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Guanidine-Selected Mutants of Poliovirus: Mapping of Point Mutations to Polypeptide 2C

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Sequence analysis of the genomic RNA of interstrain guanidine-resistant and antibody-resistant variant recombinants of poliovirus type 1 mapped the resistance of mutants capable of growth in 2.0 mM guanidine hydrochloride to a region located 3′ of nucleotide 4444. This region of the viral genome specifies the nonstructural protein 2C. The sequence of genomic RNA encoding 2C from six independently isolated mutants resistant to 2.0 mM guanidine was determined. All six isolates contained a mutation in 2C at the same position in all cases, resulting in two types of amino acid changes. Dependent mutants were examined and found to contain two amino acid changes each within 2C. Mutants resistant to 0.53 mM guanidine were isolated and found to lack the mutations seen in variants resistant to 2.0 mM guanidine. A comparison of the amino acid sequences of the 2C proteins of poliovirus, foot-and-mouth disease virus, rhinovirus types 2 and 14, and encephalomyocarditis virus revealed a strong homology over regions totaling 115 residues. All of the mutations observed in guanidine-selected mutants were contained within this region. The amino acid region containing the mutations observed in poliovirus mutants resistant to 2.0 mM guanidine was compared with the homologous region in the other picornaviruses; a strong correlation was found between the amino acid present at this position and the sensitivity of the virus to 2.0 mM guanidine.

Guanidine hydrochloride acts as a protein denaturant at concentrations of 5.0 M or higher, but at millimolar levels it selectively blocks the growth of several togaviruses (20), several plant viruses (15, 51), and many picornaviruses, including poliovirus (7, 14, 29, 38). Although guanidine has been shown to interfere with maturation of poliovirus (24), prevent capsid proteins from associating with smooth membranes (52), and cause a reduction of choline incorporation into membranes (35), the major effect appears to be blockage of synthesis of viral RNA, particularly the production of single-stranded RNA (5–7, 32). The specific site of inhibition appears to be the initiation step of RNA synthesis (5, 7, 47). However, the release of completed RNA chains might also be blocked under certain conditions (23).

Genetic and biochemical studies on mutants of poliovirus able to grow in the presence of guanidine (g^r mutants) implicated the capsid proteins as the site of action of guanidine. Cooper (12) found that g^r mutants were linked genetically to temperature-sensitive mutations that were believed to be carried by capsid proteins. In some cases the capsid protein mutants themselves exhibited increased sensitivity to guanidine, whereas strains with temperature-sensitive mutations in nonstructural genes failed to display such a change (13). Korant (26) observed capsid protein modifications in viruses made g^r by multiple passage in the presence of guanidine. The properties of deletion mutants of poliovirus, however, seem to rule out the direct involvement of capsid proteins. Due to deletions in the genome region coding for the capsid proteins (33), these mutants make no detectable capsid protein, yet still retain the ability to synthesize RNA by a mechanism that is fully sensitive to guanidine (11).

Guanidine-resistant mutants of foot-and-mouth disease virus and of poliovirus have been isolated after a minimal number of passages in the presence of drug. When virus-induced polypeptides of g^r mutants of foot-and-mouth disease virus were examined by electrofocusing and tryptic peptide fingerprinting, synthesis of an altered nonstructural polypeptide (p34) was noted in 5 of 10 mutants, an observation suggesting that p34 (the counterpart to poliovirus protein 2C) is functionally involved in the antiviral action of guanidine (41). Similar results have been described for poliovirus (1); 75% of g^r mutants contained modifications in protein 2C. (Poliovirus polypeptide 2C was formerly known as NCVPX or P2-X. For a new nomenclature, see reference 40.) Studies of genetic recombination in foot-and-mouth disease virus (27, 31) and poliovirus have concluded that g^r is contained in the noncapsid region of the genome (17, 49).

In summary, recent evidence suggests that g^r is contained in the noncapsid region of poliovirus and that protein 2C is most likely the viral gene product responsible for the guanidine trait. The question arises as to what kinds of mutations give rise to g^r, whether they indeed map to 2C, and, if so, whether they are clustered in one region of 2C or scattered throughout its sequence. We isolated six independent mutants resistant to 2.0 mM guanidine and determined the sequence of genomic RNA encoding 2C. All six isolates contained a mutation in 2C at the same position in all cases, resulting in two types of amino acid changes. Dependent mutants were examined and found to contain two amino acid changes each within 2C. Mutants resistant to 0.53 mM guanidine were isolated and found to lack the mutations seen in variants resistant to 2.0 mM guanidine.

MATERIALS AND METHODS

Viruses. Guanidine-resistant and -dependent mutants (see below) were derived from the Mahoney strain of poliovirus type 1. The parental virus represented the third cell culture passage from plaque purification (17) and was the virus sequenced by Kitamura et al. (25).

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Virus growth in cell culture. Viral mutants were grown on HeLa cell monolayers (75-cm^2 flasks) at a multiplicity of infection of 0.01 PFU/cell in the presence of the appropriate amounts of guanidine hydrochloride as described previously (17). In some cases viral mutants were grown in HeLa S_3 suspension cultures as described previously (17).

Virus plaque titration. Samples (0.1 ml) of serial 1:10 dilutions (in phosphate-buffered saline) of the virus to be titrated were used to infect, in duplicate, HeLa cell monolayers in 4.9-cm^2 wells. After adsorption, the cells in each well were overlaid with 1.0 ml of higem medium (Flow Laboratories, Inc.) containing 5% newborn calf serum, 0.9% Noble agar, and the appropriate concentration of guanidine hydrochloride. Cells were stained after 48 h with 1.0 ml of 0.01% neutral red per well. The subsequently appearing plaques were quantitated.

