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Genetic Control of Capsid Length in Bacteriophage T4

I. Isolation and Preliminary Description of Four New Mutants

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Four new mutants are described whose phenotypic expression affects the length of the head of bacteriophage T4D. All mutants produce some phenotypically normal phage particles. Mutant *pt21-34* also produces at least two size classes of phage particle which have heads that are shorter than normal. The other three mutants, *ptg19-2*, *ptg19-80*, and *ptg191*, produce, in addition to phages with normal and with shorter-than-normal heads, giant phages with heads from 1.5 to at least 10 times the normal length. All mutations are clustered near gene 23. Giant phage particles have the following properties: they are infectious and contain and inject multiple genomes as a single continuous bihelical DNA molecule of greater-than-unit length. Their frequency, relative to the total plaque-former population, increases late in the infectious cycle. They have a normal diameter, variable length, and a buoyant density range in CsCl from equal to slightly greater than that of normal phage. The arrangement of capsomers is visible in the capsids, which are composed of cleaved gene 23 protein.

One of the principal advantages of using bacteriophage as a model system in the study of morphogenesis at the molecular level results from the availability of extensive and readily manipulated genetic material. Phage T4, whose genetic organization is well known, has proved to be particularly useful in morphogenetic investigation (12). The present series of articles is focused on a group of mutants whose phenotypic expression affects the length of the capsid of that virus. One petite mutant of T4, *ptE920g*, characterized by shorter-than-normal capsids, has aleady been described (13) and investigated both genetically (10, 27) and structurally (26). The first paper in this series describes additional mutations which affect capsid length of T4 particles. Some of the mutations give rise not only to petite particles, but also to giant particles whose head lengths range from 1.5 to at least 10 times the length of normal T4 capsids. These giant particles contain concatenated T4 DNA molecules which include multiple complements of the normal genome.

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MATERIALS AND METHODS

Bacterial and phage strains. Strain CR63 of *Escherichia coli* was used whenever amber-suppressor bacteria were required. Otherwise strain B was used for stock preparation and plaque assay as well as for the host bacterium in crosses.

Several mutants of phage T4D were involved in the experiments described here. An osmotic shock-resistant mutation (*os*) was introduced into wild-type T4D. All the petite mutants isolated and described here originate from that strain and consequently carry the *os* mutation except where it may have been lost due to reverse mutation or through recombination in crosses with os^+ phages. The mutation *ptE920g*, which was described earlier (13), was available in both *os* and os^+ strains. Several T4D amber mutants, obtained from R. S. Edgar, have also been used. The T4D mutant *r48* was used in some experiments to rescue *pt* mutations from UV-irradiated stocks.

Culture techniques. Recipes for top- and bottom-layer agar, as well as for H-broth, have been described previously (3). The glucose-base medium M9 + contains the following per liter of final medium: KH_2PO_4 (3.0 g), Na_2HPO_4 (7.0 g), NaCl (0.5 g), NH_4Cl (1.0 g), $MgSO_4 \cdot 7HOH$ (0.246 g), $CaCl_2$ (0.011 g), ferric citrate trihydrate (0.6 mg), glucose (4.0 g), and Casamino Acids (10 g; Difco).

Stocks of phages which carry no *r* mutation were made by the lysis inhibition technique, a simple procedure used for several years by Kellenberger and his collaborators when higher-than-normal lysate ti-

