

A chimeric plasmid from cDNA clones of poliovirus and coxsackievirus produces a recombinant virus that is temperature-sensitive

(picornavirus/infectious cDNA clones)

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ABSTRACT We have inserted a 405-nucleotide fragment from the 5' noncoding region of the coxsackievirus B3 genome into an infectious cDNA copy of the poliovirus RNA genome. Transfection of plasmid DNA containing this hybrid genome construct into cultured monkey cells produced infectious virus. Recombinant virus stocks displayed a temperature-sensitive phenotype for growth at 37°C. We found that there is a dramatic reduction in the level of viral proteins and viral RNAs in HeLa cells infected with the recombinant at 37°C compared to that obtained at 33.5°C. Thus, insertion of a portion of the coxsackievirus genome into the poliovirus genome produces a temperature-sensitive recombinant virus. That this substitution occurs in a region of the poliovirus genome that, to date, has not been shown to have any coding function suggests that RNA sequences involved in replicase recognition or ribosome binding may contribute to the temperature-sensitive phenotype of the recombinant virus.

The genomic RNAs of members of the picornavirus family contain 500-1000 nucleotides preceding the long open reading frame that encodes a giant precursor polyprotein (1-12). In the case of poliovirus, the prototypic enterovirus of the Picornaviridae, there are several open reading frames in the viral RNA upstream of the site of initiation of polyprotein synthesis (at nucleotide 743) that could potentially give rise to peptides having molecular weights less than 10,000 (1, 3, 13). Since no proteins originating from these short open reading frames have been identified in polio-infected HeLa cells, it is likely that the RNA sequences upstream of the polyprotein initiator methionine play regulatory roles in RNA replication, protein synthesis, or packaging of viral RNA.

To determine the contribution of the 5' upstream sequences of poliovirus RNA during viral replication, we have begun a molecular genetic study using infectious cDNA clones of the polio genome. Semler *et al.* (14) and others (15-17) have described the isolation of plasmids that contain full-length cDNA copies of polio RNA. These plasmids are capable of producing infectious poliovirus after transfection of cultured primate cells. One of our plasmid constructs, pEV104, contains simian virus 40 replication and transcription signals that allow production of high levels of poliovirus type 1 (PV1) when transfected into COS-1 cells (14). We have used pEV104 as the recipient plasmid for the introduction of sequences from coxsackievirus type B3 (CB3), another enterovirus from the Picornaviridae group. The isolation of cDNA clones representing the entire genomic RNA of CB3 has recently been described (18-20). In addition, Tracy *et al.* (21) have reported the nucleotide sequence of the 5' half (≈3800 nucleotides) of the CB3 genome, and Stalhandske *et*

al. (19) have reported the nucleotide sequence of the CB3 replicase gene. Because the CB3 genome contains a 738-nucleotide 5' sequence upstream of the long open reading frame that shares ≈70% nucleotide sequence homology with the corresponding region of the PV1 genome (21), it seemed possible to derive a chimeric genome between the two viruses by *in vitro* manipulation of cloned cDNAs. This PV1-CB3 chimera could be used to analyze the function(s) of the 5' untranslated region of poliovirus RNA.

We report here the isolation of a recombinant plasmid that contains a 405-base-pair cDNA fragment from CB3 inserted into the poliovirus genome at a location corresponding to its precise position within the context of the coxsackie viral genome. Transfection of the recombinant plasmid into cultured monkey cells produces infectious virus. The recombinant virus displays a temperature-sensitive (ts) phenotype for growth at 37°C as assayed by plaque formation. In addition, we show that there is a dramatic reduction in the level of viral proteins and viral RNAs in HeLa cells infected with the recombinant at 37°C compared to that obtained at 33.5°C. Thus, it appears that insertion of a portion of the CB3 genome into a region of the PV1 genome that has no known coding function produces a ts recombinant virus.