Isolation of guanidine-resistant mutants. Independent mutants resistant to high (2.0 mM) levels of guanidine hydrochloride were isolated as follows. The parental virus was titrated in the presence and absence of 2.0 mM guanidine hydrochloride to estimate the level of the preexisting resistant population. Six HeLa cell monolayers (75-cm^2 flasks) were infected with a dilution of parental virus that contained 100-fold less virus than that which gave plaques in the presence of 2.0 mM guanidine hydrochloride. The monolayers were incubated at 37°C until all of the cells were no longer attached to the flask. After two cycles of freeze-thawing, the cell debris was removed by centrifugation, and 1.0 ml of each supernatant was used to infect a new monolayer in the presence of 2.0 mM guanidine hydrochloride. The virus-containing supernatants were harvested and titrated in the presence of 2.0 mM guanidine hydrochloride. An individual plaque was picked from each assay by removing the cells around the edge of the plaque with a sterile Pasteur pipette. The cells and agar were suspended in 1.0 ml of phosphate-buffered saline, and cell-associated virus was released by freeze-thawing twice.

Mutants resistant to intermediate (0.53 mM) levels of guanidine hydrochloride were isolated as follows. The parental virus was diluted 1:1 with phosphate-buffered saline, and 1.0 ml was used to infect an HeLa cell monolayer (75-cm^2 flasks) in the presence of 0.53 mM guanidine hydrochloride. The titer of the virus-containing supernatant was determined in the presence of 0.53 mM guanidine hydrochloride, and several plaques were picked.

Isolation of guanidine-dependent mutants. Parental virus (1.0 ml) was used to infect an HeLa monolayer in the presence of 2.0 mM guanidine hydrochloride. Two more passages were performed in the presence of 2.0 mM guanidine hydrochloride, followed by multiple passages in the presence of 4.0 mM guanidine hydrochloride. Virus titers were determined periodically by plaque assay in the absence or presence of 2.0 or 4.0 mM guanidine hydrochloride. The supernatant from passage 24 was found to contain a higher titer of virus when assayed in the presence of 2.0 or 4.0 mM guanidine hydrochloride than when assayed in its absence. A single plaque from the assay containing 2.0 mM guanidine hydrochloride was picked and labeled GD_1.

A second guanidine-dependent mutant was obtained in a single passage of guanidine mutant-free parental virus in the presence of 2.0 mM guanidine hydrochloride as described above for resistant mutants. This virus was labeled GD_2.

Virus purification and preparation of viral RNA. Viruses were grown on HeLa cell monolayers (75-cm^2 flask, five flasks per virus) as described above. After three cycles of freeze-thawing the cell debris was removed, and the supernatant was further clarified by centrifugation for 15 min at 15,000 rpm in a TY30 rotor at 5°C. Sodium dodecyl sulfate was added to the supernatant to a final concentration of 0.5%, and virus was pelleted by centrifugation for 4 h at 26,000 rpm in a TY30 rotor at 24°C. The viral pellets were suspended in 4.5 ml of 0.02 M sodium phosphate buffer (pH 7.0). CsCl (2.25 g) was added, and virus was banded by centrifugation for 22 h at 40,000 rpm in an SW50.1 rotor at 24°C. Virus was collected from the bottom of the tube after puncturing with a 24-gauge needle. The virus was then dialyzed against several changes of 0.02 M sodium phosphate buffer (pH 7.0) over a 24-h period.

Purified and dialyzed virus was phenol extracted, and viral RNA was recovered by ethanol precipitation.

Preparation of oligonucleotide primers. Primers were synthesized on a Systec 1450 with phosphoramidite chemistry by John Dunn. The crude product was purified as described previously (17). The sequence of the primers is as follows: 1b d(ACTCGTCTTGATGTC), X_1 d(TGACAGTTCTTACAC), X_2 d(GGGTGGTATAAACTCC), X_3 d(TTACCTGTTCCG GGGC), and X_4 d(ATTGGTGTATAGTTGAG).

Sequencing protocol. The viral RNAs were sequenced by dideoxy chain termination as described previously (17). Samples were loaded onto 6% buffer gradient gels (3). After electrophoresis the gels were processed for autoradiography as previously described (17).

RESULTS

Isolation of g^r mutants. Our goal was to determine the types of mutations responsible for the resistance of poliovirus to growth in the presence of 2.0 mM guanidine hydrochloride. Hence, several precautions were observed during the selection process. Our first concern was to avoid the selection of the same g^r mutant from a single stock of guanidine-sensitive virus. To circumvent this problem we first determined the resistant population in our parental virus stock by plaque assay in the presence of 2.0 mM guanidine hydrochloride. We then infected six HeLa cell monolayers with 100-fold less virus and used these preparations as independent parental stocks. These parental stocks were then used to infect HeLa cell monolayers in the presence of 2.0 mM guanidine hydrochloride. The viral stocks so generated were titrated in the presence of drug, and individual plaques were picked from each of the six assays. Our second concern was to prevent the accumulation of additional mutations by multiple passage of the resistant virus. Each plaque was used to infect a HeLa cell monolayer to generate an original stock. A second passage was performed on HeLa monolayers by infection in the presence of drug at a low multiplicity of infection (0.01 PFU/cell), and this virus was used as the working stock without further amplification.

