Genetic Control of Capsid Length in Bacteriophage T4: DNA Sequence Analysis of Petite and Petite/Giant Mutants

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The T4 gene 23 product (gp23) encodes the major structural protein of the mature capsid. Mutations in this gene have been described which disrupt the normal length-determining mechanism (A. H. Doermann, F. A. Eiserling, and L. Boehner, *J. Virol.* 12:374-385, 1973). Mutants which produce high levels of petite and giant phage (*ptg*) are restricted to three tight clusters in gene 23 (A. H. Doermann, A. Pao, and P. Jackson, *J. Virol.* 61:2823-2827, 1987). Twenty-six of these *ptg* mutations were cloned, and their DNA sequence alterations were determined. Each member of this set of *ptg* mutants arose from a single mutation, and the set defined 10 different sites at which *ptg* mutations can occur in gene 23. Two petite (*pt*) mutations in gene 23 (*pt*21-34 and *pt*E920g), which produce high frequencies of petite particles but no giants, were also sequenced. Both *pt*21-34 and *pt*E920g were shown to include multiple mutations. The phenotypes attributed to both *pt* and *ptg* mutations are discussed relative to the mechanism of capsid morphogenesis. A site-directed mutation (SD-1E) was created at the *ptg*Ng191 site, and its phenotypic consequences were examined. Plaque morphology revertants arising from a gene 23 mutant derivative of *pt*21-34 and from SD-1E were isolated. A preliminary mapping of the mutation(s) responsible for their revertant phenotypes suggested that both intra- and extragenic suppressors of the petite phenotype can be isolated by this method.

The structure of the capsid of bacteriophage T4 has been well characterized (13), and the major aspects of the capsid assembly pathway have been worked out (3). The majority of the gene products involved in capsid morphogenesis has been determined, and the relative quantities present per capsid are roughly known (13). After assembly of the prehead is completed, the prehead shell is composed primarily of gp23 and encloses a proteinaceous core.

The normal product of the capsid assembly pathway in wild-type T4 infections is a prolate icosahedron which has been elongated on its axis of fivefold symmetry. However, this is not the only capsid product in wild-type infections. A low percentage of isometric particles (petite phage) are also present (27). These particles contain only about 70% of the T4 genome and are incapable of multiplication (27).

Nonlethal, missense mutations in gp23 which modify the normal capsid assembly mechanism have been known for some years (7, 8, 14). However, the complex nature of their phenotypes has complicated their interpretation. The petite (*pt*) mutant *pt*E920g produces much higher levels of isometric phage than are seen in wild-type infections (7). This phenotype simulates the phenotypes caused by mutations in several of the core protein genes (2, 20, 33). However, *pt*21-34 expresses a new phenotype along with the high levels of isometric phage (7). It produces large numbers of particles whose length is intermediate between those of normal and isometric phage. These intermediate particles are also incapable of multiplication and contain a proportion-

ately larger fraction of the genomic DNA, about 86% (21). Fifty-five independent *ptg* mutations in gp23 have been characterized. These mutants produce giant phage in addition to intermediate-sized phage (7, 10). The *ptg* mutations have been mapped genetically to a minimum of eight sites in gene 23 (10).

DNA sequence analysis allows the assignment of specific amino acid substitutions to these mutant alleles. Mutations which lead to an alteration in the normal frequency of isometric particles and those which lead to new products in capsid assembly, giant, and intermediate particles can be correlated to specific amino acid changes in gp23. This paper describes the DNA sequence analysis of 1 site-directed, 2 gp23 petite, and 26 petite/giant mutations.

The analysis of second-site suppressors of *pt* and *ptg* mutations, as done with *ptg*Bu1980 by Doherty (11, 12), provides another attractive feature in the genetic analysis of capsid length control. Doherty characterized allele-specific, extragenic suppressors of *ptg*Bu1980, but to date no attempt has been made to select suppressors of any other capsid assembly mutation. To determine whether similar suppressors might be selected against other gp23 assembly mutations, a preliminary examination of plaque morphology revertants of *pt*21-34 and of the site-directed assembly mutation was made.

