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Production of Guanidine-Resistant and -Dependent Poliovirus Mutants from Cloned cDNA: Mutations in Polypeptide 2C Are Directly Responsible for Altered Guanidine Sensitivity

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cDNA fragments representing the region in polypeptide 2C containing mutations in a guanidine-resistant or -dependent mutant were cloned into the wild-type background of an infectious clone. Transfection of COS-1 cells with these plasmids yielded viruses that were either completely resistant to 2.0 mM guanidine hydrochloride or dependent on this concentration of drug for growth.

The three poliovirus serotypes, like other members of the *Picornaviridae* family, contain a single-stranded RNA genome of positive polarity approximately 7,500 nucleotides long (9, 18). This genomic RNA is 3′ polyadenylylated and linked at the 5′ end to a small protein called VPg (3B; for a new nomenclature, see reference 21) (9). The RNA is translated into a single continuous polyprotein with a molecular weight of 246,000 (10), which is subsequently processed by two viral proteases to yield the viral structural and nonstructural proteins (14).

The growth of many picornaviruses, including poliovirus, is selectively inhibited by guanidine hydrochloride at concentrations of 0.1 to 2.0 mM (5, 6, 12, 20). Although guanidine inhibits several virus-induced processes, the primary effect appears to be blockage of synthesis of viral RNA, particularly production of single-stranded RNA (3–5, 15). The specific site of inhibition appears to be the initiation step of RNA synthesis (3, 5, 27), although release of completed RNA chains might also be blocked under certain conditions (8).

Several lines of evidence suggest that protein 2C is the viral gene product responsible for the guanidine trait. Mutants capable of growing in the presence of guanidine were isolated from both foot-and-mouth disease virus and poliovirus. Analysis of virus-induced polypeptides in guanidine-resistant foot-and-mouth disease virus mutants showed that polypeptide 2C was altered in 5 of 10 mutants, an observation suggesting that 2C may be the target of the antiviral action of guanidine (23). Similar results have been described for poliovirus (1); 75% of guanidine-resistant mutants contained modifications in protein 2C. Recombinants between appropriate poliovirus strains unambiguously mapped the guanidine-resistant mutation(s) downstream from polypeptide $2A^{pro}$ in the region of the viral genome specifying protein 2C (7, 17). Recently we have sequenced poliovirus type 1 genomic RNA isolated from (i) six mutants resistant to 2.0 mM guanidine, (ii) one mutant resistant to 0.5 mM guanidine, and (iii) two mutants whose growth was dependent on the addition of guanidine (17). The mutants resistant to 2.0 mM guanidine all contained an amino acid substitution in 2C at the same position. Resistance to this level of drug required two nucleotide substitutions in the same codon that resullted in an exchange of either N→G or N→A. The other types of mutant each contained one or two amino acid changes also within the 2C coding region.

Racaniello and Baltimore (19) reported that a complete cloned cDNA copy of the Mahoney strain of poliovirus was infectious in mammalian cells. Semler et al. (24) showed that a similar clone containing simian virus 40 transcription and replication signals had higher specific infectivity. These observations had profound implications for the study of picornavirus genetics because they created the possibility of creating specifically designed mutant or recombinant viruses by transfecting cells with cDNA which has been modified by DNA manipulation techniques. By this technique, interstrain recombinants have been obtained for the purposes of determining the molecular basis of attenuation, as well as for further studies of antigenicity (11, 26). This technique has also been used to generate specific mutants of poliovirus with novel phenotypes (2, 22, 25). In this communication we describe the cloning of cDNA segments containing either the mutation for resistance to 2.0 mM guanidine or the mutations responsible for guanidine dependence into the wild-type background of an infectious clone. The viruses isolated from transfected cells were found to express the resistance or dependence phenotype, demonstrating that mutations in protein 2C are directly responsible for altered guanidine sensitivity.

The lack of unique restriction sites at the boundaries of the 2C region of our infectious clones (nucleotides 4124 to 5110) caused us to adopt the strategy for cloning this region from the resistant and dependent mutants depicted in Fig. 1. We synthesized double-stranded DNA from viral RNA of the guanidine-resistant (GR2) or -dependent (GD1) mutant by using reverse transcriptase as described previously (16). The cloning vector pSP-1 was prepared by addition of a *Bgl*II linker (CAGATCTG) (New England BioLabs, Inc.) at the unique *Eco*RI site of pXf3 (13). After digestion of pSP-1 with *Pst*I and *Bgl*II, the large fragment containing the gene for tetracycline resistance was purified and ligated to the double-stranded DNA which had been digested with these two enzymes. Tetracycline-resistant colonies of *Escherichia coli* HB101 were obtained after transformation with the ligation mixture and screened for the presence of poliovirus-specific inserts by rapid plasmid preparations and restriction endonuclease digestions (24). Plasmids were identified that contained poliovirus-specific inserts spanning nucleotides 3417