The production of plaques in the absence and presence of guanidine was determined for mutant viruses (Tables 1 and 2). Our parental virus stock failed to yield well-defined plaques when assayed in the presence of 2.0 mM guanidine hydrochloride. Evidence of viral replication was seen, since at a 10^{-4} dilution of virus a cytopathic effect could be observed when cells were stained with neutral red and viewed under a phase-contrast microscope. In contrast, guanidine-resistant mutants displayed similar titers when assayed in the presence or absence of drug (Table 1). Two mutants were also compared which were dependent on guanidine for growth, resulting in a 1,000-fold-higher titer in the presence of drug than in its absence.

Mapping of guanidine resistance in recombinants to the region of the poliovirus genome coding for nonstructural

TABLE 1. Characteristics of guanidine mutants of poliovirus

Mutant	Titer (PFU/ml)		Plaqueing efficiency (A/B)
	With guanidine (A)	Without guanidine (B)	
GuaS	1×10^{4}[a]	5.5×10^{10}	1.8×10^{-7}
GuaR1	2.4×10^{8}	1.3×10^{8}	1.8
GuaR2	1.7×10^{10}	1.2×10^{10}	1.4
GuaR3	1.5×10^{8}	2.4×10^{8}	0.6
GuaR4	2.8×10^{8}	4.2×10^{8}	0.7
GuaR5	4.7×10^{8}	4.8×10^{8}	0.9
GuaR6	3.2×10^{8}	4.8×10^{8}	0.7
GD_1	2.2×10^{10}	2.2×10^{7}	980
GD_2	1.4×10^{9}	1.3×10^{6}	1,100

[a] Cytopathic effects were noted at a 10^{-4} dilution. Total cytopathic effect was seen at a 10^{-3} dilution (see text).

proteins. A neutralizing monoclonal antibody-resistant variant of the Sabin vaccine strain of poliovirus type 1 and a guanidine-resistant variant (GuaR5) of the virulent parent Mahoney strain were used in a coinfection to generate recombinant virus containing both resistance markers (17). Sequence analysis of one recombinant RNA (recombinant L) revealed that the crossover occurred between nucleotides 3919 and 4116 (data not shown). We have carried out further sequence analyses of genomic RNA of recombinant A which revealed that the crossover occurred between nucleotides 4444 and 4636 (Fig. 1). Both recombinants L and A had the same guanidine-resistant parent, and antibody resistance has been mapped to the 5′ capsid-encoding region (15a). It follows that the resistance of the poliovirus mutant GuaR5 to growth in 2.0 mM guanidine hydrochloride maps to the 3′ side of nucleotide 4444 in the region of the viral genome specifying the nonstructural protein 2C (Fig. 1).

Sequence analysis of genomic RNA encoding protein 2C of the resistant mutants. Genomic RNA encoding 2C of the resistant mutants was sequenced by dideoxy chain termination with oligonucleotide primers (Fig. 2A). All six resistant mutants contained a mutation in 2C at the same position (amino acid 179, nucleotides 4658 through 4660; Fig. 2). Surprisingly, the mutation did not result in all cases in the same amino acid replacement. Nevertheless, the mutants can be grouped into two classes: (i) those containing an asparagine-to-glycine change at this position (GuaR1, -2, -3, and -5, recombinant A) and (ii) those containing an asparagine-to-alanine change at this position (GuaR4 and 6). Both types of amino acid replacement are the result of nucleotide changes at the first and second positions of this codon (Fig. 2A). This unexpected result shows that the selection to a guanidine-resistant phenotype required a double mutation.

TABLE 2. Effect of guanidine on virus titers

Virus	Titer ($\log_{10}$ PFU/ml) at the following guanidine hydrochloride concn (mM):		
	0	0.53	2.0
Wild-type Mahoney	10.7 ± 0.3[a]	9.8 ± 0.4[a]	4.0[b,c]
GuaR2	10.2 ± 0.1[b]	10.0 ± 0.1[b]	10.1 ± 0.1[b]
G50-2	9.4 ± 0.1[a]	8.9 ± 0.2[a]	7.7 ± 0.1[b]
G50-4	9.3 ± 0.1[a]	9.1 ± 0.1[a]	7.1 ± 0[b]

[a] Average of three experiments.
[b] Average of two experiments.
[c] See footnote *a* to Table 1.

Mutants resistant to intermediate concentrations of guanidine. The existence of several classes of guanidine-resistant mutants displaying different degrees of resistance has been reported previously (28). Three classes of guanidine-resistant mutants were found and designated $gua^{r/20}$ (low resistance, 0.21 mM), $gua^{r/40}$ (medium resistance, 0.42 mM), and $gua^{r/100}$ (high resistance, 1.05 mM) (42). Since all six mutants resistant to high concentrations of guanidine contained a mutation at the same amino acid in protein 2C, we sought to examine the sequence of this region in mutants resistant to intermediate concentrations of drug (0.53 mM). We could not examine mutants resistant to low concentrations of guanidine, since the titer of our parental virus was unchanged when plaqued in the presence of 20 μg of guanidine hydrochloride per ml.

The effects of intermediate (0.53 mM) and high (2.0 mM) concentrations of guanidine hydrochloride on the titer of our parental virus and a mutant resistant to high concentrations of guanidine are shown in Table 2 and Fig. 3A and B. The titer of our parental virus was inhibited by approximately 1.0 $\log_{10}$ PFU/ml at 0.53 mM guanidine, and the plaque size was significantly reduced from that seen in the absence of the drug. Further inhibition was seen at 2.0 mM guanidine; in this case plaques were not visible at a 10^{-4} dilution of the virus, but cell lysis was apparent at a 10^{-3} dilution. The mutant resistant to high guanidine displayed equivalent titers in the absence of drug and in the presence of 0.53 or 2.0 mM drug, and plaque sizes were also unaffected by the presence of the drug (Fig. 3B).