MATERIALS AND METHODS

Bacteria and phage. *Escherichia coli* B (su⁻) was used for plating T4D mutants since wild-type T4D makes large, uniform plaques when plated on B, while *pt* and *ptg* mutants make distinguishably smaller plaques (7). *E. coli* CR63 was used to prepare and titrate all T4D amber mutants. T4D strains were from the collection of A. H. Doermann. The M13 *lac* vectors mp8 and mp9 (26), mp10 and mp11 (24), and mp18 and mp19 (28) were used to clone T4 gene 23 fragments. The M13 virus was grown in *E. coli* JM103 (25). Competent cells were prepared by the method of Messing (24).

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Media. *E. coli* B, CR63, and S/6 were grown in Hershey broth (15) or M9 medium supplemented with Casamino Acids (7). YT broth (29) was used for the growth of JM103.

Enzymes and reagents. Restriction enzymes *Aha*III, *Acc*I, *Taq*I, and *Sma*I were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and the reaction conditions for each were those specified by the manufacturer. T4 DNA, containing glucosylated hydroxymethylcytosine at all cytosine positions (16, 39), was cleaved with either *Taq*I (5 U/ μ g of T4 DNA for 5 h at 65°C) or *Aha*III (3 U/ μ g of T4 DNA for 3 h at 37°C). T4 DNA ligase and DNA polymerase I Klenow fragment were obtained from Bethesda Research Laboratories. The M13 sequencing primer and the hybridization probe were obtained from New England Biolabs, Inc., Beverly, Mass. [α -³²P]dATP for DNA sequencing was purchased from New England Nuclear Corp., Inc., Boston, Mass., and [γ -³²P]dATP was obtained from Amersham Corp., Arlington Heights, Ill.

Cloning from genomic T4 DNA and DNA sequence analysis. Electrophoresis was carried out on 50 μ g of *Taq*I- or *Aha*III-restricted T4 DNA for 5 h at 10°C and 10 V/cm or 16 h at room temperature and 1 to 2 V/cm through a 5% polyacrylamide gel containing 89 mM Tris, 89 mM sodium borate, and 1 mM EDTA, pH 8.0. Ethidium bromide-stained DNA fragments of appropriate size were cut out of the gel and purified by the method of Smith (37). DNA fragments, in 5 μ l of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, were combined with 40 ng of *Acc*I- or *Sma*I-digested mp10 replicative form DNA in 10 μ l of 50 mM Tris hydrochloride (pH 7.5)–5 mM MgCl₂–1 mM dithiothreitol–100 μ M ATP and incubated with 5 U of DNA ligase for 6 h at 30°C or for 16 h at 10°C. Reaction products were used to transfect competent JM103 and plated into a 3-ml YT soft-agar overlay containing 0.33 mM IPTG (isopropyl- β -D-thiogalactopyranoside), 0.8 mM X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside), and 2×10^8 cells of overnight-grown JM103. IPTG and X-Gal were included in these platings to demonstrate the fraction of plaques containing T4 DNA (25). After incubation overnight at 37°C, plaque lifts and hybridizations were performed (18) with M13 clones containing appropriate wild-type gene 23 fragments (31) as the probe. Hybridizing, noncolored plaques were picked, and the recombinant M13 phage were eluted and replated. Preparation of template DNA for dideoxy sequencing was essentially that of Oliver and Crowther (29). Precipitation of M13 phage was carried out in 0.5 M NaCl–4% polyethylene glycol 6000 at 0°C for 1 h or at 4°C overnight. Dideoxy DNA sequencing was performed essentially by the method of Sanger et al. (34) as adapted for M13 by Schreier and Cortese (35).

Site-directed mutagenesis. Site-directed mutagenesis was carried out by an adaptation of published methods (17, 40). A synthetic oligodeoxynucleotide (5'-ATGCTGTTGCTG-3') was prepared by Brian Reid. The mutant oligonucleotide, containing a single nucleotide mismatch at base pair (bp) 860 in gene 23 (31), was annealed to an M13 template DNA in which the complementary wild-type T4 DNA strand was present. A 10- μ l hybridization mix containing 0.4 pmol of template DNA and 1 nmol of oligonucleotide in hybridization buffer (6 mM Tris hydrochloride [pH 7.5], 50 mM NaCl, 6 mM MgCl₂, 1 mM dithiothreitol) was heated to 65°C for 10 min and rapidly cooled to -70°C. The frozen hybridization mix was placed on ice and allowed to thaw after addition of 10 μ l of reaction buffer (20 mM Tris hydrochloride [pH 7.5], 8 mM MgCl₂, 8 mM dithiothreitol) containing 100 nmol of each deoxynucleoside triphosphate and 100 nmol of ATP. Once it had been thawed and mixed, 6 U of DNA ligase and

0.25 U of Klenow fragment were added. The reaction mix was incubated at 0°C for 2 h, followed by reaction at 11°C for 18 h. The enzymes were heat inactivated at 65°C for 10 min. A 2- μ l portion was used to transfect 0.3 ml of competent JM103 cells, and 50- μ l and 200- μ l samples were plated. Plaques were transferred to nitrocellulose from plates containing 200 to 500 plaques and prepared for hybridization (18, 23).