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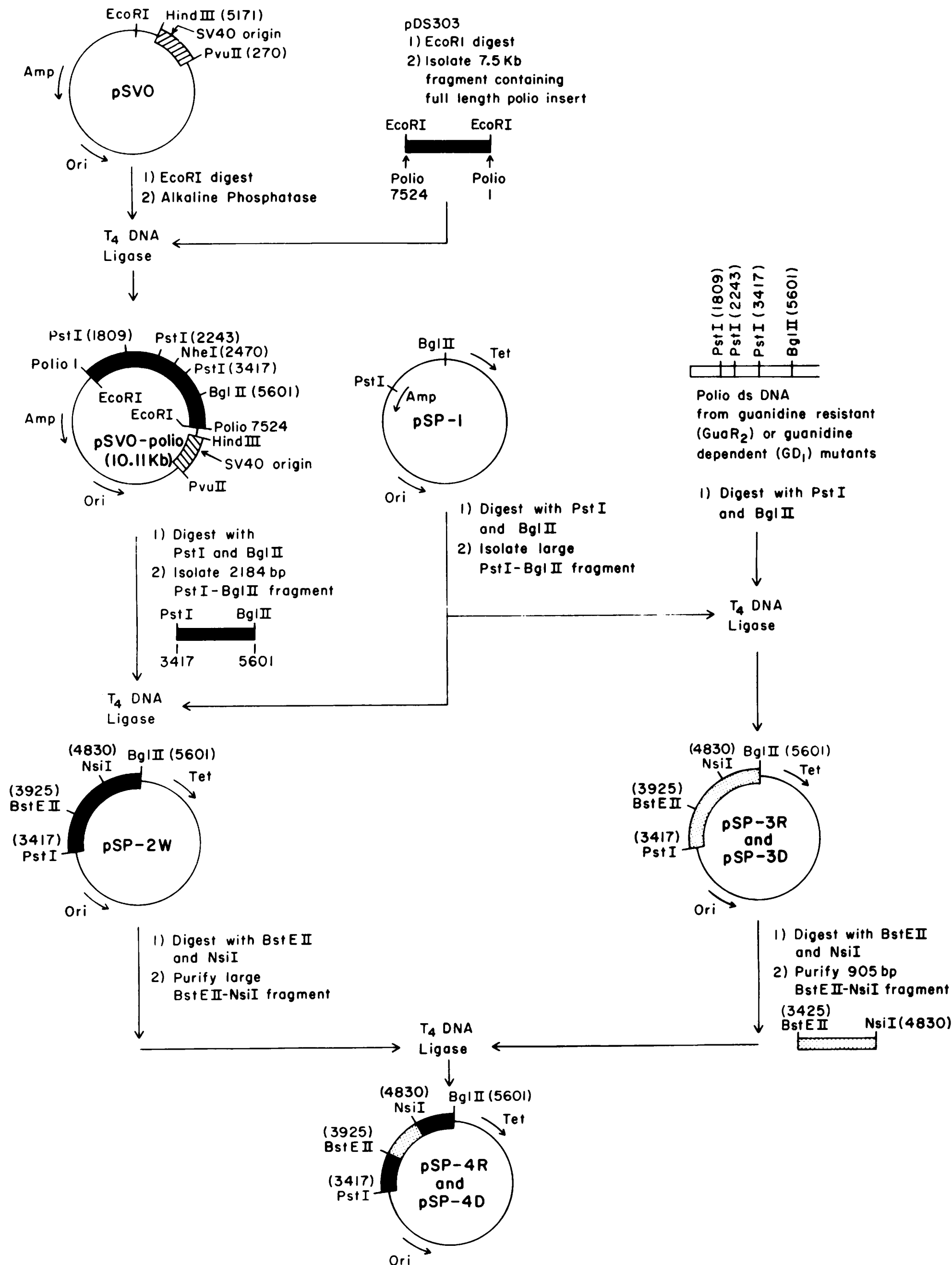

FIG. 1. Cloning of poliovirus double-stranded DNA encoding polypeptide 2C from a guanidine-resistant (GR2) or -dependent (GD1) mutant.

to 5601 of either the resistant (pSP-3R) or the dependent (pSP-3D) mutant genome.