Two mutants resistant to intermediate concentrations of guanidine were selected after a single passage in the presence of 0.53 mM guanidine (see Materials and Methods) and titrated in the presence and absence of this concentration of drug. Both mutants (G50-2 and G50-4) grew better in the presence of 0.53 mM guanidine than the parental virus; G50-4 was reduced in titer by 0.2 $\log_{10}$ PFU/ml in the presence of 0.53 mM guanidine, whereas G50-2 was reduced in titer by 0.5 $\log_{10}$ PFU/ml at this concentration of drug (Table 2). The plaque sizes observed in the presence of 0.53 mM guanidine with both G50-4 (Fig. 3C) and G50-2 (data not shown) were similar to that seen in the absence of drug. These two mutants were partially resistant to growth in 2.0 mM guanidine; G50-2 was reduced in titer by 1.7 $\log_{10}$ PFU/ml, whereas G50-4 was reduced by 2.2 $\log_{10}$ PFU/ml (Table 2). Genomic RNA encoding 2C of these two mutants

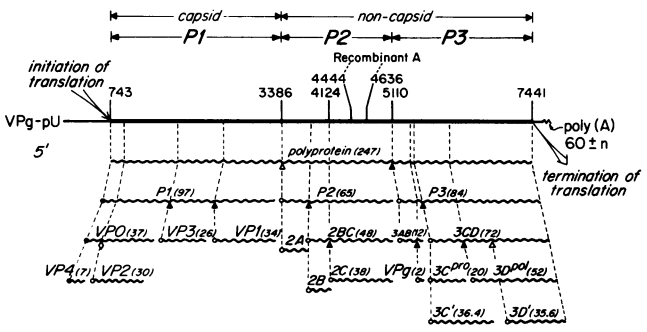


FIG. 1. Genome organization of poliovirus. Viral polypeptides are designated according to a newly adopted nomenclature (40), e.g., 2C is P2-X; 3AB is P3-9, 3C is P3-7c, etc. Values in brackets are molecular weights. Nucleotide coordinates for the N terminus and C terminus of protein 2C are indicated. The crossover event generating recombinant A occurred within the region of nucleotides 4444 to 4636 as indicated (see text).