ters are desirable. The method as used here includes the following steps. Cells (preferably strain B) at a titer of about 4×10^8/ml are infected with about 0.1 plaque-former phage per cell and incubated at 30 C with vigorous aeration. Dow-Corning Antifoam A or propylene glycol monolaurate is added as needed to reduce foaming. After one round of infection and lysis, all remaining cells become infected. Some of these lyse on schedule, liberating phage which superinfect the majority of cells which are still unlysed. This superinfection causes inhibition of lysis (9). Starting at 150 min, samples (5 ml) are removed periodically and shaken with chloroform to test for the degree of lysis which can be induced. When that test shows that most of the cells can be induced to lyse so that clearing is nearly complete (usually at about 180 min after initial infection), the culture is chilled in an ice bath and the pregnant bacteria are pelleted by centrifugation at about 5,000 rpm for 10 min in a cold centrifuge. The pellet is resuspended in 1/20 to 1/10 the original volume of culture medium by using a magnetic stirring bar. After the cells are resuspended, several milliliters of chloroform are added, followed immediately by DNase and RNase at a concentration of at least 10 μg/ml and additional Mg^{2+} to assure a final concentration of no less than 10^{-3} M. After 30 min, the lysate is centrifuged for 10 min at 4,000 to 5,000 rpm in the SS34 rotor of the Sorvall RC2-B centrifuge. If the preparation contains giant phages, centrifuge speeds of over 4,000 rpm are avoided. The supernatant fluid is centrifuged a second time to remove additional debris. T4 wild type ordinarily gives a titer of 10^{12} particles/ml or higher after this procedure.

Phage assays are made with exponentially growing plating bacteria (14).

Selection and identification of petite mutants. Petite mutants were obtained by using several variations of a basic method, and only a general procedure is given here. In all cases, T4D*os* was grown in the presence of a chemical mutagen. The properties of petite particles (lower density and slower sedimentation through sucrose gradients than normal phages) make possible a powerful selective technique. Because it was anticipated that phenotypic mixing (28) might strongly reduce the frequency of particles with the petite phenotype, the mutagenized lysates were subcultured in a cycle of single infection and lysis in which the multiplicity of infection (MOI) was lower than 0.5 particles per cell. Density gradients using cesium formate in heavy water were used to select lower-density phages, or, alternatively, sucrose gradients were used to select slowly sedimenting particles.

The petite particles have incomplete genomes, making it necessary to rescue the presumptive *pt* mutation into whole phage capable of multiplication. Eiserling et al. (13) had shown that *ptE920g* is located between *amB17* in gene 23 and *amN65* in gene 24. On the assumption that new petite mutations would also be located in this region, the doubly mutant strain *amN53amE355* (gene 21 and gene 24 amber mutants, respectively) was used as the helper phage in the rescue procedure. Cells of *E. coli* B were infected with 1 to 3 double-amber phage per cell and much less than 1 particle per cell from the gradient samples. The lysates were plated on B, which eliminates any phage which carries either amber mutation. Demanding both am^+ genes assures that the vast majority of plaques will be formed from particles which received the segment between genes 21 and 24 from the mutagenized phages which have been selected for low-density or slow sedimentation.

In picking plaques from these platings, the morphology of the plaque was used as an additional selective criterion; smaller than normal plaques were selected because *ptE920g* makes a distinctly smaller plaque than the wild type. Such plaques were subcultured to produce small-volume lysates.

Among the stocks obtained in this way, some petite mutants were identified. They were recognized by one of two techniques. One method was to titrate the stocks by the usual plaque-count assay, and compare the titer obtained in that way (plaque former titer) with the titer obtained from a complementation assay (27). Alternatively, bacteria-killing ability could also be used to estimate the titer of total phage particles for comparison with the plaque former assay (see second paper of this series for details of that method). The second method used to recognize petite mutant stocks was to examine a sample of each lysate in the electron microscope.

Elimination of irrelevant mutations. Mutations were selected after vigorous mutagenization by using small-plaque morphology as one criterion in the selection process. It would not be surprising to find that the products of such a method would frequently carry additional mutations unrelated to the petite characteristic. To eliminate irrelevant mutations, a mutant stock was UV-irradiated to give plaque former survival between e^{-20} and e^{-25}. The *pt* mutation was rescued from the UV-killed phages by mixed infection with an *os* wild type or, in one case, with T4D*r48*. In the latter case, the *r48* was again removed by further crosses with wild type. A mutant stock was regarded as clean when it yielded only two plaque types in test crosses with wild type.

