JOURNAL OF VIROLOGY, Oct. 1989, p. 4441–4444
0022-538X/89/104441-04$02.00/0

Vol. 63, No. 10

Guanidine-Resistant Mutants of Poliovirus Have Distinct Mutations in Peptide 2C

ROBERT F. BALTERA, JR., AND DANIEL R. TERSHAK*

Microbiology Program, Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received 27 March 1989/Accepted 29 June 1989

In previous work in our laboratory, 12 guanidine-resistant (*gr*) mutants of poliovirus were selected from 12 separate stocks of plaque-purified guanidine-sensitive (*gs*) viruses (K. Anderson-Sillman, S. Bartal, and D. R. Tershak, J. Virol. 50:922–928, 1984). Peptide mapping of protein 2C and evaluation of virus growth at different temperatures enabled us to subdivide these mutants into several distinct groups (D. R. Tershak, Can. J. Microbiol. 31:1166–1168, 1985; Anderson-Sillman et al., J. Virol.). Studies by Pincus et al. indicate that two nucleotide changes in codon 179 of protein 2C leads to an Asn-to-Gly or Asn-to-Ala change and that these missense modifications account for guanidine resistance (S. E. Pincus, H. Rohl, and E. Wimmer, Virology 157:83–88, 1987; S. E. Pincus and E. Wimmer, J. Virol. 60:793–796, 1986). In the present report, we confirm their findings but also show that a single amino acid change of Phe to Tyr in residue 164 of protein 2C or a Met-to-Leu replacement in amino acid 187 coupled with mutations in codons 233 or 295 and 309 could confer guanidine resistance.

Guanidine inhibits the growth of numerous picornaviruses at concentrations that do not adversely affect host cells (3, 8, 12). Although guanidine inhibits several viral processes, the primary effect appears to be blockage of the initiation step of RNA synthesis (2, 3, 15). However, the release of completed RNA chains from the replication complex is also depressed under certain conditions (6).

Isoelectric focusing and peptide fingerprinting of polypeptides of guanidine-resistant (*gr*) mutants of foot-and-mouth disease virus (14) and poliovirus (1) initially suggested that viral protein 2C is responsible for the guanidine trait (nomenclature of picornavirus peptides is described by Rueckert and Wimmer [13]). Subsequent experiments involving nucleotide sequence analyses of *gr* and guanidine-dependent (*gd*) variants of the Mahoney type 1 poliovirus identified mutations in peptide 2C (9, 10), and all occurred in a region of the 2C locus that is highly conserved among picornaviruses and cowpea mosaic virus (10). Furthermore, transfection of COS-1 cells with plasmids that were engineered from infectious clones of guanidine-sensitive (*gs*) virus and cDNA fragments that correspond to regions of 2C containing *gr* or *gd* mutations produced *gr* and *gd* viruses, respectively (11). On the basis of their observations, Pincus et al. concluded that a mutation of asparagine to glycine or asparagine to alanine at amino acid 179 of 2C is required for resistance of viral growth to 2.0 mM guanidine, while a methionine-to-leucine change at amino acid 187 coupled with a mutation in the amino acid at position 142, 225, or 227 generates *gd* viruses with variable degrees of dependence on guanidine for growth (10). In fact, mutations in two amino acids of peptide 2C appear to produce viruses that are *gd*. The observation by Pincus et al. that all seven *gr* mutants of poliovirus that were resistant to 2.0 mM guanidine harbor an asparagine-to-glycine or alanine replacement at amino acid 179 of 2C strongly supports the conclusion that this modification confers guanidine resistance to viral growth (9).

Twelve *gr* variants that are resistant to 1 mM inhibitor were previously isolated in our laboratory from separate stocks of plaque-purified *gs* viruses and were classified by subgroup according to peptide maps of 2C that were obtained with *Staphylococcus aureus* V8 protease (1). Further experiments showed that growth characteristics of these mutants in the presence of guanidine varied with temperature of incubation and suggested that *gr* mutations clustered at several sites in the 2C locus (16) rather than at one distinct site, which is anticipated if the codon for amino acid 179 is alone responsible for resistance to the drug. Consequently, the following study was undertaken to determine whether amino acid changes other than asparagine to glycine or alanine at position 179 confer guanidine resistance on the Mahoney strain of type 1 poliovirus. The data suggest that a single amino acid change at residue 164 or 179 or 187 in combination with additional mutations in the 2C locus can control guanidine resistance.

