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Isolation of Poliovirus 2C Mutants Defective in Viral RNA Synthesis

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Two poliovirus mutants were isolated that contain an oligonucleotide linker insertion in the 2C-coding region of the viral genome. One, 2C-31, has a strongly temperature-sensitive phenotype and the other, 2C-32, forms small plaques on HeLa cell monolayers at all temperatures. Both mutants have a severe temperature-sensitive defect in viral RNA synthesis but little effect on the types of viral protein that are made. Temperature shift experiments showed that the 2C function is continuously required for viral RNA synthesis to proceed. The 2C mutants could be complemented in *trans* by mutants with mutations in other viral proteins. Protein 2C is also the locus of the guanidine resistance and dependence mutants, a drug whose action also affects viral RNA synthesis. Thus, protein 2C is one that is needed continually for viral RNA synthesis and, at least with these temperature-sensitive alleles, can be provided in *trans*.

Poliovirus, a member of the picornavirus family, contains a single plus-strand RNA genome of about 7.5 kilobases. The viral genome encodes a single polyprotein that is proteolytically cleaved to give rise to various viral proteins (13, 14, 22, 32). The structural proteins, which form the viral capsid, are located at the amino-terminal P1 region, while the viral polymerase (3D), a protease (3C), and the genome-linked small peptide (VPg) are located at the carboxyl P3 region (see map in Fig. 1). The central P2 region of the viral polyprotein contains several polypeptides with poorly delineated functions. Protein 2A appears to be involved in the shutoff of host cell protein synthesis by infecting poliovirus (3) and also contains a protease activity that is required for the cleavage of the P1-P2 junction (35). Protein 2B is involved in RNA synthesis (2). Mutations providing resistance to and dependence on guanidine map to the 2C-coding region of the viral genome (15, 23–25). Guanidine hydrochloride can completely inhibit poliovirus replication at millimolar concentrations, and the major effect of guanidine appears to be blockage of viral RNA synthesis (7, 8, 21), although the mechanism of this effect remains obscure.

The genome of poliovirus type 1 (Mahoney) was the first picornavirus genome to be cloned (26) and sequenced (16, 26). The finding that the cDNA clone was infectious when transfected into mammalian cells (27) offered an opportunity to study the biology of poliovirus through well-defined mutants made by in vitro mutagenesis of the cDNA clone, and several such mutants have been obtained (2, 3, 28, 30). To study the function of polypeptide 2C, we made several cDNA constructs that carried a linker insertion in the 2C-coding region. When transfected into HeLa cells, two viable mutants were recovered, both of which were defective in viral RNA synthesis. Viral RNA synthesis at the permissive temperature rapidly ceased when the temperature was shifted to the nonpermissive condition. The inability of one of the mutants, 2C-31, to grow at the nonpermissive temperature was reversed by coinfection with other poliovirus mutants, suggesting that the function of 2C can be provided in *trans*.

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MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension culture in the Joklik modification of minimal essential medium supplemented with 7.5% horse serum (GIBCO Laboratories, Grand Island, N.Y.). Wild-type poliovirus was isolated from a single plaque derived from HeLa cells transfected with a pSV2-polio plasmid as described previously (3, 27, 30).

