 and control cell line 107. Plates containing 5×10^5 of the indicated cells were infected with wild-type or HF121 virus at a multiplicity of 5 plaque-forming units (pfu) per cell. Cells were harvested at the indicated times, virus stocks were prepared, and virus titer was determined by plaque assay on HeLa cells. The titers are plotted semilogarithmically as a function of time after infection. o, Wild-type poliovirus; •, HF121 mutant.

nonretrovirus RNA viruses through a DNA copy has not been substantiated (26). Retroviruses, of course, use the same strategy but are much more efficient in that they continually produce virus from all persistently-infected cells. Poliovirus, being lytic in its growth, and released by cell lysis, cannot both grow and be maintained in the same cell. Therefore, there may be selection against high expression during the establishment of the cell lines because poliovirus encodes powerful inhibitors of cellular protein synthesis and transcription, which might produce lethality at low levels. So far, we were unable to detect poliovirus-specific RNA in the persistently infected cells by RNA blot analysis (5) of total cellular RNA.

To examine whether there is any expression of the viral genome, we used the sensitive tool of complementation. We infected a persistently infected cell line with mutant HF121. This mutant is very tight, producing only very small plaques on HeLa cells at any temperature. The mutation is located in protein 2A (also called protein 8; ref. 27) and fails to selectively inhibit cell protein synthesis. It is known to be complemented by many other poliovirus mutants (H.D.B. and D.B., unpublished observations). Its complementation in cell lines 40 and 35 indicates that they produce at least a little viral protein in some cells. The complementing protein would be one involved in inhibition of cellular protein synthesis. Whether there is some selective inhibition of cellular protein synthesis in the persistently infected cells is not clear. Preliminary experiments showed that the cap-binding complex protein P220 (28) is cleaved at low levels in the persistently infected cell lines. This cleavage might facilitate the cap-independent translation of the mutant RNA molecules. Alternatively, protein 2A might have multiple functions, and the complementing activity might not involve inhibition of cellular protein synthesis.

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