The plaque produced by *ptg19-80*, even after elimination of extraneous mutations by the procedure described above, seemed to be unreasonably small when compared with any of the other petite mutants found. It seemed possible that a closely linked but otherwise nonrelated second mutation might still be present and be responsible for the unusually small plaques. An additinal test was therefore made. A stock of the apparently pure *ptg19-80* was made in the presence of a highly mutagenic concentration of nitrosoguanidine. This lysate produced some particles whose plaques showed typical wild-type morphology. Twenty such particles were picked and suspended in broth. Platings of the diluted suspensions gave plaques from which heterozygous particles were largely eliminated. The latter plaques were used to make lysates from each of the 20 original revertants. Of the 20 stocks, 19 showed a complementation titer which did not differ significantly from the plaque former titer. One case did consistently show a complementation titer at least twice as high as the plaque former titer, presumably indicating the presence of petite particles. Single-plaque subcultures of that revertant showed the same result. The fact that 19 of

20 stocks simultaneously reversed both the tiny plaque morphology and the petite phenotype leads to the conclusion that a single mutation is probably responsible for both characteristics. The exceptional case can be attributed to the accidental induction of a new *pt* mutation as a result of the nitrosoguanidine mutagenization.

Electron microscopy (EM). Samples were prepared for negative contrast in uranyl acetate (UA) by simple adhesion of phage particles to a carbon-coated Parlodion film on a 200- or 400-mesh copper grid. The adhering phage were rinsed briefly with distilled water and allowed to air-dry in a thin layer of 1% (wt/vol) UA. The pH of the solution was not adjusted and usually was about 4.5.

A significant improvement in preservation of shape was noted when the rinsed, adhering phage particles were first soaked for 15 to 30 min in saturated UA and then air-dried from 1% UA as described above. From 20 to 50% or more of the phage heads on the film appear gray, i.e., partially penetrated by the stain. These have straighter sides and better defined edges and angles than do the wider, more electron-lucent images normally seen by negative staining. Perhaps the saturated UA acts as an in situ DNA fixative under these conditions.

Quantitative particle counts were done by using the agar filtration technique (18) with added polystyrene latex particles as a concentration reference standard. This method was used whenever absolute particle counts or length distributions of giant phage were measured, because the simple adhesion method may be selective and is certainly nonquantitative in phage recovery. The agar filtration method was used to establish the degree of selectivity and thereby to determine the reliability when the adhesion method was used for length distribution studies.

DNA molecules were released from purified giant phage by using 5 M $NaClO_4$ (15). The molecules were prepared for microscopy by using the formamide technique described by Davis et al. (7). Lengths were determined by measuring the contour lengths on tracings of micrographs at 10 or 20 times the EM magnification of X 5,000.

Mutant-infected cells were examined by thin sectioning. Prefixation was in 0.5% glutaraldehyde (1:100 dilution, Fisher Biological Grade) for 5 min. Prefixed cells were washed once in Veronal acetate buffer at pH 6.0, followed by overnight fixation in 1% OsO_4 as described by Kellenberger et al. (20). After 2 h of further fixation in 0.5% UA in Veronal acetate, cells (in agar blocks) were dehydrated in acetone and embedded in Vestopal W.

Phage purification. For experiments requiring large amounts of phage, separation and purification of petite, normal, and giant particles were done by rate-zonal centrifugation on preformed sucrose gradients. Quantities of 50 to 100 ml of concentrated lysate prepared by the lysis inhibition technique were centrifuged on 15 to 40% (wt/vol) sucrose gradients in the Spinco Ti-15 zonal rotor at 24,000 rpm for 60 min (petite phage), or the Sorvall SZ-14 reorienting zonal rotor at 14,000 rpm for 60 min (giant phage). Fractions were examined by EM, assayed for viable phage, and further concentrated.