Linker-insertion mutagenesis. All enzymes and linkers were purchased from New England BioLabs, Inc. (Beverly, Mass.) unless noted and used according to the protocols of the manufacturer. The general method of linker-insertion mutagenesis has been described previously (18, 30, 31). Briefly, the plasmid pSV2-polio that contains the intact infectious poliovirus type 1 (Mahoney) cDNA sequence was digested partially with *Xba*I, *Bal*I, *Bam*HI, *Nsi*I, *Sph*I, or *Xmn*I. Alternatively, a plasmid containing only the poliovirus cDNA sequence from nucleotide 3660 to nucleotide 6056 was used for *Nco*I or *Rsa*I partial digestion. In both cases, the full-length linearized plasmid fragments with a single cut were then isolated from agarose gel for subsequent ligation with a linker. The DNA fragments with 5′ protruding ends were first filled in with the Klenow fragment of DNA polymerase I in the presence of all four deoxyribonucleotides, and those with 3′ protruding ends were treated with S1 nuclease (Sigma Chemical Co., St. Louis, Mo.). They were then ligated with a DNA linker and T4 DNA ligase. The DNA fragments with blunt ends were directly ligated with the linker. After mapping with various restriction enzymes, a *Bst*EII (3925)-*Bgl*II (5601) fragment containing a linker insertion in the 2C-coding sequence was cut from a plasmid and ligated into the corresponding region of a second plasmid identical to pSV2-polio except that it does not contain the *Eco*RI site present in pSV2-polio in the pBR-derived sequence. All plasmids containing the inserted linker were then selected on the basis of the newly introduced linker site and recircularized through the unique linker sequence to ensure a single-copy insertion. These plasmids were mapped again with various restriction enzymes before they were used to transfect HeLa cells. The sequences of the two plasmids that gave mutant viruses 2C-31 and 2C-32 were later verified by direct DNA sequencing through the region that contains the inserted linker.

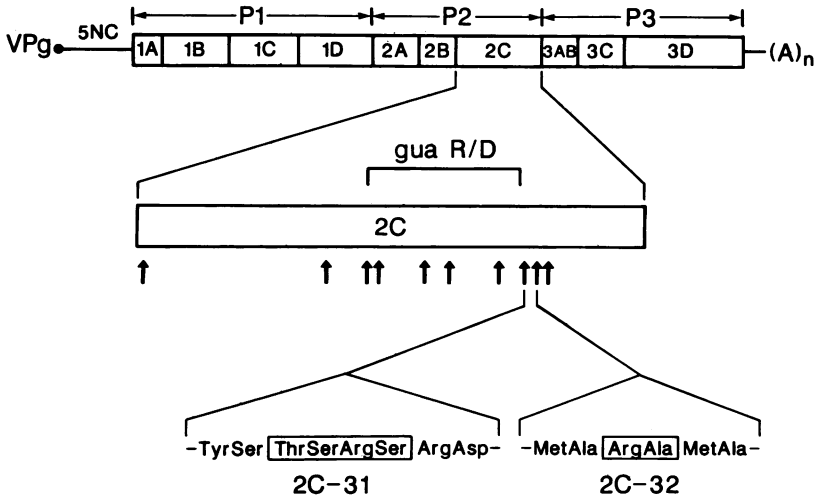

FIG. 1. Locations of the inserted DNA linker in each of the 10 poliovirus cDNA constructs. Arrows indicate their relative positions in the 2C-coding region. The corresponding restriction sites in the cDNA sequence (23) are (from left to right): *Sph*I (4154), *Rsa*I (4502 and 4585), *Bam*HI (4600), *Xmn*I (4684), *Nco*I (4728), *Nsi*I (4830), *Xba*I (4886), *Bal*I (4908), and *Nco*I (4911). An 8-bp *Eco*RI linker (5'-GGAATTCC-3') was used for ligations with *Bam*HI, *Nco*I, or *Xba*I fragments (pretreated with the Klenow fragment of DNA polymerase I), and a 10-bp *Eco*RI linker (5'-CGGAATTCCG-3') was used for ligations with *Sph*I or *Nsi*I fragments (pretreated with S1 nuclease). For ligations with the blunt-ended fragments created by *Rsa*I, *Xmn*I, or *Bal*I digestion, a 6-bp *Sma*I linker (5'-CCCGGG-3') was used. The resultant insertions in the amino acid sequence of 2C mutants are shown in boxes. gua R/D, Region in which guanidine-resistant and -dependent determinants were mapped (15, 23, 24).