The oligonucleotide was labeled with ³²P at the 5' end with polynucleotide kinase. A 70- μ l reaction mix containing 20 pmol of oligonucleotide, 100 mM Tris hydrochloride (pH 8.0), 10 mM MgCl₂, 200 μ Ci of [γ -³²P]dATP, and 20 U of polynucleotide kinase was incubated at 37°C for 1 h. The heat-inactivated reaction mix was loaded onto a Sephadex G-25 column (0.5 by 5 cm) equilibrated with 1 $\times$ SSC (0.15 M NaCl, 15 mM sodium citrate, 1 mM EDTA, pH 7.6). The void volume was collected and contained 2×10^6 dpm. Hybridization was carried out for 1 h at 20°C (18). The filters were washed three times in 6 $\times$ SSC at 20°C, dried at room temperature, and autoradiographed at -70°C with an enhancing screen (23).

Determination of phenotype for mutations identified by DNA sequencing. The marker rescue technique described elsewhere (K. Johnson, B. Condie, D. T. Mooney, and A. H. Doermann, manuscript in preparation) was used to characterize the plaque morphology of progeny arising from the mixed infection of JM103. Bacteria actively producing gene 23-containing recombinant M13 virus were infected at a ratio of 5 T4 gene 23 amber phage per cell. The ratio of wild-type to smaller plaque types among the progeny was found to be inversely proportional to the distance between the test mutation and the amber allele for all but one of the combinations tested. The exception was *amC208*, which lay 3 bp from the *Taq*I site used to clone the cluster III *ptg* mutants, at bp 1279 (31) (Fig. 1). This proximity is probably responsible for the much reduced frequency of *am*⁺ progeny observed when *amC208* was used (36).

Genetic mapping and marker rescue. The methods for UV mapping and UV rescue followed those outlined by Doermann (6). The complementation assay used to estimate the number of defective particles in the lysate of the site-directed mutant was done as described by Doherty (12).

RESULTS

Two factors greatly facilitated the cloning and sequencing of *pt* and *ptg* mutants. First, the genetic mapping of Doermann et al. (10) localized the 55 independently isolated *ptg* mutations to specific double amber intervals within gene 23. Second, the DNA sequence of gene 23 by Parker et al. (31) and their assignment of all 15 known amber alleles in gene 23 allowed a correlation of the genetic mapping data to specific *Taq*I DNA fragments which were expected to bear each mutation. These *Taq*I fragments were purified and cloned into M13 as described in Materials and Methods. Recombinant M13 phage, generally containing 10 to 30% colorless plaques, were screened by plaque hybridization (18). In 2 to 5% of the colorless M13 plaques, T4 DNA was found to hybridize. These plaques were eluted and replated at low plaque density. After overnight growth, an entire M13 plaque was used to infect an exponentially growing, 2- to 5-ml culture of JM103. At 5 h postinfection, a 10- μ l portion was removed from each culture and its T4 gene 23 DNA content was determined by the rescue of *gp23 am*⁺ alleles known to be present on the *Taq*I fragment of interest (29). At 7 to 12 h postinfection, the cells were removed by centrifugation.

FIG. 1. DNA sequence map of gene 23 *pt* and *ptg* mutations. The positions of the 10 *ptg* mutations and three *pt* mutations were determined by DNA sequence analysis (Table 1) and are plotted relative to the 1,563-bp coding sequence of gene 23. The positions of the *TaqI* restriction sites used in cloning these mutations are designated by solid triangles.

gation and template DNA was prepared from those M13 supernatants which were positive for hybridization to cognate T4 sequences and also rescued the predicted *am*⁺ alleles. The T4 DNA sequences of the mutant clones were determined (Table 1).