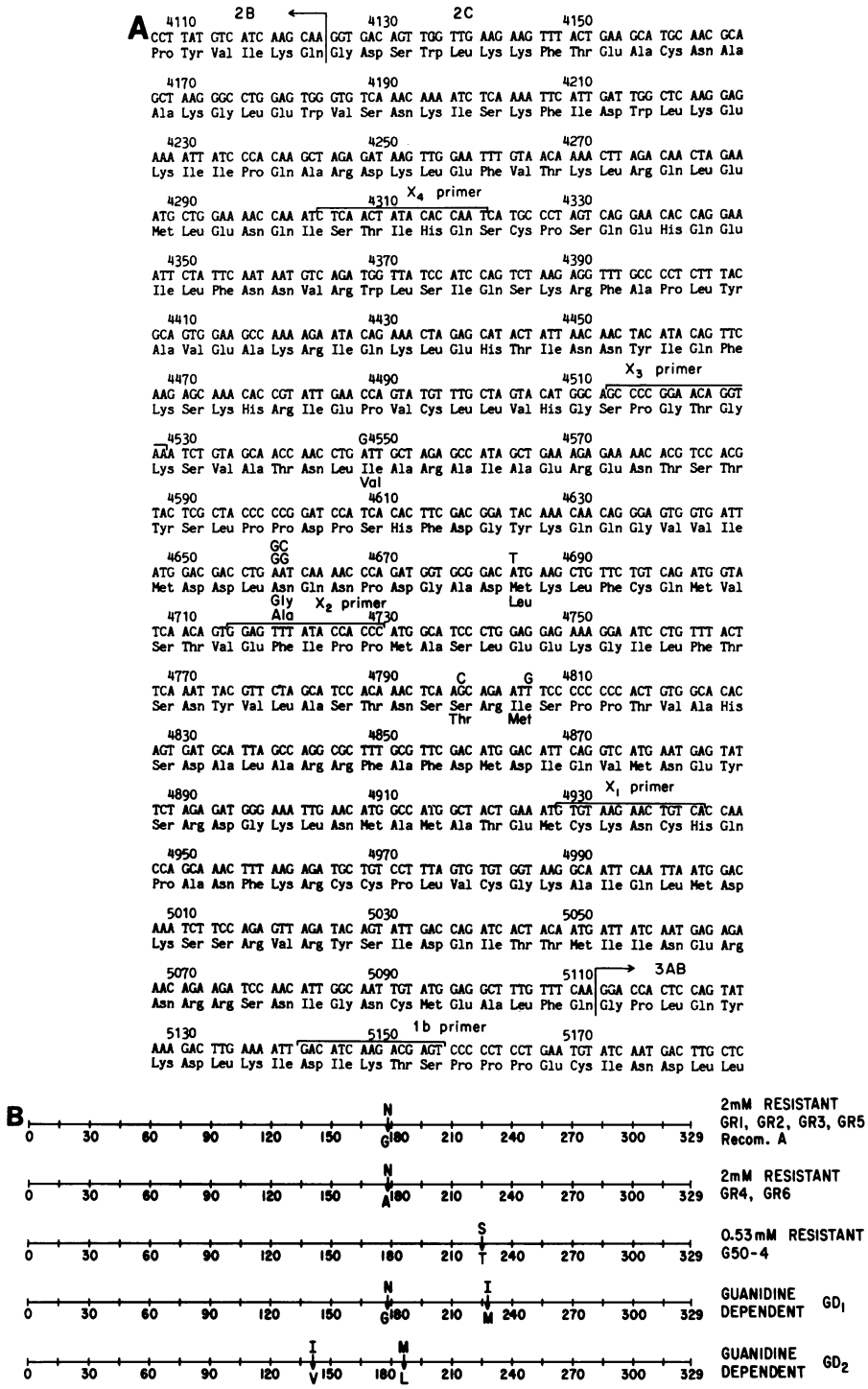
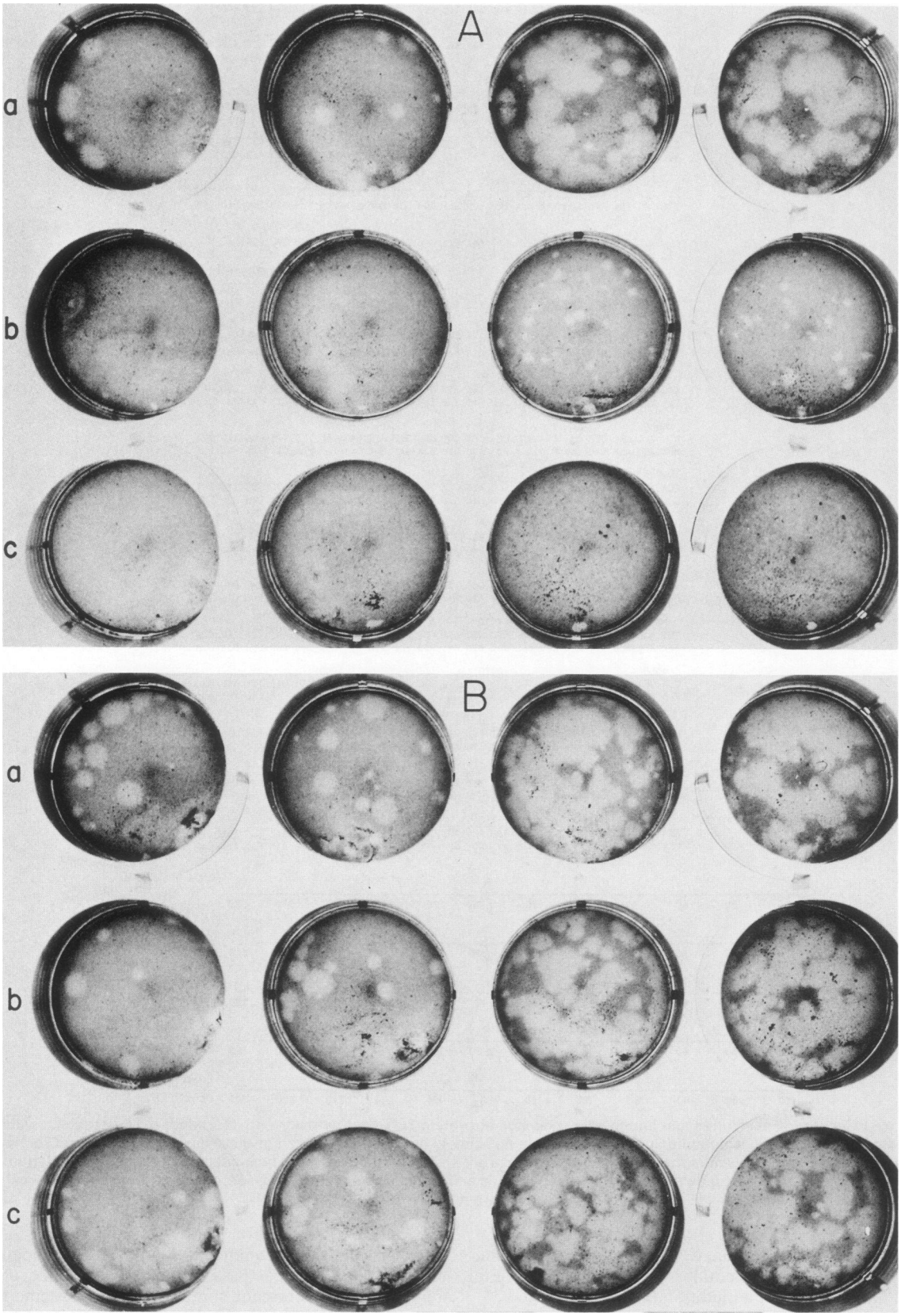


FIG. 2. (A) Consensus nucleotide and amino acid sequence of protein 2C in poliovirus type 1 (Mahoney). The sequence determined by Kitamura et al. (25) contained a glutamine and arginine at amino acids 13 and 14 of 2C instead of the asparagine and alanine shown here. The location of nucleotide primers used in the sequencing of mutants are shown by the bars above the nucleotide sequence. Mutations occurring in guanidine-selected variants are indicated for both the nucleotide and amino acid sequences. (B) Summary of the mutations seen in different classes of mutants. Numbers indicate the amino acid positions from the N terminus of p2C.

was sequenced. G50-4 contained a serine-to-threonine mutation (amino acid 225, nucleotides 4796 through 4798; Fig. 2). G50-2 did not contain a mutation in this region.