DNA transfection. At 24 h before transfection, HeLa cells were harvested from suspension culture and plated onto 60-mm petri dishes at about 5×10^5 cells per dish. About 5 μg of DNA from both the wild-type and linker-insertion-mutagenized pSV2-polio plasmids was used in a single transfection by the DEAE-dextran procedure (20). Immediately after transfection, HeLa cell dishes were overlaid with 1% agar in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum (GIBCO) and incubated at 32, 37, or 39.5°C for up to 7 days. Individual plaques were then isolated from the agar overlayer and plaque purified again before propagation of the virus in liquid culture.

Virus infections and metabolic labeling of infected cells. For HeLa cell infections, about 0.5×10^6 to 1×10^6 cells growing in suspension were collected by centrifugation and resuspended in 0.2 ml of minimal essential medium. Virus was then added to the cell suspension and allowed to adsorb for 30 min at room temperature. After adsorption, the cells were diluted in fresh medium and incubated at various temperatures for periods of time specified for each experiment. For protein labeling with [^{35}S]methionine, medium was removed by centrifugation several hours after infection and cell pellets were resuspended in methionine-free minimal essential medium. After a brief incubation, [^{35}S]methionine (>800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) was added to a final concentration of 50 μCi/ml. Incubation of cells was continued for 30 min, and then incorporation was stopped by placing the culture on ice. After being washed three times with ice-cold phosphate-buffered saline, the cell pellets were collected by centrifugation and lysed in phospholysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS] in phosphate-buffered saline). Chromatin was removed by centrifugation, and the supernatants were used for immunoprecipitation with an anti-2C antiserum as described previously (3) or they were used directly for SDS-polyacrylamide gel electrophoresis essentially by the method of Laemmli (17).

For analysis of viral RNA synthesis, [^{3}H]uridine (50 μCi/ml; Dupont, NEN Research Products) was added several hours after infection and the cells were then incubated in the presence of 5 μg of actinomycin D per ml for various periods. They were then lysed in phospholysis buffer, and RNA was prepared from the cell lysates for analysis by agarose gel electrophoresis (3).

Genetic complementation analysis. The methods for genetic complementation among poliovirus mutants and the calculation of the complementation index have been described previously (2).

RESULTS

Isolation and growth of poliovirus 2C mutants. Two small-plaque mutants in protein 2C were created by site-directed linker insertion: 2C-31 and 2C-32. Figure 1 shows the locations of linker insertions into the 2C region of poliovirus cDNA constructs and the corresponding changes in amino acid sequence in the two viable mutants. Eight other insertions were apparently lethal to viral replication because no virus was recovered when these constructs were used in the transfection assay. The cDNA of 2C-31 contained a 12-base-pair (bp) insertion (see legend to Fig. 1 for details) at position 4886 (according to reference 26) which resulted in a four-amino-acid insertion in the 2C sequence. The construct of 2C-32 contained a 6-bp insertion at position 4908 resulting in a two-amino-acid insertion. When the two cDNA constructs were transfected into HeLa cells, they gave rise to viable virus after 6 to 7 days of incubation at 32°C. At 39.5°C, no virus was recovered from HeLa cells transfected with 2C-31 cDNA. The temperature-sensitive (*ts*) phenotype of 2C-31 was confirmed by single-cycle virus infection of HeLa cells. At 39.5°C, the yield of 2C-31 in a single cycle of infection was about 10^{-3} to 10^{-4} of the yield at 32°C (Fig. 2). Furthermore, the relative titer of a viral stock at 39°C was also about 10^{-3} to 10^{-4} of the titer at 32°C (data not shown). Even at the permissive temperature, the yield of 2C-31 was about 100-fold lower than that of wild-type poliovirus (Fig. 2). A given stock of 2C-32 gave similar titers of small plaques at all temperatures (data not shown); the yield at 39.5°C in a