MATERIALS AND METHODS

Cells and Virus. Virus for propagation and plaque assays was grown on HeLa cell monolayers originally obtained from J. Wilkinson and E. J. Stanbridge, University of California, Irvine. Transfections with infectious cDNA plasmids were carried out on COS-1 cells (14, 22). The wild-type poliovirus stock used for experiments reported here was derived from a single plaque isolate produced after transfection of 293 cells with a cDNA clone of the type 1 (Mahoney) RNA as described (14). The recombinant virus isolates were designated PCV for polio-coxsackie virus.

Transfections and Plaque Assays. Cells were transfected with CsCl gradient-purified plasmid DNAs using a calcium phosphate coprecipitation method (23) that employs a 3- to 4-min glycerol boost step (24, 25). Transfections were carried out on 60-mm plates of subconfluent COS-1 cells using a plasmid DNA/salmon sperm carrier DNA mixture that totaled 10 μg per plate (14).

Plaque assays were carried out on 60-mm plates of HeLa cell monolayers under a semisolid agar overlay containing Dulbecco's modified Eagle's medium, 6% fetal calf serum, and 0.45% agarose (SeaKem Laboratories, Rockland, ME). Cells incubated at 33.5°C were stained with crystal violet (26)

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Abbreviations: PV1, poliovirus type 1; CB3, coxsackievirus type B3; ts, temperature-sensitive; PCV, polio-coxsackie virus; kb, kilobase(s).

3–4 days after infection while those incubated at 37°C were stained 2–3 days after infection.

Plasmids and Cloning Procedures. The scheme for isolation of plasmids pMV1.8, pCP2, and pCP110 is given in Fig. 1. Relevant cloning procedures and the derivation of plasmid pDS14 are given in ref. 14. The small [2.1-kilobase (kb)] plasmid vector designated pMV7 is a derivative of pBR322 (27). It was obtained by digestion of pBR322 DNA with restriction endonucleases *Cla*I and *Nde*I, treatment with the Klenow fragment of DNA polymerase I, ligation of the newly generated blunt ends, and transformation of *Escherichia coli*. Plasmid pCBIII51 is a 5' cDNA clone derived from the Nancy strain of CB3 RNA (18, 21). The construction of plasmid vector pES131 (containing the simian virus 40 transcription and replication signals) has been reported (14). Plasmid pPV16 contains the full-length 7.5-kb PV1 cDNA insert from the infectious clone pDS303 cloned into the *Eco*RI site of vector pNT4 (14).

Labeling of Infected Cells with [³⁵S]Methionine. HeLa cell monolayers in 60-mm plates ($\approx 1 \times 10^6$ cells) were infected with wild-type or recombinant virus isolates (see below) at a multiplicity of infection of 25 in methionine-free minimal essential medium and 8% newborn calf serum. Infected cells were incubated at 33.5°C or 37°C. At specific times after infection (see Fig. 2), cells were pulse-labeled with [³⁵S]methionine (50 μ Ci per plate; 1 Ci = 37 GBq; Amersham) for 2 hr. 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 (29). Samples were boiled and electrophoresed on 12.5% polyacrylamide gels containing NaDodSO₄ (29). Protein gels were fluorographed (30) and exposed to XAR film.

Labeling of Infected Cells with [³H]Uridine. The protocol for infection was as described above except that the cell culture media contained unlabeled methionine. Three hours after infection, actinomycin D (5 μ g/ml) was added to the infected cultures. Cultures were labeled beginning 3.5 hr after infection with [³H]uridine (50 μ Ci per plate; Amersham). At various times (see Fig. 3), cells were harvested as above and then suspended in 1 ml of phosphate-buffered saline. Portions of the resuspended cell extracts were treated with trichloroacetic acid and the precipitates were collected on glass fiber filters. Radioactivity on the filters was determined in a liquid scintillation counter.