Petite/giant mutants. Twenty-six *ptg* mutants were sequenced, and 10 mutations were identified. Mutant designations are as described by Doermann et al. (10). Nine of the 10 *ptg* mutations were found in at least two independently isolated mutants. However, the mutation which gave rise to *ptgSp95* was found only once among the mutants cloned. Type-mutant designations are used to indicate the specific *ptg* mutant allele. Nine transition mutations were responsible for these alterations; *ptgSp95* again provided the lone exception, having arisen from a transversion mutation. A genetic map for the *ptg* mutations determined by DNA sequence analysis is shown in Fig. 1. The positions of the amber mutations used in cloning and marker rescue experiments are shown (31).

The strong correlation between the locations of the *ptg* mutations identified by sequence analysis and the genetic map derived from genetic recombination data (10) suggests that each of the mutations is by itself sufficient to confer the *ptg* phenotype. The characteristic plaque morphologies of *ptg* mutants (10) allowed another test of this conclusion. These mutations were individually rescued from recombinant M13 phage by mixed infection with T4 gene 23 amber mutants. From 10 to 40% of the rescue progeny produced plaques which were smaller than wild type. The small

plaques derived from each *ptg* clone were identical to those of the original T4 *ptg* mutant from which the clone had been derived.

The Ala-66 to Asp mutation in *ptgSp95* causes an alteration of the gp23 cleavage site (19) from Glu-65-Ala-66 to Glu-65-Asp-66. Sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis of proteins extracted (22) from *ptgSp95* revealed that gp23 was cleaved in this mutant to produce a protein with a mobility indistinguishable from that of the mature wild-type gp23 (data not shown).

The mutants *ptgBuJS17* and *ptgBuA* arose from mutations in adjacent base pairs within the Ala-94 codon (Table 1). These mutants produced similar amounts of petite and giant phage in lysates grown at 37°C. While the plating efficiency of *ptgBuA* (Val-94) was not affected when grown at 42°C, the presence of Thr-94 was lethal when *ptgBuJS17* was grown at that temperature.

Petite mutant *ptE920g*. Gene 23 *pt* mutants have been characterized which produce no giant phage (7, 14). The original mutation *ptE920g* was mapped to the distal third of the gene (8). UV rescue experiments showed that *ptE920g* carried two mutations and that both components mapped between *amC208* and *amE1236*. When *amC208/amE1236* was used as the helper phage to rescue the UV-irradiated *ptE920g*, the rescue plaques all resembled the original strain, *ptE920g*. However, when *amA489/amC208* was the helper, three kinds of plaque were seen: typical wild type, typical *ptE920g*, and a new tiny plaque that was present on plates incubated at 37°C but absent from 42°C platings. With

TABLE 1. DNA sequence analysis of *ptg* mutants

<i>ptg</i> cluster	Type mutant ^a	Identical mutants	Alteration (base no.) ^b	Amino acid alteration (residue no.) ^b
I	Sp95		C to A (197)	Ala to Asp (66)
	BuJS17	Ng38A	G to A (280)	Ala to Thr (94)
	BuA	Ha13	C to T (281)	Ala to Val (94)
	Bu19-2	Sp47, Em55	G to A (289)	Gly to Ser (97)
II	Em65	Em74, Ha21	C to T (803)	Ala to Val (268)
	Bu1980c	Ng212, Ap12	C to T (824)	Ala to Val (275)
	Ap24	Sp96	A to G (854)	Asp to Gly (285)
	Ng191	BuB7d, Ha20, Em92	G to A (859)	Asp to Asn (287)
III	Ng719	Ap19	C to A (1378)	Arg to Cys (460)
	BuD10	Em41, Sp101, Ha2	G to A (1381)	Gly to Ser (461)

^a The type mutants listed identify *ptg* mutants whose designation is used to describe a particular mutation.

^b gp23 amino acids are numbered from the amino-terminal methionine of the nascent gp23, and the gene 23 nucleotides are numbered from the A in the codon for that methionine (31).