Sequence analysis of genomic RNA of GR2 and GD1 from nucleotides 4048 to 5138 revealed an asparagine-to-glycine change within 2C (amino acid 179, nucleotides 4658 to 4660) in both mutants and an isoleucine-to-methionine change within 2C in GD1 (amino acid 227, nucleotides 4802 to 4804) (17). We have previously shown that the resistance of another mutant containing the N→G change maps to the 3′ side of nucleotide 4444 (17). Thus, pSP-3R and pSP-3D contain regions of the mutant genomes that should not be needed for expression of the altered guanidine phenotypes. The following strategy was used to eliminate these additional regions.

A full-length poliovirus insert was isolated from pDS303 (24) and cloned into the *Eco*RI site of pSVO, which contained the simian virus 40 origin and enhancer sequences. The resulting clone (pSVO-polio) was found to have as high a specific infectivity as pEV104 (24; Putnak and Wimmer, unpublished data). A *Pst*I-*Bgl*II fragment from pSVO-polio was ligated to the *Pst*I-*Bgl*II fragment from pSP-1 described above resulting in a plasmid (pSP-2W) containing wild-type poliovirus sequences from nucleotides 3417 to 5601. pSP-2W was digested with *Bst*EII and *Nsi*I, and the large fragment

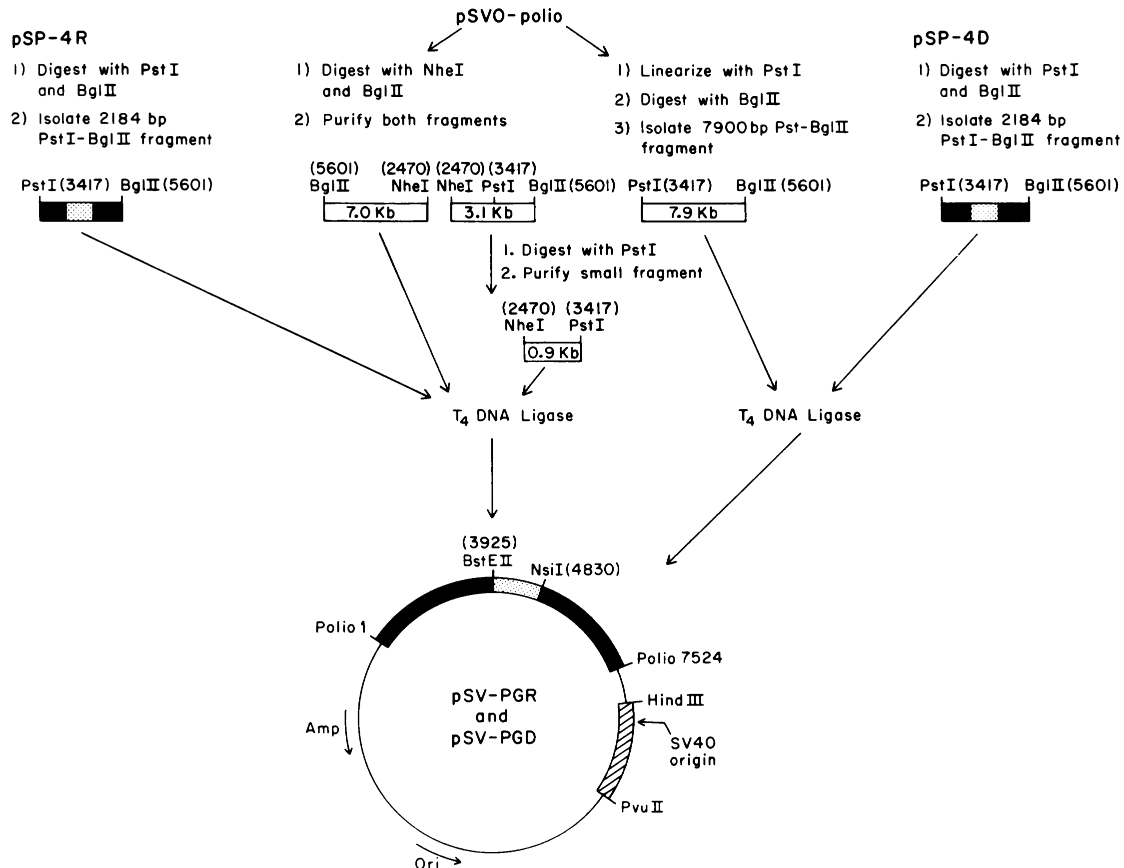

FIG. 2. Scheme for construction of full-length cDNA clones of poliovirus containing the 2C region from a guanidine-resistant or -dependent mutant.

was purified and ligated to the *Bst*EII-*Nsi*I fragment (nucleotides 3925 to 4830) from pSP-3R or pSP-3D. Plasmids were identified that contained the resistance (pSP-4R) or dependence (pSP-4D) region in a wild-type background. Since the region from nucleotides 3925 to 4048 had not been sequenced in the dependent mutant, we had not ruled out the possibility that a mutation required for dependence was contained within this region. A *Hinc*II-*Nsi*I fragment (nucleotides 3913 to 4830) was isolated from pSP-4R or pSP-4D and sequenced in the M13 system (Bethesda Research Laboratories, Inc.); no additional mutations were found in either plasmid within this region.