RESULTS

Isolation of a Recombinant Plasmid Containing Poliovirus and Coxsackievirus cDNA. The scheme for isolation of a polio-coxsackie recombinant cDNA clone is outlined in Fig. 1. Briefly, the insert from a 5' subgenomic clone of PV1 (pDS14; ref. 14) was cloned into a 2.1-kb plasmid (pMV7) that is a shortened derivative of pBR322. The resulting recombinant plasmid was digested with restriction endonucleases *Bam*HI (partial) and *Bal*I to remove a 407-base-pair fragment (corresponding to PV1 nucleotides 220–627). Into this plasmid, we inserted a 405-base-pair *Xmn*I/*Bal*I fragment originating from the 5' CB3 cDNA clone (pCBIII51; ref. 18) to generate plasmid pCP2 (Fig. 1). A 1.1-kb fragment from pCP2 was then isolated and used in a three-fragment ligation as shown at the bottom of Fig. 1. The origins of vector pES131 and plasmid pPV16 are given in ref. 14 and *Materials and Methods*, respectively. The final plasmid construct containing the 5' CB3 fragment inserted into the full-length PV1 cDNA clone is called pCP110. In addition to the picornaviral sequences, pCP110 also contains the simian virus 40 regulatory sequences necessary for the efficient expression of polio-specific sequences from plasmid DNAs

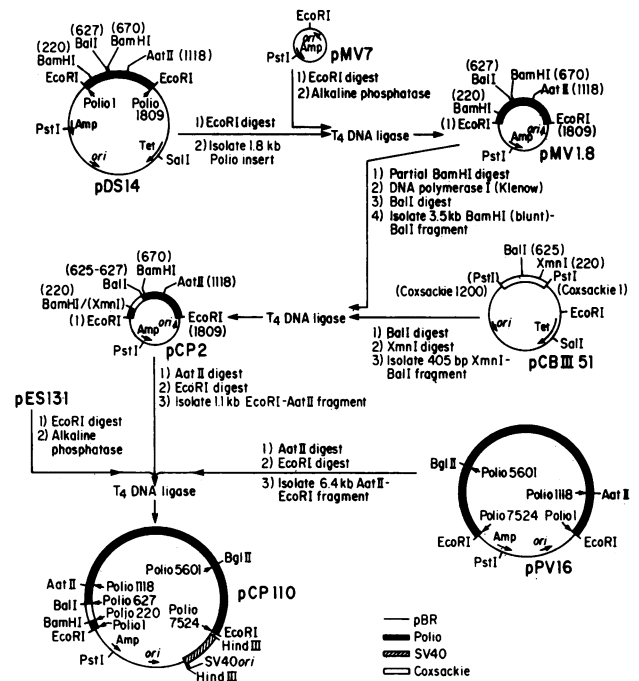


FIG. 1. Scheme for the construction of a recombinant plasmid containing cDNA sequences of poliovirus and coxsackievirus. The origin of plasmids pDS14 and pES131 is given in ref. 14. The construction of plasmids pMV7 and pPV16 is described in *Materials and Methods*. The *Pst*I site in pCBIII51 and the *Xmn*I site in pCP2 are enclosed with parentheses to indicate that these sites were not regenerated during cloning.

that are transfected into COS-1 cells (14, 22, 28, 31). We have confirmed the structure of pCP110 as shown at the bottom of Fig. 1 by extensive restriction endonuclease mapping studies (data not shown).

Transfection of COS-1 Cells with pCP110 DNA and Infectivity of the Recovered Virus. To test whether the recombinant plasmid pCP110 would produce infectious virus, we carried out DNA transfections of COS-1 cells. Transfected cultures were incubated under liquid media for 5–6 days at either 33.5°C or 37°C. The cell culture medium was then harvested and analyzed for virus growth on HeLa cells by plaque assay. Plaque assay cultures were incubated at temperatures corresponding to those used during growth of COS-1 cells following transfection.