TABLE 2. *pt21-34c*-rev UV rescues of intra- and extragenic amber alleles^a

Amber combination ^b	Plaque type (%)					
	<i>pt21-34c</i> -RevA			<i>pt21-34c</i> -RevB		
	WT	S	MED	WT	S	MED
<i>2am23</i>	3	97	0	5	95	0
<i>2am23/amlys8-82</i>	4.8	95.2	0	3.3	93.8	2.9
<i>2am23/2am24</i>	2	98	0	2	98	0
<i>2am23/am22</i>	1.8	98.2	0	1	99	0
<i>2am23/am1</i>	3.8	96.2	0	6	94	0

^a The *pt21-34c* revertants were each irradiated to 68 phage lethal hits and used to rescue the indicated multiple amber mutants. Wild-type (WT), small (S), and medium (MED) were the only plaque types observed in these rescue experiments. A minimum of 852 plaques were tabulated in each case, and the rescue experiments were carried out simultaneously.

^b The term *2am23* indicates the use of the double amber mutant *amE506/amE1270*. These amber alleles flank the *pt21-34c* mutation, and therefore two double-crossover events are required to rescue the *am⁺ pt⁺ am⁺* genotype. The terms *amlys8-82*, *2am24*, *am22*, and *am1* indicate the use of amber mutations located in those genes.

amE1236/amE355 (gene 23/gene 24) as the helper, three kinds of plaque were again observed. In addition to the wild-type and the *ptE920g*-type plaques, another tiny type was seen. It was not temperature sensitive. Stocks of the two kinds of tiny-plaque phage were made and crossed to each other. One of the segregants in this cross was a typical *ptE920g* phage. The 3' *TaqI* fragment in gene 23 was cloned from *ptE920g*, and two single-base transition mutations were found (Fig. 1). The mutation of the A at position 1369 to G led to the alteration of Thr-457 to Ala, and Val was substituted for Ala-495 due to the mutation of C to T at position 1454. From the locations of the amber mutations in the gene 23 map, it is likely that the temperature-sensitive segregant had Thr-457 changed to Ala and that the tiny segregant from the second UV rescue had Ala-485 changed to Val. It is interesting that these two mutations, separated by only 18 amino acids in the same protein, were mutually suppressive. Combined, they produced *ptE920g*, whose plaques were slightly smaller at 42°C than at 30°C. At the latter temperature, it made a plaque only a little smaller than the typical wild-type plaque.

Petite mutant *pt21-34*. Mutant *pt21-34*, first described by Doermann et al. (7), was not tightly mapped at that time due to large numbers of plaque morphology revertants in lysates of the mutant. More recently, UV rescue mapping (data not shown) suggested that this mutation lay between *amE506* and *amE1270* in the *ptg* cluster II region. The *TaqI* fragment that covers this segment was cloned from *pt21-34*. A single G to A transition mutation was observed at bp 823 (Fig. 1). It led to the alteration of Ala-275 to Thr in *pt21-34*. An M13 recombinant carrying this mutation was used to rescue T4 *amE1270⁺*. No plaques typical of *pt21-34* were observed among the rescue progeny. However, a small plaque type was frequently rescued. Phage from these plaques were isolated and replated on *E. coli* B cells. Electron microscopic examination of a lysate prepared with these phage revealed the presence of a high percentage of petite phage in its lysate, but no giant particles. This derivative of the parent *pt21-34* was therefore also recognized as a petite mutant and designated *pt21-34c*. UV rescue experiments with three gene 23 double amber helpers gave results consistent with the conclusion that *pt21-34c* carries a single mutation located close to the position known from sequencing data.

Revertants of *pt21-34c*. Doherty (11) demonstrated that the

phenotypic effects of the Val-275 mutation (*ptgBu1980c*) can be used to select extragenic mutations which suppress the *ptg* phenotype. These suppressor mutations were recognized as large-plaque revertants and mapped to genes 22 and 24. Lysates of both *pt21-34c* and its parent, *pt21-34*, were found to contain large numbers of plaque morphology revertants. Two such revertants of *pt21-34c* displayed a medium-plaque morphology which was similar to the medium plaque size of *pt21-34*. Lysates prepared from representative revertant plaques contained petite particles in levels significantly higher than in the wild type. Again, no giant particles were observed by electron microscopy.