Petite phage zonal gradient fractions in sucrose containing 95% petite phage by EM were combined and centrifuged to a pellet in polycarbonate tubes at 25,000 rpm for 60 min in a Spinco Ti-60 rotor at 20 C, and were allowed to resuspend overnight in a small volume of M9 salts, which consists of M9+ medium from which the glucose and Casamino Acids have been omitted. A portion (1 ml) of this sample was applied to a linear 15 to 40% (wt/vol) sucrose gradient in the Spinco SW27 rotor and centrifuged 50 min at 19,000 rpm at 20 C. Fractions wre obtained by collecting drops from the bottom of the punctured nitrocellulose tube. The band containing petite phage was concentrated by centrifugation and used for further experiments. The same procedure was followed when purifying normal phage. Microscope examination of petite phage preparations showed a very clean preparation of intact, isometric phage. The ratio of plaque-former titer to particle titer (from EM count by agar filtration) was 2×10^{-3}, indicating greater than 99% petite phage. Samples from sucrose gradients which contained giant phages (greater than unit length as determined by EM) were pooled, dialyzed against phosphate-magnesium buffer, and concentrated by centrifugation onto a 3-ml shelf of Renografin (Squibb) at 11,500 rpm for 50 min using polycarbonate tubes in the Sorvall HB-4 rotor at 20 C. The concentrated phage were dialyzed and used in those experiments which required purified giant phages.

Buoyant density determination. Purified samples of normal, petite isometric, and giant phages were prepared as described above and then diluted in phosphate-magnesium buffer to give about the same amount of light scattering. Equal volumes of the three samples were mixed, and stock CsCl (optical grade, Stanley H. Cohen Co., Yonkers, N.Y.) at 1,857.1 g plus 1,000 g of distilled water (ρ = 1.905 g/cm^3) was added very slowly to give an average density of about 1.501 g/cm^3 (refractive index 1.3819). Three 4-ml samples were overlaid with 1 ml of mineral oil and centrifuged at 25,000 rpm in the SW39 Spinco rotor for 31 h at 20 C. Gradients were removed and photographed, and five-drop samples were collected by puncturing the bottoms of the nitrocellulose tubes. Sample density was estimated by refractive index measurements. Each sample, still in CsCl, was prepared for UA negative staining as described above, except that 0.1% cytochrome *c* was added to the rinse to insure spreading of the stain.

Gel electrophoresis. The method of Laemmli (22) was used for sample and gel preparation. Purified phages were dissociated with 2% (wt/vol) sodium dodecyl sulfate and 2% (vol/vol) β-mercaptoethanol at 100 C for 1 to 3 min. This material was run as described earlier (8).

UV radiation. Samples to be irradiated were diluted at least 200 fold in M9+ medium from which the glucose and Casamino Acids were omitted and to which gelatin was added at a concentration of 10 μg/ml. The UV source was a 15-W low-pressure mercury vapor lamp powered by a Sola constant-voltage transformer. Suspensions of phage were exposed in watch glasses placed 1 meter from the center of the lamp. All assays of UV-irradiated phages were

carried out in dim yellow light to avoid photoreactivation.

RESULTS AND DISCUSSION

Qualitative characteristics of the mutants. By using the methods described, more than 10 new petite mutants have been isolated. Of these, four have been selected for the study described here. Some of their principal features are summarized in Table 1. In our hands, mutant *ptE920g* gives low numbers of intermediate-length petite heads, although Mosig et al. (26) report conditions under which they are increased. Petite particles have been reported at low frequency in wild-type lysates by Mosig (25), who separated them from normal T4 particles on the basis of their low density. Occasional petite particles were also observed in electron micrographs of wild-type populations by several investigators (2, 19, 24). Under our growth conditions, however, such particles are quite rare in wild-type lysates. Figure 1 shows the plaque morphologies of various mutants and Fig. 2 shows electron micrographs of normal, intermediate, and isometric particles as well as giants. To date it has not been possible for us to distinguish two sizes of capsid in the intermediate category as would be expected on the basis of the data presented by Mosig et al. (26). A quantitative study of petite head lengths encountered here will be presented in a future paper of this series.