The results of three transfection experiments with recombinant pCP110 are shown in Table 1. Transfection with pCP110 produced virus having a titer of ≈ 4 log units per ml of culture fluid at 33.5°C while no infectious virus was recovered from the 37°C incubations. In control transfections with the plasmid harboring the wild-type PV1 cDNA insert (pEV104), virus having a titer of ≈ 6 log units per ml was obtained at both 33.5°C and 37°C. Note that in the third experiment shown in Table 1, virus yields from transfection with a plasmid designated pCP108 are presented. This plasmid was derived from a second clone isolated after the same ligation and transformation that produced pCP110. Plasmid pCP108 is identical in structure to pCP110 and, after transfection, produces infectious virus in yields equal to those of pCP110. Our results show that a chimeric plasmid containing both polio and coxsackie cDNA sequences is infectious in cultured cells and that the growth of the recombinant virus is impaired at 37°C.

To further investigate the temperature-sensitivity of the recombinant virus, we picked a number of individual plaques after assaying culture fluids from the above transfections with pCP110 DNA at 33.5°C. These individual plaque isolates

Table 1. Recovery of recombinant virus from transfected COS-1 cells

Exp.	Plasmid	Incubation temperature, °C	Titer, log ₁₀ pfu/ml
1	pCP110	33.5	3.83
	pCP110	37	0
	pEV104	33.5	6.00
2	pEV104	37	6.43
	pCP110	33.5	4.57
3	pCP110	37	0
	pCP110	33.5	4.18
3	pCP110	37	0
	pCP108	33.5	4.51
	pCP108	37	0
	pEV104	33.5	6.23
	pEV104	37	6.20

Cells were transfected using 2–4 μg of plasmid DNA per plate for pCP110 and pCP108 and 0.05–0.1 μg of plasmid DNA per plate for pEV104. The total amount of DNA added to each plate was then adjusted to 10 μg using salmon sperm DNA as carrier. pfu, Plaque-forming units.

were used to infect fresh HeLa cell monolayers at 33.5°C. The culture fluids were harvested after 48 hr and then titered for virus growth by plaque assays on HeLa cells at both 33.5°C and 37°C. The results of the plaque assays from two such experiments are shown in Table 2. All of the plaque isolates shown in Table 2 yielded virus having a titer of ≈8 log units per ml at 33.5°C. The virus yields at 37°C were reduced considerably, producing recombinant virus having a titer of 5–6 log units per ml. Thus, the recombinant virus has a *ts* phenotype that results in virus titers that are reduced by 2–3 log units per ml at the nonpermissive temperature (37°C) compared to those obtained at the permissive temperature (33.5°C).

[³⁵S]Methionine Labeling of HeLa Cells Infected with the Recombinant Virus. To examine the effect (if any) that the cDNA insert had on the proteins synthesized in cells infected with the recombinant virus, we labeled infected cells with [³⁵S]methionine at 33.5°C and 37°C. The source of recombinant virus used in this experiment was a stock derived from a first-passage pool of plaque isolate 2 (Table 2). We have designated this recombinant polio-coxsackie virus stock as PCV110(2). The labeled protein pattern displayed on a polyacrylamide gel by extracts of cells infected with PCV110(2) was compared to that obtained after infection with a transfection-derived wild-type stock PV1(14). Infected cells were labeled at 33.5°C or 37°C with [³⁵S]methionine for 2 hr beginning at the times indicated in Fig. 2. At the end of the labeling period, cells were harvested and extracts were prepared for NaDodSO₄/polyacrylamide gel electrophoresis as described (32).

The results (Fig. 2) show that the viral-specific proteins synthesized in PCV110(2)-infected HeLa cells at 33.5°C are both qualitatively and quantitatively the same as those

Table 2. Reproductive capacity of recombinant virus isolates assayed at different temperatures

Exp.	Plaque no.	Titer, log ₁₀ pfu/ml		Log difference (titer at 33.5°C/ titer at 37°C)
		33.5°C	37°C	
1	2	8.34	5.54	2.80
	3	7.96	5.20	2.76
	4	8.48	5.60	2.88
2	2	8.32	5.74	2.58
	4	8.45	5.70	2.75

pfu, Plaque-forming units.