UV rescue experiments were carried out to determine the location of the mutation(s) responsible for the revertant medium-plaque phenotype. *pt21-34c* revertants A and B (*pt21-34c*-RevA and -RevB) were irradiated with UV to 68 phage lethal hits and used to rescue a series of intra- and extragenic amber alleles (Table 2). The use of the double amber *amE506/amE1270* mutant in all rescue experiments guaranteed the rescue of the *pt21-34c* mutation in essentially all progeny. The mutation leading to the medium-plaque phenotype was not close to the *pt21-34c* site, since medium plaques were not observed when *amE506⁺/amE1270⁺* was rescued. Marker rescue of three other gene 23 double amber mutants by the *pt21-34c* revertants (data not shown) also failed to produce medium-plaque formers among a minimum of 852 plaques scored in each rescue. Thus, the mutation leading to the medium-plaque phenotype of these revertants is probably outside gene 23. The second pair of ambers in each helper ensured the rescue of a second segment of the T4 genome (Table 2). UV rescue experiments with selected amber alleles outside gene 23 in combination with *amE506/amE1270* demonstrated that the mutation responsible for the *pt21-34c* A revertant was not located in gene 22 or 24 and was not close to either gene 1 or the lysozyme gene. However, the rescue of *amlys8-82⁺* by *pt21-34c*-RevB yielded a sufficient number of medium-plaque formers (2.9%) to suggest that the medium-plaque phenotype of *pt21-34c*-RevB may be due to a mutation located in the vicinity of the lysozyme gene.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis was carried out as described in Materials and Methods. Autoradiography of M13 plaques, potentially containing the mutation in T4 DNA, revealed that all plaques had retained some isotope, but 1% of the plaques were three- to fourfold more radioactive. Phage from these plaques were eluted and replated. Template DNA was prepared, and DNA sequence analysis of three clones revealed that the predicted transversion mutation, A to T at bp 860, had been made.

Expression of this mutation led to the alteration in gp23 of Asp-287 to Val. To determine the effects of this mutation on capsid morphogenesis, an M13 clone containing the mutation was used to rescue the T4 *amE1270⁺* allele. A non-wild-type plaque morphology was present in 6% of the progeny. These plaques were minute in size and could best be observed after 48 h of incubation at 37°C. While plaques arising from wild-type infections contained approximately 10⁹ PFU under these conditions, these minute plaques yielded about 5 × 10⁴ PFU per plaque. Many plaque morphology revertants were present in lysates prepared in the normal manner from 48-h minute plaques.

Lysate preparation of the minute-plaque former. In preparing a lysate of this mutant, 1 ml of an *E. coli* B culture, grown to 5 × 10⁷ cells/ml in M9⁺ medium, was infected with one 48-h minute plaque and shaken vigorously at 37°C for 1 h. Serial twofold dilutions with fresh, prewarmed M9⁺ were

then carried out every 30 min for 2.5 h. In this way the bacterial concentration was held near 5×10^7 cells/ml while the phage in the culture were allowed to adsorb and multiply to the point where all cells could be productively infected. At 6.5 h the cultures were chilled on ice for 10 min, lysed with CHCl_3 , and clarified by centrifugation at $5,000 \times g$ for 10 min. Its titer was found to be 2×10^7 minute PFU/ml, and the stock contained fewer than 0.2% large-plaque formers. This mutant was designated SD-1E, and the phenotypic effects of its site-directed mutation were examined.

Complementation assay of SD-1E. Since petite phage contain only a fraction of the T4 genome, they are not viable after single infection (14). However, the fraction of defective phage can be approximated from the frequency with which an amber mutation is complemented in mixed infections (32). The complementation ratio of wild-type phage, when rescuing the gene 37 amber mutation *amN52*, was 1.3, and the ratio for *ptgNg191* was 7.9. A ratio of 24 was observed for SD-1E. This suggests the presence of more than 24 petites for each plaque-former phage in its lysate.

Revertants of mutant SD-1E. Large numbers of plaque morphology revertants were present in normal lysates of SD-1E. The high frequency of petite phage in the SD-1E lysate suggested that there was a strong selection for mutations whose phenotypic effect was to reduce the frequency of defective phage. Two revertants of SD-1E were examined. The first revertant, termed SD-1E-RevA, produced plaques which were essentially the same as wild type. A lysis-inhibited stock was prepared, and a portion was UV irradiated. UV rescue experiments (data not shown) suggested that two or more suppressor mutations were responsible for its phenotype. A second plaque morphology revertant of SD-1E, designated SD-1E-RevB, produced medium-sized plaques which were indistinguishable from those of *ptgNg191*. A lysis-inhibited stock was prepared and examined by electron microscopy. Petite particles were observed in levels comparable to *ptgNg191*, but no giant particles were present. The mutation responsible for the revertant phenotype was mapped after UV-irradiating SD-1E-RevB to a dose of 204 PLH. These inactivated phage were used to rescue a series of contiguous gene 23 double amber mutations (Table 3). The medium-plaque former phenotype mapped to a region very close to the known location of the SD-1E allele, but in no rescue was the minute-plaque phenotype of SD-1E observed. The mutation which leads to the formation of medium plaques is therefore very close to or allelic with the Val-287 mutation present in SD-1E.