Clustering of the mutants in the genetic map. Four of the five mutants described in Table 1 were crossed in all pairwise combinations. The fifth, *pt21-34*, reverted to wild-type (or pseudo-wild-type) too frequently to allow dependable estimates of recombinant frequency. Crosses were performed as described by Doermann and Parma (10) except that *E. coli* strain B was used as the host bacterium. The crosses are not ideal because it is difficult to assess the input multiplicity ratios precisely due to a combination of two factors: (1) plaque morphologies of *ptE920g*, *ptg191*, *ptg19-2* are not sufficiently different to permit reliable distinction. That prevents estimation of equality of parental inputs from the yields obtained in all crosses except those in which the distinguishable *ptg18-80* is one of the parents. The input titers on the other hand are not very reliable because isometric and intermediate petite particles inject only fractional genomes, whereas giant phages inject multiple genomes. The following method was used to obtain crosses with approximately equal contributions of two parents indistinguishable by plaque morphology. Each was first crossed to *ptg19-80* where plaque-type distinction of progeny allows evaluation of equality of input. Then the two indistinguishable parents were crossed by using inputs identical to those which achieved equality in the crosses with *ptg19-80*. Uniparental multiple-infection experiments (in which the MOI of the single parent was doubled in comparison to its multiplicity in biparental crosses) were included to show that reversions are not a significant source of error.

The main conclusion from the crosses given here is that the mutations are all located quite near the original *ptE920g* (Table 2). The two mutations *ptg191* and *ptg19-80* are significantly closer to one another than are any other pair of mutations. It should be noted that the recombination values are probably exaggerated to a significant degree by the presence of heterozygotes in the progeny of the cross. Not only recombinant heterozygotes (11), which are in fact only half-recombinants, but also the nonrecombinant heterozygotes will amplify the frequency of apparent recombinants. This is pre-

TABLE 1. *Characteristics of head-length mutants compared with wild-type phage*[a]

Mutant	Mutagen[b] used	Plaque size	Isometric petite particles	Intermediate petite particles	Normal-length phage	Giant[c] phage	Long[c] giant phage
ptg19-2	BrDU	Medium	+	+	+	+	–
ptg19-80	BrDU	Tiny	+	+	+	+	R
ptg191	*NG*	*Small*	*R*	+	+	+	–
pt21-34	BrDU	Small	+	+	+	–	–
ptE920g	AP	Small	+	R[d]	+	–	–
T4D wild type	None	Large	R[d]	R[d]	+	–	–

[a] Head length classification is by electron microscopy and sedimentation velocity. Symbols: +, class of particle is found with high frequency ($\geq 10^{-2}$); R, class is rare ($< 10^{-2} > 10^{-3}$); –, class was not seen ($< < 10^{-3}$).

[b] BrDU, 5-bromo-2'-deoxyuridine; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; AP, 2-aminopurine.

[c] Giant phages have head lengths which are 1.5 to 8 times normal; long giants have head lengths greater than 8 times normal.

[d] Data from the following references: Mosig et al. (26), Kellenberger et al. (19), and Eiserling et al. (13). See text for discussion.