Plaque selection of *gr* mutants of poliovirus from the Mahoney type 1 parent, growth and titration of viruses, and cell culture conditions for HeLa cells were previously described (1). Nucleotide sequences of the entire 2C regions of poliovirus RNA were obtained with the dideoxy-chain termination procedure as described for RNA extracted from purified virions (5) or for mixtures of viral and cell RNA obtained from cells 7 to 8 h after infection (7). Both procedures provided results analogous to those with reverse transcriptase (Bio-Rad Laboratories and Boehringer Mannheim Biochemicals) and the five primers described by others (9). Oligodeoxy primers were synthesized by Donald A. Bryant (The Pennsylvania State University) with an oligodeoxynucleotide synthesizer (Applied Biosystems, Inc.) and used without further purification. Sequencing procedures incorporated two modifications: the primer-to-RNA ratio was 20:1 or 50:1, and terminal deoxynucleotidyltransferase (Boehringer Mannheim) at 5 U per reaction was used during the chase period as recommended by DeBorde et al. (4). Approximately 1 to 2 mg of purified virus was obtained via CsCl density gradients after growth of *gs* virus in 15 monolayer cultures of HeLa cells in 150-cm^2 flasks. Sequence analyses of *gr* mutants and *gs* virus were performed with RNA obtained from infected cells (7). The exact amount of

* Corresponding author.

TABLE 1. Plaque-forming efficiencies of *gr* mutants of poliovirus

Mahoney type 1 polio virus	PFU/ml after treatment with:			Guanidine/control ratio		Plaque size[a] after treatment with:		
	Control	1 mM guanidine	2 mM guanidine	1 mM guanidine	2 mM guanidine	Control	1 mM guanidine	2 mM guanidine
gs	1.8×10^9	3.2×10^4	2.7×10^4	1.8×10^{-5}	1.5×10^{-5}	L	S	S
*gr*1	7.1×10^9	9.3×10^9	8.1×10^9	1.3	1.1	I	L	I
*gr*2	5.3×10^8	6.2×10^8	6.7×10^8	1.2	1.3	I	I	I
*gr*3	2.0×10^8	6.0×10^8	5.9×10^8	3.0	3.0	S	L	L
*gr*4	1.2×10^8	4.5×10^8	3.2×10^8	3.8	2.7	S	I	I
*gr*5	1.4×10^9	1.2×10^9	1.3×10^9	0.9	0.9	L	L	L
*gr*6	9.0×10^8	1.0×10^9	7.5×10^8	1.1	0.8	I	I	I
*gr*7	1.1×10^8	2.8×10^8	3.6×10^8	2.5	3.3	I	L	I
*gr*8	1.8×10^9	2.9×10^9	3.6×10^9	1.6	2.0	S	I	I
*gr*9	2.1×10^9	1.0×10^8	1.3×10^8	5×10^{-2}	6×10^{-2}	I	I	L
*gr*10	6.0×10^8	6.5×10^8	7.3×10^8	1.1	1.1	I	L	L
*gr*11	4.9×10^8	1.2×10^9	9.0×10^8	2.4	1.9	S	I	I
*gr*12	6.5×10^8	6.5×10^8	5.3×10^8	1.0	0.8	I	L	I

[a] Plaques were measured after 4 days of incubation at 35°C. S, 1 to 2 mm; I, 2 to 4 mm; L, 3 to 7 mm.

viral RNA in cellular extracts was not measured. Consequently, we used approximately 2 μg of total RNA per reaction mixture. Sequences obtained with RNA from purified *gs* virus served as a control for sequences obtained with RNA from infected cells. Purified RNA and RNA in cellular extracts produced analogous results.

gr mutants were originally derived in our laboratory with 1 mM inhibitor in agar overlay medium instead of with the 2 mM inhibitor used by others (1). Consequently, the 12 *gr* mutants we previously isolated were assayed by plaque procedures with 1 or 2 mM guanidine to determine whether they were sensitive to the higher concentration of the compound. Plaque sizes of both groups were evaluated on day 4 of titrations. Guanidine at 1 and 2 mM inhibited the parent *gs* virus approximately 100,000-fold, while plaque-forming efficiencies of *gr* variants were comparable at both concentrations of inhibitor (Table 1). Five mutants, *gr*3, *gr*4, *gr*7, *gr*8, and *gr*11, produced two- to threefold more plaques in the presence of guanidine than did controls which lacked the inhibitor. This increase in efficiency is negligible compared with that of *gd* mutants, which generally display several-order-of-magnitude-higher plaque-forming efficiencies when guanidine is incorporated into the medium (1, 9, 10). *gr*3, *gr*4, *gr*7, *gr*8, and *gr*11 viruses are characteristically resistant rather than dependent variants. Plaque formation by one mutant, *gr*9, was depressed about 95% by guanidine, but this virus is still categorized as a *gr* variant when compared with the wild-type virus. In a previous report, *gr*9 produced equal numbers of plaques in control medium and medium with 1 mM inhibitor (1). The virus in the present study underwent three additional passages at about 0.1 PFU per cell without guanidine, and during that period, selection for partially resistant virus apparently occurred. In the majority of cases (8 of 12 mutants), guanidine stimulated formation of large plaques. Although the data are not recorded in Table 1, plaques appeared a day earlier in the presence of guanidine than they did in the control, possibly because of higher yields of virus.