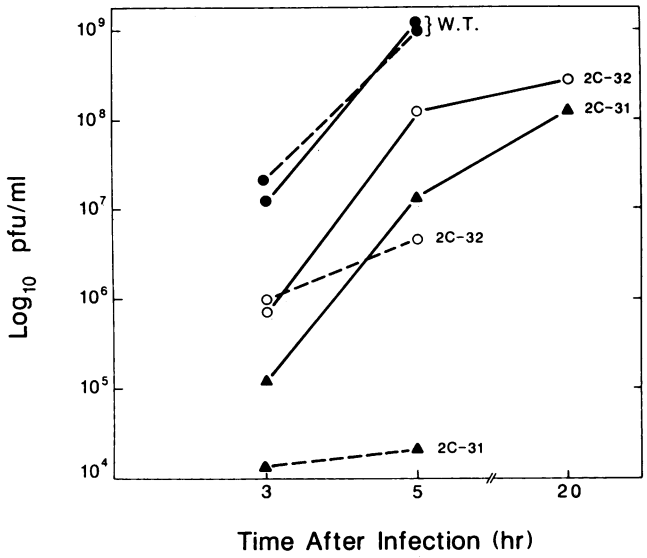

FIG. 2. Growth curves of mutant and wild-type (W.T.) polioviruses in a single cycle of infection. All infections were carried out in a HeLa cell suspension (10^6 cells per ml) with a multiplicity of infection of 10. At each time point, 1 ml of cell suspension was taken, virus was released by three cycles of freeze-thawing, and titers were determined by standard plaque assay on HeLa cells. ——, Infections done at 32°C; - - - - -, infections done at 39.5°C.

single-cycle infection was at least 10-fold less than that at 32°C, indicating a significant temperature sensitivity, although not one sufficient to alter the efficiency of plaque formation.

Protein synthesis in mutant-infected cells. The pattern of viral protein synthesis and the concomitant shutoff of cellular protein synthesis by 2C mutants was examined by labeling infected cells with [^{35}S]methionine. At 39.5°C, a temperature that significantly lowered the rate of growth of 2C-32 and completely restricted the growth of 2C-31, various viral proteins including 2C were synthesized, albeit at a lowered rate. Neither the cleavage of the various precursors nor the mobility on SDS-polyacrylamide gels of the 2C protein was affected by the mutations (Fig. 3). This was further confirmed by SDS-polyacrylamide gel electrophoretic analysis of mutant 2C proteins immunoprecipitated by an anti-2C antiserum (Fig. 4). The low amount of viral proteins being synthesized in the mutant-infected cells was explained by the minimal amount of viral RNA made at 39.5°C (see below). The shutoff of cellular protein synthesis by both mutants at 39.5°C was somewhat delayed, but both mutants were able to shut off host cell protein synthesis completely by late during the infection cycle (Fig. 3; data not shown). At 32°C, the overall pattern of viral protein synthesis by both mutants was very similar to that of wild-type virus (data not shown).

Viral RNA synthesis. To examine viral RNA synthesis by the 2C mutants, HeLa cells were labeled with [^{3}H]uridine at 4.5 h (39.5°C) or 6 h (32°C) after infection and RNAs were prepared from cell lysates 30 min later and analyzed by agarose gel electrophoresis (Fig. 5). At 32°C, both 2C-31 (Fig. 5A, lane 2) and 2C-32 (Fig. 5A, lane 1) synthesized an amount of viral RNA severalfold less than that of wild-type virus (Fig. 5A, lane 3). Both single-stranded and double-stranded viral RNAs were apparent. At 39.5°C, however, there was very little if any viral RNA synthesis in 2C-31-infected cells (Fig. 5B, lane 2). This correlated well to the *ts*