Mutants dependent on guanidine for growth. Genomic RNA encoding 2C of two mutants dependent on guanidine for growth was sequenced. GD_1 is a dependent mutant isolated by multiple passages in the presence of 2.0 mM guanidine. This mutant contained the asparagine-to-glycine mutation observed in the highly resistant mutants (amino acid 179, nucleotides 4658 through 4660; Fig. 2) as well as a


A
a
b
c
B
a
b
c

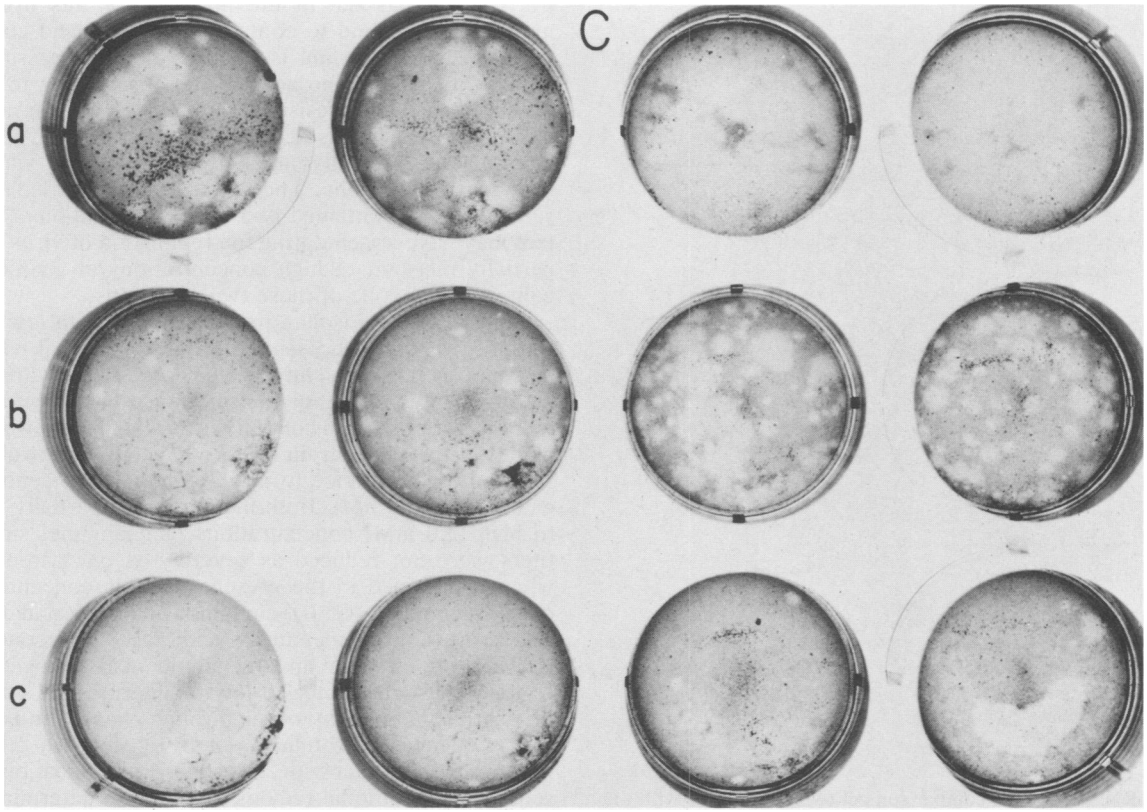


FIG. 3. Effects of intermediate (0.53 mM) and high (2.0 mM) guanidine hydrochloride concentrations on plaque size and number. Rows contained (a) no guanidine hydrochloride, (b) 0.53 mM guanidine hydrochloride, and (c) 2.0 mM guanidine hydrochloride. (A) Parental Mahoney strain, 10^{-9} dilution (first two wells in each row) or 10^{-8} dilution (third and fourth wells in each row). (B) Gr2 mutant, 10^{-9} dilution (first two wells in each row) or 10^{-8} dilution (third and fourth wells in each row). (C) G50-4 mutant, 10^{-7} dilution (first two wells in each row) or 10^{-6} dilution (third and fourth wells in each row).

second mutation, isoleucine to methionine (amino acid 227, nucleotides 4802 through 4804) not observed in the resistant mutants (Fig. 2 and 4). GD_2 is a dependent mutant isolated in a single passage in the presence of 2.0 mM guanidine. This mutant contained an isoleucine-to-valine mutation (amino acid 142, nucleotides 4547 through 4549) as well as a methionine-to-leucine mutation (amino acid 187, nucleotides 4682 through 4684; Fig. 2).

DISCUSSION

In the present study we have sequenced a segment of genomic RNA encoding protein 2C from eight guanidine-resistant and two guanidine-dependent mutants of the Mahoney strain of poliovirus type 1 and have identified mutations in protein 2C in all except one of the mutants. These results extend the finding in foot-and-mouth disease virus and poliovirus that mutants resistant to guanidine contain modifications in nonstructural proteins (1, 17, 41, 49) and does not agree with the observation of capsid protein modifications in poliovirus mutants made guanidine resistant by multiple passage in the presence of drug (26). An analysis of recombinants of poliovirus has narrowed the range of the genome to which the site of guanidine resistance can be mapped (17). Further nucleotide sequencing of recombinants, discussed here, has demonstrated that mutations leading to resistance to inhibition of growth of poliovirus in 2.0 mM guanidine hydrochloride reside 3′ of nucleotide 4444.