Nucleotide sequence analysis of the 2C region of genomic RNA by dideoxy-chain termination procedures broadly divided the 12 mutants into two categories (Fig. 1). Group 1 includes *gr*1, *gr*3, *gr*4, and *gr*6, while group 2 includes the remaining eight variants. All four viruses in group 1 contained mutations in a single codon, in contrast to the viruses in group 2, in which several codons were modified. *gr*3, *gr*4, and *gr*6 contained mutations in both the first and second bases of the codon responsible for amino acid 179 of peptide 2C. These mutants show an asparagine-to-glycine modification and are analogous to those described by Pincus et al. (10). However, *gr*1 is unusual because a single nucleotide change in the second base of codon 164 caused a phenylalanine-to-tyrosine switch. Furthermore, peptide maps of protein 2C of *gr*1 are markedly different from fingerprints obtained with *gr*3, *gr*4, and *gr*6 viruses (1). Growth characteristics of *gr*1 are also distinct compared with those of *gr*3, *gr*4, and *gr*6 (16). Growth of all four viruses of group 1 is inhibited 1,000- to 10,000-fold more at 41°C than at 36°C, but 1 mM guanidine completely counteracts inhibition with *gr*1 (16). Conversely, guanidine depresses growth of *gr*3, *gr*4, and *gr*6 at least 10-fold at 41°C.

All eight variants in group 2 exhibit a mutation in the first nucleotide of codon 187 which results in a methionine-to-leucine change. This modification is coupled with other mutations in the first nucleotides of codons 228, 233, 248, 295, and 309. Peptide maps of protein 2C of these mutants and growth properties in the presence or absence of guanidine show these mutants to be different from those in group 1 (16; unpublished data). Others have shown that a mutation in codon 187 in conjunction with mutations in codon 142, 225, or 227 modulates the degree of guanidine dependence (10). Surprisingly, all eight of the mutants we examined proved to be *gr* rather than *gd*. The isoleucine-to-valine replacement at residue 248 of five of the variants described in Fig. 1 probably does not influence the guanidine trait, because this mutation is not detectable in the genomes of *gr*2 and *gr*7. Nonetheless, changes in both codon 187 and codon 248 could produce *gr* variants, even though we have not observed this pair of mutations in the present study. We have not observed viruses with mutations at position 187 alone, nor have others observed a change in this region of the genome without companion mutations (9, 10). It appears that a missense change in amino acid 187 must be associated with a concurrent mutation elsewhere to confer guanidine resistance or dependence. The position of the second change and the specific missense modification are probably crucial in determining characteristics of virus. Pincus et al. (10) found that a methionine-to-leucine change at amino acid residue 187 and a serine-to-threonine change at amino acid 225 produced virus that was dependent on intermediate or high concentrations of guanidine for growth. Following the same nucleotide numbering system as these investigators (9, 10), we noticed that a methionine-to-leucine change in amino