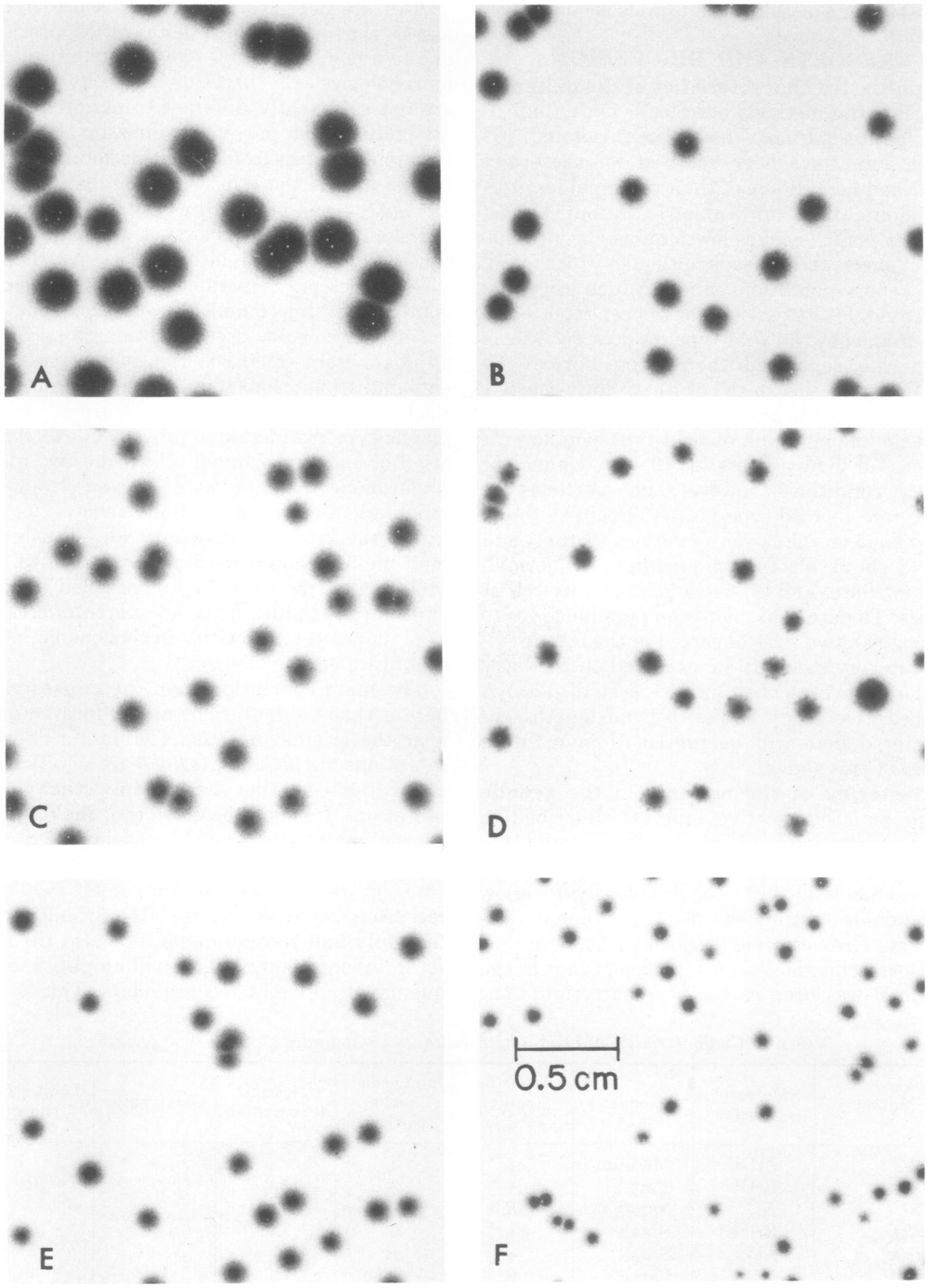

FIG. 1. *Plaque photographs of the five petite mutants and of wild type. The genotypes are as follows: A, wild type; B, ptg19-2; C, ptE920g; D, pt21-34; E, ptg191; F, ptg19-80. All magnifications are identical and can be determined from the bar shown in F. The one large plaque in D presumably resulted from a genetic revertant already present in the phage sample plated, or was caused by a reversion which occurred at an early stage in the growth of the plaque.*