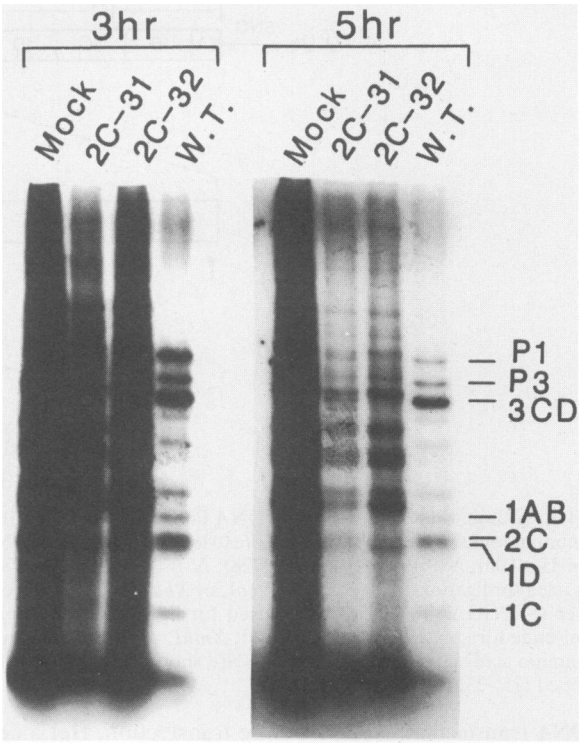

FIG. 3. Pattern of protein synthesis in virus-infected cells at 39.5°C. About 10^6 HeLa cells were used in a single infection with a multiplicity of infection of 10, and 1/200th of the total cell lysates prepared at 3 or 5 h after infection was loaded on each lane of an SDS-12.5% polyacrylamide gel. W.T., wild type; mock, mock infection.

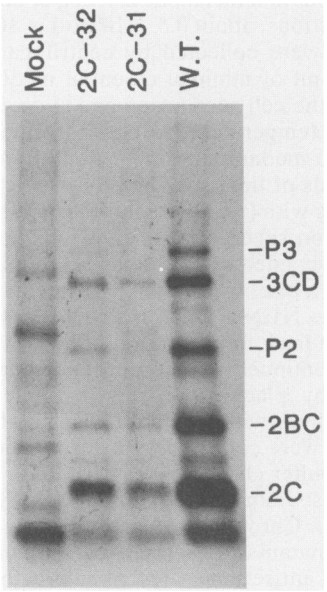

FIG. 4. Immunoprecipitation of viral protein from HeLa cells infected for 5 h. Infection was at 39.5°C. An anti-2C antiserum which also contains anti-P3 activity (3) was used. See the legend to Fig. 3 for details.

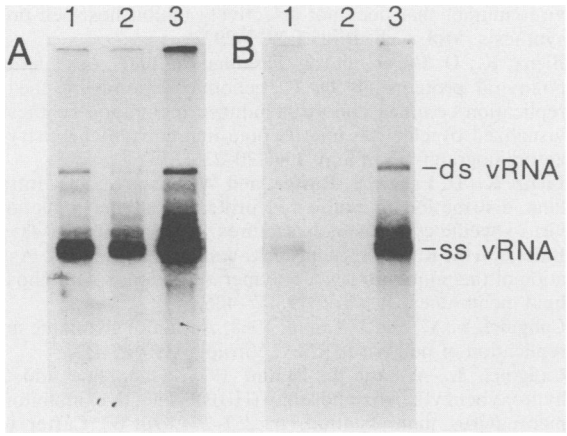

FIG. 5. Viral RNA synthesized in infected HeLa cells analyzed by electrophoresis through a native 1% agarose gel. Infections were carried out at 32°C (A) or 39.5°C (B) for 6 or 4 h, respectively, with a multiplicity of infection of 50. Samples analyzed were 1/10th of the total RNA from 10^6 HeLa cells. Lanes 1, 2, and 3, RNA from 2C-32-, 2C-31-, and wild-type virus-infected HeLa cells, respectively. ds vRNA, Double-stranded viral RNA; ss vRNA, single-stranded viral RNA.

phenotype manifested by this mutant and suggested that protein 2C is directly involved in the process of viral RNA synthesis. This was supported by 2C-32-infected cells (Fig. 5B, lane 1) which showed a drastic reduction in viral RNA synthesis correlating with temperature sensitivity of the yield of 2C-32 in a single cycle of infection (Fig. 2).