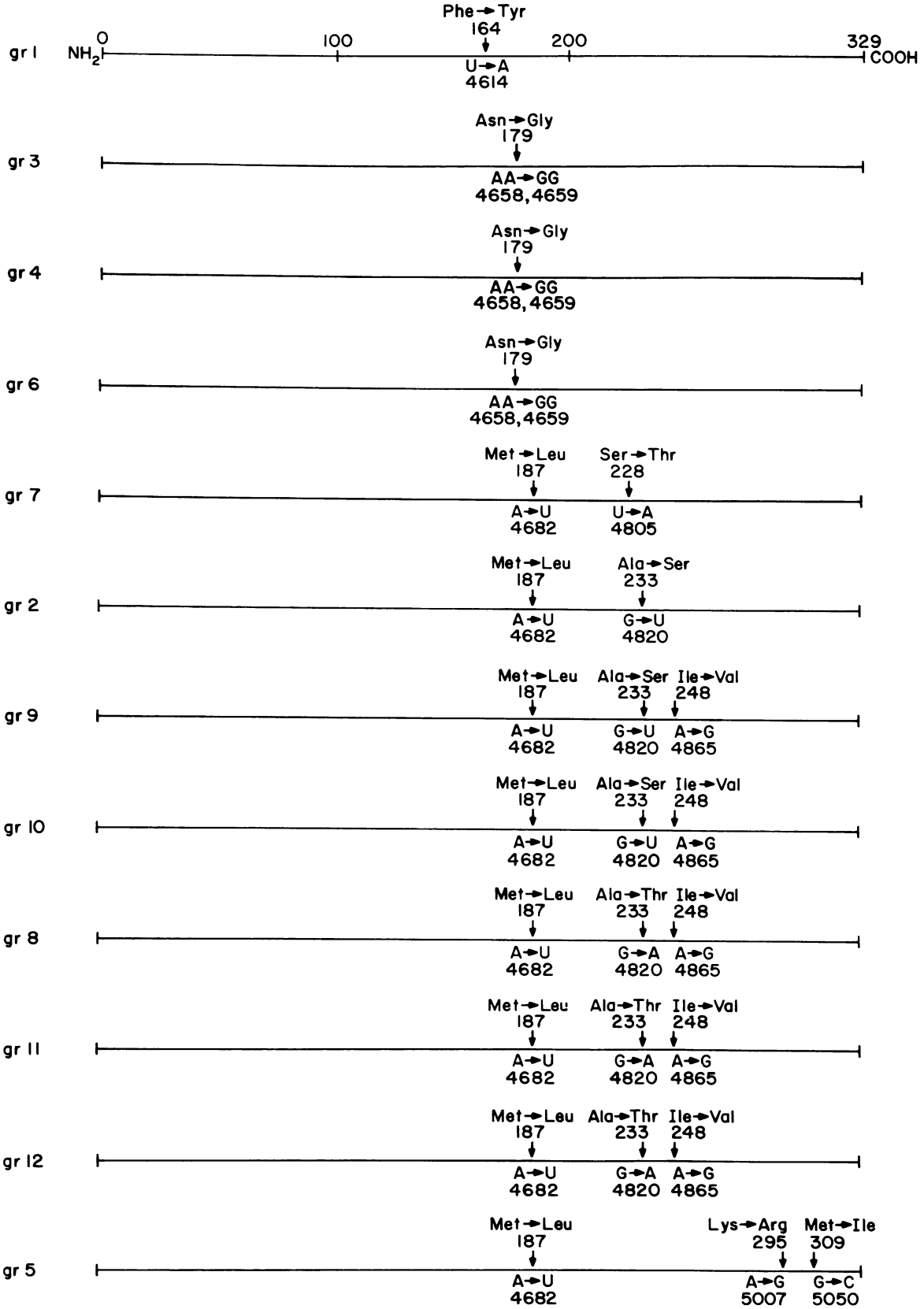

FIG. 1. Map of *gr* mutants of poliovirus. Numbers above the drawings indicate amino acid numbers from the amino terminus of protein 2C. Numbers below the drawings indicate nucleotide numbers from consensus nucleotide sequences (9, 10) in which mutations were detected. The specific base changes and amino acid replacements are indicated by arrows.

acid 187 in conjunction with a serine-to-threonine switch at position 228 (*gr*7) yielded virus that is *gr*. It might be argued that *gr*7 is slightly guanidine dependent, since approximately threefold more plaques are produced during titrations with guanidine in medium than are produced in controls lacking inhibitor (Table 1). However, this increase in plaque-forming efficiency is at least 1 order of magnitude lower than increases found by others (9, 10).

Mutant *gr*9 is unusual because it displays an intermediate degree of resistance to guanidine. In this respect, *gr*9 is similar to mutants G50-2 and G50-4 described by others (9). However, amino acid changes were observed at positions 187, 233, and 248 of peptide 2C with *gr*9. Since identical mutations were detected with *gr*10 (Fig. 1), it seems likely that a rare mutation outside peptide 2C can modulate guanidine resistance. In fact, growth of *gr*6 was not enhanced in the presence of guanidine compared with that of *gr*3 and *gr*4, even though mutations in 2C are identical. This also supports the thesis that mutations in other loci can temper the guanidine phenotype. The G50-4 variant described by other investigators contained a serine-to-threonine mutation at amino acid 225 of peptide 2C, whereas the G50-2 variant did not contain an amino acid change in 2C (9). The data for *gr*9, *gr*6, and G50-2 suggest that mutations in proteins that interact with peptide 2C during viral growth can affect the guanidine trait. However, phenotypically expressed mutations in these proteins appear to occur infrequently in comparison with mutations in the 2C locus.