DISCUSSION

DNA sequence analysis of 26 *ptg* mutants identified 10 mutated sites in gene 23. These mutations were grouped in three clusters which constituted no more than 10% of the length of the uncleaved gp23. Identical mutations were observed in several cases, and each of the mutagens used led to substitutions of G:C for A:T base pairs. With other mutagens, reciprocal substitutions might be found at other sites which could alter the capsid length-determining mechanism in T4.

To establish more firmly that these mutations were by themselves sufficient to lead to expression of the *ptg* phenotype, a series of UV rescue experiments were carried out. Mutant T4 DNA fragments, cloned into M13, were used to rescue adjacent gene 23 *am*⁺ alleles in T4 phage. In all cases, the plaque morphology of each *ptg* mutant was a major

TABLE 3. UV rescue of contiguous gene 23 double amber intervals by SD-1E-RevB^a

Gene 23 double amber allele	% Medium-plaque formers
<i>amB272/amE509</i>	39
<i>amE509/amE506</i>	62
<i>amE506/amE1270</i>	100 ^b
<i>amE1270/amE389</i>	78
<i>amE389/amE1236</i>	65

^a SD-1E-RevB was irradiated to 204 PLH and used to rescue the indicated gene 23 double amber alleles.

^b Only medium plaque formers were observed among 166 *E. coli* B-viable progeny produced by the rescue of this amber interval. Rescues involving the other amber intervals yielded only wild-type plaque formers in addition to the medium plaques.

component of the *E. coli* B-viable rescue progeny. Based on data from genetic crosses, DNA sequence analyses, and marker rescue experiments, the phenotypes of the *ptg* mutants described are concluded to arise from the consequences of single mutations in gene 23.

The Ala-66 to Asp mutation observed in *ptgSp95* had a characteristic of particular interest. During capsid assembly the Glu-65-Ala-66 peptide bond is cleaved by the action of the gp21 proteinase (19, 30). That bond in *ptgSp95* linked Glu-65 to Asp-66, but SDS gel analysis (data not shown) suggested that the mutant gp23 was nevertheless cleaved at or near this position. An analysis of the cleavage reactions of this mutant may determine a new dipeptide which can be cleaved by the gp21 proteinase.

The observation that phenotypically different *ptg* mutants can be generated by the mutation of Ala-94 to either Thr or Val (Table 1) allows direct comparison of the effects of three alternative amino acid residues at one site on the *ptg* phenotype. The Thr mutation (*ptgBuJS17*) was temperature sensitive, and its lysates contained very few isometric particles (9). This can be contrasted with significantly higher levels of isometric phage observed for *ptgBuA* (Val-94) as well as for all *ptg* mutations that lay 5' to the *ptgBu1980* allele (9). Both in vivo and in vitro analyses of *ptgBuJS17* prehead structures at 37 and 42°C, relative to *ptgBuA* and the wild type, may suggest the stage at which capsid assembly of *ptgBuJS17* is blocked at 42°C.

Two petite mutations in gene 23 were characterized which produced no giant phage (7, 14), and the gene 23 amino acid substitutions involved were identified. The mutant *ptE920g* produced high levels of isometric particles but few particles of the intermediate size class (7). Therefore the phenotype of this gene 23 double mutant is analogous to those of a group of mutants carrying mutations in the core proteins, which also form high levels of isometric phage (2, 20, 33). This suggests that the mutations responsible for the *ptE920g* phenotype may alter a gp23 structure which binds to the core proteins during assembly.

The phenotype of petite mutant *pt21-34* can be contrasted with *ptE920g*; the former produced high levels of both intermediate and isometric particles (7). DNA sequence analysis and marker rescue experiments demonstrated that *pt21-34* also carried at least two mutations. However, only one was located in gene 23. The gene 23 mutant derivative of the parental *pt21-34*, *pt21-34c*, was of interest because its mutation (Ala-275 to Thr) led to the formation of no giant particles. But when Val was substituted for Ala-275 (*ptgBu1980c*), both the giant and petite phenotypes were observed. These results reveal that at least one amino acid in

gp23 can be substituted for in such a way that it either enhances the level of petite particles or forms both giant and petite phage particles.