Full-length clones containing the resistance or dependence regions were generated by the strategy depicted in Fig. 2. A 7,900-base-pair *Pst*I-*Bgl*II fragment was isolated from pSVO-polio by partial digestion with *Pst*I and complete digestion with *Bgl*II. This fragment was ligated to the *Pst*I-*Bgl*II poliovirus specific insert from pSP-4D, and after transformation a full-length clone (pSV-PGD) was identified. Our attempts to obtain a full-length resistant clone by this strategy were unsuccessful for reasons that are not clear. We overcame this problem by performing a three-fragment ligation. pSVO-polio was digested with *Nhe*I and *Bgl*II, and the resulting fragments (7.0 and 3.1 kilobases) were purified. The 3.1-kb fragment was digested with *Pst*I, and a 0.9-kb fragment was purified. Ligation of the 7.0-kb and 0.9-kb fragments and the poliovirus-specific insert from pSP-4R resulted in a full-length clone (PSV-PGR). The N→G mutation results in loss of a *Hin*FI site. We verified that PSV-PGD and PSV-PGR contained mutant sequences by comparing the *Hin*FI pattern of these clones to that obtained with pSVO-polio.

To test whether our full-length clones containing the resistance or dependence region would produce infectious virus on introduction of plasmid DNA into primate cells, we carried out transfection of COS-1 cells by using the modified calcium phosphate coprecipitation technique (24). COS-1 cells were grown to confluency in 6-cm (diameter) plastic dishes and transfected in duplicate with 50 ng of pSVO-polio, PSV-PGD, or PSV-PGR with a 20% glycerol shock. The plates were incubated at 37°C for 2 days after which half received 2.0 mM guanidine hydrochloride. Addition of guanidine before this time caused total cell death in mock-transfected plates. After 7 days at 37°C, those plates in which the cells were no longer attached were subjected to three cycles of freeze-thawing, the cell debris was removed by centrifugation, and the virus-containing supernatants were titrated in the absence or presence of 2.0 mM guanidine hydrochloride as previously described (17).

The results of two separate experiments are presented in Table 1. Virus obtained by transfection of COS-1 cells with pSVO-polio in the absence of guanidine was as sensitive to 2.0 mM guanidine as our parental Mahoney strain of poliovirus. Addition of 2.0 mM guanidine on day 2 to cells transfected with this plasmid prevented the appearance of any cytopathic effect (data not shown). Cells transfected in the absence of guanidine with pSV-PGR produced virus that displayed similar titers when assayed in the presence or

TABLE 1. Characteristics of viral stocks derived upon transfection of COS-1 cells with recombinant polio-virus plasmids

Expt no. and plasmid	Titer (PFU/ml) With guanidine (A)	Titer (PFU/ml) Without guanidine (B)	Plaquing efficiency (A/B)
1			
pSVO-polio	1.0×10^{2}[a]	1.7×10^{8}	5.9×10^{-7}
pSV-PGR	5.8×10^{7}	6.6×10^{7}	0.9
pSV-PGD	8.2×10^{8}	2.9×10^{6}	280
2			
pSVO-polio	1.0×10^{2}[a]	1.9×10^{8}	5.3×10^{-7}
pSV-PGR	7.0×10^{7}	9.0×10^{7}	0.8
pSV-PGD	2.0×10^{8}	3.5×10^{5}	570

[a] Cytopathic effects were noted at a 10^{-2} dilution. Total cytopathic effect was seen at a 10^{-1} dilution.

absence of drug, indicating that this virus was as resistant to guanidine as was GR2. Virus produced by transfection of this plasmid in the presence of guanidine was equally resistant (data not shown). Transfection of cells in the absence of guanidine with pSV-PGD failed to produce any cytopathic effect (data not shown). Cells transfected with PSV-PGD in the presence of guanidine produced virus that displayed a 280- to 570-fold higher titer in the presence of drug than in its absence, indicating that this virus was as dependent on guanidine as was GD1.

In conclusion, the results presented here demonstrated that the N→G mutation in GR2 was directly responsible for its resistance to 2.0 mM guanidine hydrochloride and addition of the I→M mutation in GD1 resulted in a virus that required guanidine for growth. The results demonstrated that transfection of cells with cDNA clones is a powerful tool for identifying the genome location of mutations in poliovirus and for demonstrating that a mutation is responsible for an observed phenotype.

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