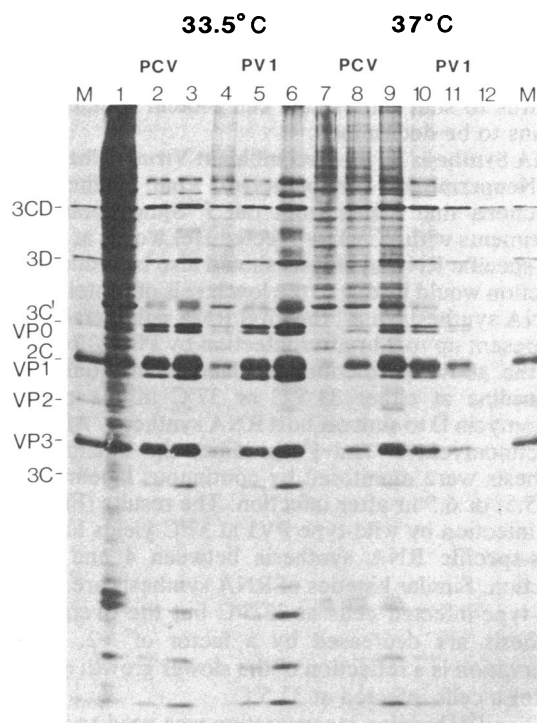


FIG. 2. [³⁵S]Methionine labeling of HeLa cells infected with recombinant virus PCV110 and wild-type poliovirus. Labeling periods were from 3 to 5 hr after infection (lanes 1, 4, 7, and 10), from 5 to 7 hr after infection (lanes 2, 5, 8, and 11), and from 7 to 9 hr after infection (lanes 3, 6, 9, and 12). PCV, samples derived from PCV110(2)-infected cells; PV1, samples derived from wild-type poliovirus-infected cells. Lanes M: [³⁵S]methionine marker lysates from wild-type poliovirus-infected HeLa cells that had been labeled between 4 and 7 hr after infection at 37°C. The figure displays an autoradiograph of a NaDodSO₄/12.5% polyacrylamide gel obtained after electrophoresis of extracts from the samples. Polio-specific proteins (3CD–3C) are indicated on the left.

synthesized in PV1-infected cells (compare lanes 2 and 3 with lanes 5 and 6, respectively). The earliest labeling time points (lane 1 for PCV and lane 4 for PV1) show that neither virus effects complete shutoff of cellular protein synthesis at 3–5 hr after infection at 33.5°C. Presumably, this is a reflection of the fact that a poliovirus infection proceeds more slowly at 33.5°C compared to 37°C.

When the protein labeling pattern of cells incubated at 37°C is analyzed, there are major differences between the PCV-infected cells and PV1-infected cells. As shown in Fig. 2 (lanes 7, 8, and 9), a clearly distinguishable pattern of polio-specific polypeptides for PCV-infected cells is not observed until 7–9 hr after infection. In the wild-type (PV1) infection at 37°C, however, a typical viral-specific pattern of polypeptides without any observable background of labeled, cellular proteins is observed 3–5 hr after infection (lane 10). Note that 7–9 hr after infection (lane 12) there are almost no detectable proteins recovered because the wild-type virus at 37°C has caused lysis of nearly all infected cells and release of intracellular material into the culture media. During the times chosen for labeling in this experiment, there does not appear to be a complete shutoff of host cell protein synthesis by the recombinant virus even when the polio-specific proteins such as 3CD, 3D, 2C, and VP3 have accumulated to easily detectable levels (Fig. 2, compare lane 9 to lane 3) in cells infected at 37°C. Since the experiment shown in Fig. 2 used pulse labeling (rather than continuous labeling) for 2 hr at the above indicated times after infection, the host cell proteins seen in lane 9 are the result of *de novo* synthesis between 7 and 9 hr after infection. Our data suggest that,

following infection, recombinant virus PCV110 is delayed in the synthesis of viral proteins at the nonpermissive temperature. Whether this is the cause or the result of the failure of the virus to shut off all host cell protein synthesis at 37°C remains to be determined.