Sequence analysis of the genomic RNA encoding 2C from six independent mutants resistant to growth in 2.0 mM guanidine revealed a mutation in all cases at the same amino acid (amino acid 179), which was either asparagine to glycine or asparagine to alanine. It has been proposed that a high degree of resistance to guanidine is accomplished after several mutational steps which are additive (28). We have observed that the mutation frequency of poliovirus to a high degree of resistance to guanidine ranges from 1.8×10^{-7} for poliovirus type 1 (Mahoney) to 4×10^{-8} for the Sabin 1 strain of poliovirus type 1 (Table 1) (17). These values are in good agreement with a mutation frequency ranging from 1.8×10^{-5} to 7.5×10^{-8} reported previously (1, 8, 21). Since the error level per genome doubling at given base positions in the RNA genome is between 10^{-3} and 10^{-4} (22), the observed mutation frequency of poliovirus to a high degree of resistance to guanidine is consistent with a requirement for several mutational steps which are additive. This could mean that high resistance requires nucleotide changes in one position of several codons, resulting in several amino acid changes or nucleotide changes in more than one position of a particular codon to generate a particular amino acid change. The mutations observed here require two nucleotide changes AAU→GNU (where N is either G or C). All six mutants could have arisen from the same parent (a mutant) containing GAU (aspartic acid) at this locus. This mutant might be resistant to low or intermediate concentrations of guanidine; since these types of mutants arise at a higher frequency than high-resistance mutants (28, 42), the procedure employed to isolate high-resistance mutants would not

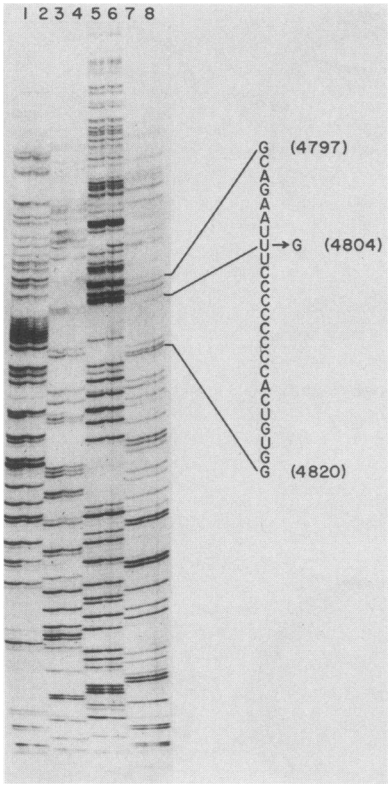


FIG. 4. RNA sequence comparison gel of Gr2 and GD_1 with the X_1 primer. Lanes 1, 3, 5, 7, are the Gr2 "C", "T", "A", and "G" lanes, respectively. Lanes 2, 4, 6, 8, are from GD_1 in the same order. The sequence on the right identifies the U→G mutation in GD_1, not found in gr2.

preclude the presence of this parent in the stocks used to generate high-resistance mutants. During the sequencing of the 2C region from these mutants we observed a number of nucleotide mutations in the third position of amino acid codons which did not result in an amino acid change. The six mutants could be grouped into three classes based on these silent mutations. Thus, for the six mutants to be generated from the same parent, mutations would have had to occur at the three silent positions and at the second position of amino acid 179. Since there does not seem to be any selective pressure to accumulate silent mutations, it seems more likely that these mutants arose from a series of parents containing aspartic acid at this position and various silent mutations. Alternatively, the mutants could have arisen from parents (mutants) resistant to low or intermediate concentrations of guanidine-containing AGU (serine) or ACU (threonine) at this position and various silent mutations. This suggests that resistance to 2.0 mM guanidine can occur through a very limited number of types of mutations.

Guanidine-dependent strains of poliovirus can be obtained after repeated passage of highly resistant populations in the presence of high concentrations of the drug (28, 30). One question that arises is whether mutation to guanidine dependence in a given virus particle requires that the genome be in a state which confers a high degree of resistance or whether dependence can also be acquired directly during multiplication of sensitive or low-degree resistance particles as has been proposed for coxsackie virus A9 (16). We have described in this report the isolation of dependent mutants either by multiple passages in the presence of guanidine or by a single passage in the presence of this drug. Both mutants were found to contain two amino acid changes in protein 2C. The mutant isolated by multiple passages contained the same amino acid change (asparagine to glycine) observed in highly resistant mutants as well as a second amino acid change (isoleucine to methionine). This dependent mutant probably arose from virus that was highly resistant to guanidine. The dependent mutant isolated in a single passage contained two different mutations. This mutant may have arisen during the replication of virus that was partially resistant to high concentrations of guanidine and contained only one of these two mutations.

Our attempts to isolate mutants resistant to low concentrations were unsuccessful since our parental virus was resistant to this concentration of drug. Large variations in mutation rates for guanidine resistance have been encountered in various strains of poliovirus and also between sister clones of the same strain (8). We have studied two mutants selected for resistance to an intermediate concentration of guanidine (0.53 mM). Both mutants were partially resistant to high (2.0 mM) concentrations of guanidine, since their titers were not reduced as severely as our parental virus when determined in the presence of this concentration of drug. One mutant (G50-4) contained an amino acid change in 2C (serine to threonine, amino acid 225). The second mutant (G50-2) did not contain any amino acid changes in this region. Thus, resistance to low or intermediate concentrations of guanidine may occur through changes in the amino acid sequence of protein 2C or by changes in other viral proteins that interact with 2C during viral replication. We are sequencing additional regions of G50-2 to determine where the mutation occurs.