When infections were done at the permissive temperature for 6 h and then the temperature was shifted to the nonpermissive condition, viral RNA synthesis in 2C-31-infected cells rapidly ceased (Fig. 6B), while similar shifting had little effect on RNA synthesis by wild-type virus (Fig. 6A). This result indicates that the 2C protein accumulated during infection at the permissive temperature in 2C-31-infected cells could maintain viral RNA synthesis for only a short period after the switch of temperature, suggesting that the involvement of 2C in viral RNA synthesis is probably not just an early event during poliovirus replication. Rather, the continual presence and perhaps synthesis of this protein is required to complete the replication cycle.

Genetic complementation analysis. To examine whether the potential function of 2C could be provided in *trans*, we coinfected HeLa cells with mutant 2C-31 and each of two small-plaque mutants with lesions elsewhere in the viral genome. The first, 2B-11, contains a six-nucleotide insertion at position 3933 in the 2B-coding region of the viral genome (J.-P. Li and D. Baltimore, unpublished data). The second, 3D-3, contains a lesion in the sequence encoding the viral polymerase, 3D (2). The growth of these two mutants is not complementable by other mutants (2; unpublished observations). The virus yield for 2C-31 in a single cycle of infection at 39.5°C could be increased several hundredfold by coinfection with one of the two small-plaque mutants (Table 1). The increase in titer of 2C-31 in the mixed infection was demonstrated by examining the phenotype of progeny virus in individual plaques derived from the mixed infections: more than 70 and 50% of the individual plaques derived from 2C-31 plus 2B-11 and 2C-31 plus 3D-3, respectively, contained progeny virus with a *ts* phenotype. These results clearly indicate that the function of 2C can be provided in *trans*. With mutant 3D-3, the complementation was so efficient that the yield of 2C-31 was only threefold less than that of wild type.

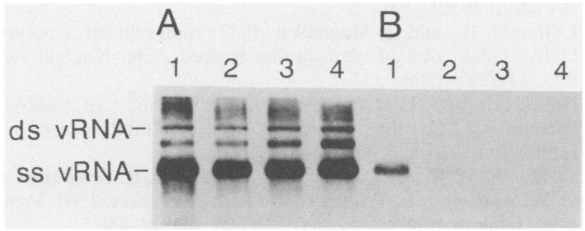

FIG. 6. Effect of a temperature upshift on viral RNA synthesis. Infections were first carried out at 32°C for 6 h, and then the temperature was shifted to 39.5°C for 15 min (lane 1), 30 min (lane 2), 45 min (lane 3), and 60 min (lane 4). [^{3}H]uridine was added 15 min before each sample was taken. (A) RNA samples from wild-type virus-infected cells. (B) RNA samples from 2C-31-infected cells. ds vRNA, Double-stranded viral RNA; ss vRNA, single-stranded viral RNA.

DISCUSSION

By isolating mutants with a recognizable phenotype in protein 2C, we partially characterized the function of the protein. The protein is necessary for viral growth and appears to be involved in viral RNA synthesis. Its function must be performed continuously through the infection cycle, and it can be provided in *trans* to the viral genome.

Previous data suggested that protein 2C is involved in RNA synthesis. The high degree of amino acid sequence conservation of protein 2C among picornaviruses (1, 10, 23) argues for its importance, and the demonstration that 2C is associated with the membrane-bound viral replication complexes in poliovirus-infected cells (5, 6, 33) suggests that it is inherent in the replication process. This perspective is further supported by the observation that several guanidine-resistant and guanidine-dependent poliovirus mutants map to within the region that encodes protein 2C (15, 23–25). Guanidine hydrochloride can block the growth of many picornaviruses, including poliovirus, at millimolar concentrations (8, 9, 19, 29). The mechanism of guanidine action has been a subject of debate, but one result of guanidine action is a blockage of viral RNA synthesis, particularly that of single-stranded RNA (7, 8, 21).