RNA Synthesis by the Recombinant Virus at the Permissive and Nonpermissive Temperatures. One of the biological predictions that arises from our [³⁵S]methionine labeling experiments with PCV110-infected HeLa cells at 37°C is that viral-specific RNA synthesis should also be reduced. Such a reduction would be due to the low levels of proteins involved in RNA synthesis (e.g., the viral RNA polymerase, 3D) that are present up to 7 hr after infection by PCV110 at 37°C. To test the above prediction, we labeled infected cells with [³H]uridine at either 33.5°C or 37°C in the presence of actinomycin D to shut off host RNA synthesis. After addition of actinomycin D and [³H]uridine, the kinetics of RNA synthesis were monitored by continuous labeling for up to 4.5, 5.5, or 6.5 hr after infection. The results (Fig. 3) reveal that infection by wild-type PV1 at 37°C yields high levels of virus-specific RNA synthesis between 4 and 6 hr after infection. Similar kinetics of RNA synthesis are observed in wild-type-infected cells at 33.5°C but the overall levels of synthesis are decreased by a factor of >2. Again, this observation is a reflection of the slower growth of poliovirus in HeLa cells infected at 33.5°C.

When [³H]uridine incorporation was used to measure the relative rates of viral RNA synthesis in HeLa cells infected with the recombinant virus PCV110, the data differed markedly from that obtained after wild-type infection. As shown in Fig. 3, the recombinant virus infection at 33.5°C yields readily detectable levels of viral RNA synthesis between 4 and 6 hr after infection. When compared to RNA synthesis by the wild-type virus (PV1) at 33.5°C, the levels detected in PCV110-infected cells are reduced by factors of 2 and 3. However, when the relative rate of RNA synthesis by the recombinant virus at 37°C is compared to that of recombinant viral RNA synthesis at 33.5°C, the level of viral RNA synthesis at the nonpermissive temperature (37°C) is reduced

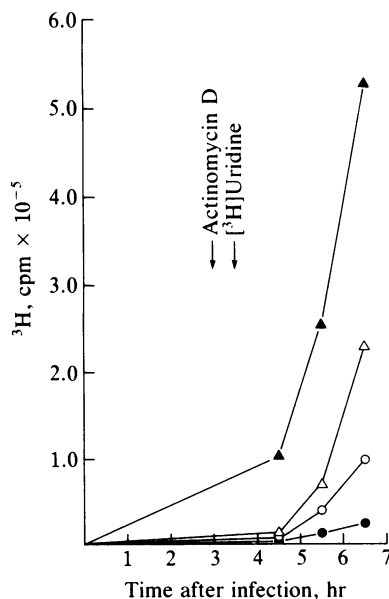


FIG. 3. Analysis of viral-specific RNA synthesis in HeLa cells infected with recombinant virus PCV110 and wild-type poliovirus. The PCV110 recombinant virus stock used in this experiment was from plaque isolate no. 4 as shown in Table 2. ▲, Wild-type poliovirus at 37°C; △, wild-type poliovirus at 33.5°C; ●, recombinant virus PCV110 at 37°C; ○, recombinant virus PCV110 at 33.5°C.

by a factor of almost 5, as measured 6.5 hr after infection. In addition, compared to that of the wild-type virus at 37°C, the rate of synthesis of viral RNA by the recombinant virus at 37°C (as measured 6.5 hr after infection) is reduced by a factor of >20. It therefore appears that the temperature-sensitive defect in the recombinant virus (PCV110) causes a reduced rate of viral RNA synthesis at the nonpermissive temperature, either as a consequence of the reduced levels of viral-specific proteins in infected cells or as a result of an altered (decreased) recognition of the recombinant RNA template by viral proteins involved in RNA replication.