Extragenic suppressors of the *ptg*Bu1980 phenotype were isolated as plaque morphology revertants (11), and this suppression was found to be strongly allele specific (12). The presence of significant levels of plaque morphology revertants in lysates of both the parental *pt*21-34 and *pt*21-34c suggested that suppressors similar to those affecting *ptg*Bu1980 might be selected. While UV mapping of the medium-plaque phenotype of *pt*21-34c-RevA has not yet revealed its location, the revertant phenotype of *pt*21-34c-RevB was occasionally corescued with the *amlys*8-82⁺ allele. A rescue with the same helpers was used by Doherty (11) to isolate the originally existing *ptg*Bu1980c suppressor mutant, Sup-OR. The Sup-OR mutation appears to be located in or near the genes encoding the core proteins IpII and IpIII. It can therefore be tentatively concluded that a suppressor of the *pt* phenotype can also be selected in a region of the genome which is known to generate a suppressor of the *ptg* phenotype.

The mutations observed at Ala-94 and Ala-275 suggest that a range of alterations at a particular site may lead to viable phage production. To examine this possibility, the transversion mutation in SD-1E was constructed by oligonucleotide-directed mutagenesis at the site of the *ptg*Ng191 mutation. The formation of extremely minute plaques by this mutant was correlated with a high frequency of petite phage in its lysates (>95%). No electron microscopic observation of this mutant was made due to the very low titers achieved in the stocks of SD-1E, and therefore the presence of giant particles has not been assessed. The high frequency of plaque morphology revertants observed for SD-1E reflects a strong selection for genomes producing fewer defective phage than the parent. The mutation in one such plaque morphology revertant (SD-1E-RevB) was located very close to or was perhaps a site allele of, that in SD-1E. High levels of petite phage but no giant particles were observed, and SD-1E-RevB was thus recognized to be a petite mutant. If this revertant allele does not alter Val-287 itself, then SD-1E-RevB can be considered an intragenic suppressor of the SD-1E phenotype. The mutations in *pt*E920g also appeared to be mutual intragenic suppressors of the petite phenotype, since each individually produced plaques which were smaller than those of the double mutant.

These mutations in *pt*E920g and others that could, perhaps, be found by further study of *pt*21-34 may provide a useful experimental method for studying the protein-protein interactions in which gp23 is involved. Furthermore, the isolation of extragenic suppressors of capsid assembly mutations (11, 12) can be the foundation of a system for investigating the interactions between different proteins that are instrumental in building a geometrically defined capsid. The analysis of mutations which enhance or suppress the phenotypes of core protein mutations may lead to a better understanding of the structural relationships among the constituents of the core and between the core and the shell proteins.

A comparison of the amino acid sequences of gp24 (the vertex pentamer protein [1]) and gp23 (G. K. Yasuda, M. L. Parker, G. Churchill, and D. T. Mooney, manuscript in preparation) revealed several regions of strong homology. In gp23 these regions included all but one (*ptg*Sp95) of the *pt* and *ptg* mutations reported here. These regions are proposed to play a direct role in the assembly process, perhaps as binding sites through which gp23 and gp24 interact with the

core proteins in the assembling prehead. By the methods of Chou and Fasman (4, 5), the secondary structure of the gp23 region which encompasses *ptg* cluster II is expected to be altered when gp23 mutations are compared with the wild type. While the wild-type amino acid sequence from residues 260 to 293 was predicted to be α -helical throughout this sample, each of the *pt* and *ptg* mutations in cluster II was predicted to lead to a helix break. The *pt* and *ptg* mutations are proposed to induce structural changes in gp23 which alter the characteristics of its interactions with the core proteins (11, 12).

The gp23 mutant-specific intermediate particles reported by Doermann et al. (8, 9) may result from a change in gp23 structure which increases the likelihood that distal capping (13) will occur at the intermediate length. In this view, mutants carrying mutations in the core proteins (2, 20, 33, 38) failed to form intermediate capsids because termination of elongation cannot be achieved at the intermediate stage without an alteration in gp23, and only isometric petite and normal capsids are produced.

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