The mutations in protein 2C described in this report are clustered in a region encompassing approximately 25% of the amino acid sequence (amino acids 142 through 227 of 2C). A strong amino acid homology was previously observed between the 58-kilodalton polypeptide of cowpea mosaic virus and the 2C proteins of encephalomyocarditis virus, foot-and-mouth disease virus, and poliovirus (2, 19) over regions totaling 115 residues (amino acids 119 through 231 in the poliovirus 2C sequence). In Fig. 5 we have extended the comparison to include the amino acid sequences of rhinovirus types 14 and 2 (43, 44). The asparagine-to-glycine mutation in mutants resistant to 2.0 mM guanidine is contained within an amino acid sequence MDDLNQNP that is completely conserved between poliovirus and rhinovirus type 14 and differs in encephalomyocarditis and foot-and-mouth disease viruses only in the substitution of a glycine for the asparagine. A strong correlation exists between the amino acid at this position and the sensitivity of the picornavirus to 2.0 mM guanidine. Both poliovirus and rhinovirus type 14 are strongly inhibited by 2.0 mM guanidine, whereas encephalomyocarditis virus and foot-and-mouth disease virus strain $A_{12}119$ are resistant to this concentration of drug (36, 46). Rhinovirus type 2 (in which this sequence is MDDIMQNP) is less severely inhibited by 2.0 mM guanidine than is rhinovirus type 14, but nevertheless this virus is sensitive to guanidine (data not shown). This suggests that methionine at this position may function in the same manner as asparagine. The guanidine sensitivity of cowpea mosaic virus has not been determined. Cowpea mosaic virus contains an alanine at this position; thus we predict that this virus would be guanidine resistant. It remains to be explained why mutations from an asparagine to glycine or to alanine result in the guanidine-resistant phenotype. Secondary structure analysis of this region by

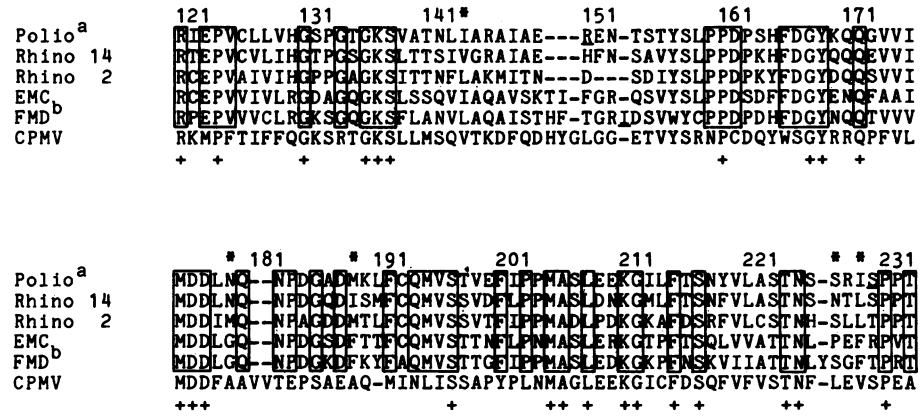


FIG. 5. Alignment of the amino acid sequences of the 2C regions of encephalomyocarditis virus (EMC), poliovirus, foot-and-mouth disease virus (FMD), rhinovirus (Rhino) types 14 and 2, and the 58K polypeptide encoded by cowpea mosaic virus B-RNA. Numbers above the poliovirus sequence represent the amino acid positions from the N-terminus of poliovirus polypeptide 2C. Thus the third amino acid in the poliovirus sequence (glutamic acid) represents amino acid 121. Boxed sequences identify those amino acids conserved among the viruses indicated. The a superscript indicates the consensus sequence of poliovirus type 1 (Mahoney) and the Sabin 1, 2, and 3 strains. The underlined amino acids are different in Sabin 2 and Sabin 3. Arginine (amino acid 149) is a lysine in these two strains, and serine (amino acid 228) is a threonine (25, 34, 39, 50). The b superscript indicates the consensus sequence of foot-and-mouth disease virus strains O1K, A_{10}, 61, and $A_{12}119$ (9, 18, 39). The underlined isoleucine is a threonine in $A_{12}119$. Asterisks indicate locations of mutations observed in guanidine-selected mutants. Pluses indicate that the residue is conserved in all six sequences.

the method of Chou and Fasman (10) failed to reveal a correlation between secondary structure and response to guanidine.

The strong amino acid homology of this region suggests that it is of vital importance for the growth of picornaviruses. This region might be involved in interacting with other viral proteins required for replication. Membrane-bound replication complexes contain protein 2C, although this protein does not contain hydrophobic regions (4, 45). It has been suggested that 2C is associated with membranes by its affinity to the N-terminal portion of protein 3AB (45). Guanidine could inhibit initiation of poliovirus replication by preventing this interaction. Attempts to demonstrate an inhibitory effect of guanidine on RNA synthesis in vitro by utilizing membrane fractions have been unsuccessful (Takeda, Kuhn, and Wimmer, unpublished data). Virus-inhibitory concentrations of guanidine do not dissociate 2C from cellular membranes in vitro, and membranes isolated from cells in which viral protein synthesis was inhibited 80% by guanidine show no difference in the association of 2C (48). Guanidine could act by inducing conformational changes in the proteins of the replication complex in vivo and affect the ability to catalyze reinitiations, but fail to act in vitro because of distortions in the replication complex caused by isolation procedures.

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