DISCUSSION

We have isolated a chimeric plasmid that contains the poliovirus polyprotein coding region and a hybrid polio-coxsackievirus 5' untranslated region. When such a plasmid (i.e., pCP110) was introduced into cultured monkey cells by transfection procedures, infectious virus was produced. To our knowledge, PCV110 represents the first isolation of a viable recombinant derived from two different picornaviruses. Although the sequence homology between the PV1 and CB3 5' untranslated genomic regions is ≈70%, it was somewhat surprising that replacing ≈400 nucleotides within the polio genome by a CB3 segment that contains nearly 100 nucleotide differences would yield an infectious recombinant virus. Our own attempts to make small deletions (5–15 base pairs) in the 5' untranslated region of a cDNA clone (pEV104) of the polio genome have, to date, yielded no infectious virus (B.L.S., V.H.J., and M. F. Ypma, unpublished data). Perhaps it is the spacing of various blocks of nucleotides rather than the precise sequence of those nucleotides that is important in the ribosome binding/replicase recognition functions that may be supplied by the 5' untranslated regions of picornaviral genomes.

The results presented here show that recombinant virus PCV110 is temperature-sensitive in the production of viral-specific proteins as well as in the synthesis of viral RNAs. The temperature sensitivity is a unique property of the recombinant virus since both PV1 and CB3 grow to high titers in human cells at 37°C (33–35). The actual nature of the defect in protein/RNA synthesis is at present unknown. It is possible that the defect is at the level of initiation of viral RNA synthesis, perhaps at the step of polymerase binding to the 3' ends of minus-strand RNAs. Thus, at 37°C, the ts defect of PCV110 may interfere with genome amplification early in the infectious cycle of the virus and would eventually lead to lower levels of viral-specific proteins and RNAs in infected cells. It has been previously shown that nucleotide changes in the 3' extracistronic region of bacteriophage Qβ RNA produces changes in *in vitro* RNA replication (36) as well as in *in vivo* replication (37, 38). A conditional mutation in the 3' noncoding region of poliovirus RNA has also been reported (39).

The mechanism by which the polio-coxsackie virus exhibits a ts phenotype is unclear. One possible explanation is that the recombinant RNA molecule itself assumes an "incorrect" or less thermodynamically stable structure at 37°C because of the presence of ≈30% mismatched nucleotides in the recombinant RNA. The high degree of homology (≈70%) between the corresponding PV1 and CB3 sequences may allow the conservation of the most important secondary structures (in terms of biological function) in the 5' untranslated region at 33.5°C. However, the alteration of such structures at 37°C may result in a decreased efficacy of ribosome/replicase recognition of 5' proximal nucleotide sequences. Although the presence of secondary structures in the 5' ends of PV1 and CB3 RNAs has been suggested from partial RNA sequence analysis (21, 40) as well as from computer-assisted RNA structure analysis (2, 41), no clear

demonstration exists to date for such structures in any of the molecular events that occur during a picornavirus infection.

One of the differences that we observed in the growth of PCV110 at 33.5°C and 37°C was a failure to effect complete shutoff of host protein synthesis at the nonpermissive temperature. This preliminary finding suggests that the 5' untranslated region of the poliovirus genome may have a role in host shutoff. It may be argued that the lack of complete host shutoff simply reflects the delayed time course of viral infection at 37°C. However, the lack of amplification of viral genomes early in infection by PCV110 at 37°C cannot fully explain the defect in host shutoff because labeled host proteins are present even when large amounts of viral-specific polypeptides are detected at later times after infection (Fig. 2, lane 9). The unlikely possibility exists that a structural modification of the 5' untranslated region of the recombinant RNA affects the ability of the virus to completely shut off host protein synthesis.

Finally, the data presented here show that there are no strictly polio-specific sequences in the nucleotide 220- to 627-region of the genomic RNA since the CB3 genome in this region provides a functional replacement. It will be of interest to determine whether sequences more 5' proximal than nucleotide 220 contain the polio-specific signals required for RNA replication. In addition, it will be important to determine whether recombinant virus PCV110 is neurovirulent in monkeys since recent data (41, 42) suggest that the 5' untranslated region of poliovirus RNA may contribute to the expression of neurovirulence determinants.

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