Our results also imply that protein 2C function is required for effective viral RNA synthesis, but no studies prove that it is directly involved in any particular step of viral RNA

TABLE 1. Complementation of 2C-31 by other mutants[a]

Virus	Titer (PFU/ml)[b]		CI[c]
	32°C	39°C	
Wild type	1.3×10^8	1.4×10^8	
2C-31	5×10^4	5×10^2	
2B-11	6.5×10^6 (*ts*, 0%)	9×10^6	
3D-3	2×10^7 (*ts*, 0%)	1.5×10^7	
2C-31 + 2B-11	3×10^7 (*ts*, ≥70%)	7×10^6	460
2C-31 + 3D-3	6×10^7 (*ts*, ≥50%)	2.2×10^7	760

[a] All single and mixed infections were carried out at 39.5°C for 4 h with a multiplicity of infection of 5 for each virus.

[b] Titers of the viruses harvested from single or mixed infections containing 10^6 HeLa cells each in 2 ml of minimal essential medium. All titrations were done on HeLa cell dishes at both 32 and 39.5°C.

[c] CI (complementation index) values for mutant 2C-31 from a mixed infection were calculated as described previously (2).

synthesis. Because elongation of RNA chains appears to require only protein 3D (the polymerase [36]), a likely role for 2C is in initiation of RNA synthesis, a process whose mechanism is still shrouded.

Guanidine inhibition has been ascribed both to inhibition of release of RNA chains from the replication complex (11), a process about which little is known, and to inhibition of initiation of RNA chains (7, 8, 34). Because temperature shift experiments with mutant 2C-31 showed no excess of double-stranded RNA, while guanidine inhibition does produce such an excess, the two mechanisms of inhibition could be different. Thus, there may be multiple functions performed by 2C.

It has been observed that protein 2C (or its precursors) is first free in the cytoplasm after being synthesized in the virus-infected cells and then becomes associated with membranous structures in which viral RNA synthesis proceeds (4). One function of 2C, therefore, may be to attach the viral RNA polymerase (6), the viral RNA, or the viral replication complex as a whole onto membranes (4).

The nature of the lesions in protein 2C caused by the 2C-31 and 2C-32 mutations is unclear. They do not affect the cleavage of 2C from its precursors but could affect its folding pathway or its functional sites. The loss of RNA synthesis after a shift to nonpermissive temperature late in the infection cycle points to a continual need for 2C, but that could be either a consequence of a denaturing effect on 2C or a need for newly made 2C. Viral RNA synthesis proceeds normally even if viral protein synthesis is seriously curtailed (3), so relatively small amounts of protein 2C should suffice for normal RNA synthesis. However, larger amounts may be needed when protein synthesis is normal to maintain the stoichiometry of possible protein-protein complexes.

With mutant 2C-31, the requirement for protein 2C could be provided very efficiently in *trans*. This contrasts to certain guanidine-dependent (resistant) alleles that are virtually noncomplementable (12). It could be that 2C is needed in *cis* for one function but can be provided in *trans* for others. We previously noted that many of the loci involved in RNA synthesis are *cis* active (2), possibly because of an involvement in initial negative-strand synthesis.

Of 10 cDNA constructs that carried a linker insertion in the 2C-encoding sequence, only the two reported here gave rise to viable viruses (Fig. 1). All the other introduced mutations apparently were lethal to viral replication. It is possible that this region of the viral genome, given the fact that it is highly conserved among various picornaviruses, is extremely sensitive to changes. Alternatively, perturbation of polyprotein secondary structure by the mutations could prevent proper cleavage.

ACKNOWLEDGMENTS

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