Remarkably subtle changes in the folding of peptide 2C must occur in the presence of millimolar levels of guanidine to explain the differences in growth characteristics of viruses with identical amino acid changes over a distance of only three amino acids (amino acid 225 as described by Pincus and co-workers [9] and amino acid 228 in this report). The interaction of protein 2C with other peptides during synthesis of viral RNA must be highly ordered to explain the expression of guanidine resistance and dependence over short domains of the 2C protein. We are presently programming site-directed mutagenesis studies to determine the effects of mutations in codon 187 alone and in combination with other amino acid replacements, and we also plan to sequence segments of the genome that are adjacent to peptide 2C to identify ancillary mutations that could affect the guanidine trait.

This study was supported by Public Health Service grant AI23717 from the National Institutes of Health.

LITERATURE CITED

1. **Anderson-Sillman, K., S. Bartal, and D. R. Tershak.** 1984. Guanidine-resistant poliovirus mutants produce modified 37-kilodalton proteins. J. Virol. **50**:922–928.
2. **Caliguiri, L. A., and I. Tamm.** 1968. Action of guanidine on the replication of poliovirus RNA. Virology **35**:408–417.
3. **Caliguiri, L. A., and I. Tamm.** 1973. Guanidine and 2-(β-hydroxybenzyl)-benzimidazole (HBB): selective inhibitors of picornavirus multiplication, p. 257–294. *In* W. A. Carter (ed.), Selective inhibitors of viral functions. CRC Press, Inc., Boca Raton, Fla.
4. **DeBorde, D. C., C. W. Naeve, M. L. Herlocher, and H. F. Maassab.** 1986. Resolution of a common sequencing ambiguity by terminal deoxynucleotidyl transferase. Anal. Biochem. **157**: 275–282.
5. **Emini, A. E., J. Leibowitz, D. C. Diamond, J. Bonin, and E. Wimmer.** 1984. Recombinants of Mahoney and Sabin strains of poliovirus type 1. Analysis of *in vitro* phenotypic markers and evidence that resistance to guanidine maps in the nonstructural proteins. Virology **137**:74–85.
6. **Huang, A. S., and D. Baltimore.** 1970. Initiation of polysome formation in poliovirus-infected HeLa cells. J. Mol. Biol. **47**: 275–291.
7. **Kuge, S., and A. Namoto.** 1987. Construction of viable deletion and insertion mutants of the Sabin strain of type 1 poliovirus: function of the 5′ noncoding sequence in viral replication. J. Virol. **61**:1478–1487.
8. **Loddo, B., W. Ferrari, G. Brotzu, and A. Spanedda.** 1962. *In vitro* inhibition of infectivity of poliovirus by guanidine. Nature (London) **193**:97–98.
9. **Pincus, S. E., D. C. Diamond, E. A. Emini, and E. Wimmer.** 1986. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J. Virol. **57**:638–646.
10. **Pincus, S. E., H. Rohl, and E. Wimmer.** 1987. Guanidine-dependent mutants of poliovirus. Identification of three classes with different growth requirements. Virology **157**:83–88.
11. **Pincus, S. E., and E. Wimmer.** 1986. Production of guanidine-resistant and -dependent poliovirus mutants from cloned cDNA: mutations in polypeptide 2C are directly responsible for altered guanidine sensitivity. J. Virol. **60**:793–796.
12. **Rightsel, W. A., J. R. Dice, R. J. McAlpine, E. A. Timm, I. W. McLean, G. J. Dixon, and F. M. Schabel.** 1961. Antiviral effect of guanidine. Science **134**:558–559.
13. **Rueckert, R. R., and E. Wimmer.** 1984. Systematic nonmenclature of picornavirus proteins. J. Virol. **50**:957–959.
14. **Saunders, K., and A. M. Q. King.** 1982. Guanidine-resistant mutants of aphthovirus induce the synthesis of an altered nonstructural polypeptide, p34. J. Virol. **42**:389–394.
15. **Tershak, D. R.** 1982. Inhibition of poliovirus polymerase by guanidine in vitro. J. Virol. **41**:313–318.
16. **Tershak, D. R.** 1985. Effect of temperature on guanidine-resistant mutants of poliovirus. Can. J. Microbiol. **31**:1166–1168.