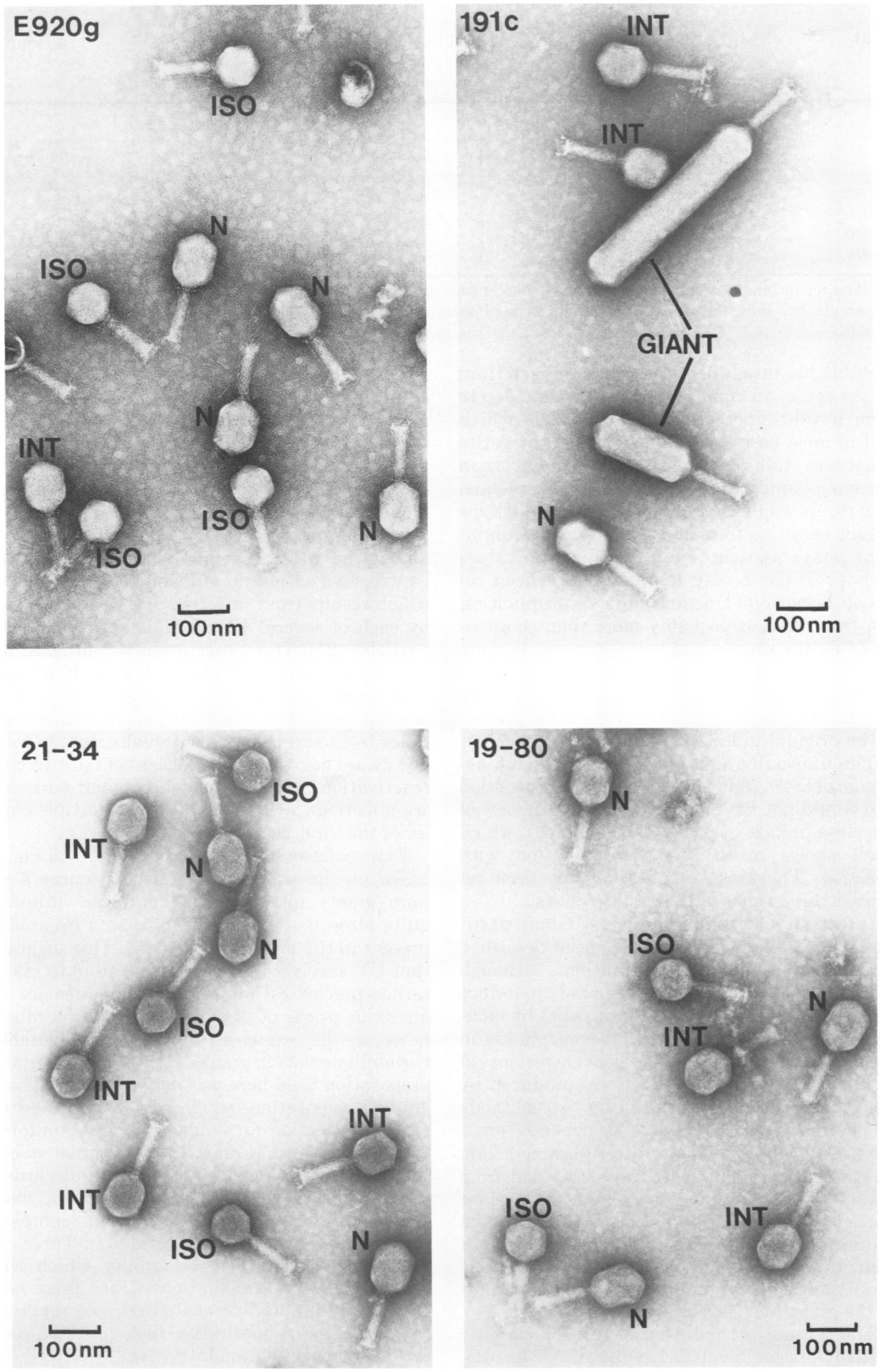

FIG. 2. *Selected micrographs of mutant lysates prepared by treatment with saturated uranyl acetate in situ. Mutant number is at upper left. Head classification is indicated as follows: ISO, isometric petite; INT, intermediate length petite; N, normal length.*

TABLE 2. *Recombination values[a] from crosses between pairs of petite mutants*

Parent II	Parent I			
	ptE920g	*ptg19-80*	*ptg191*	*ptg19-2*
ptE920g	<0.1 (0)	10.3 (550)	4.4 (167)	4.5 (81)
ptg19-80		<0.1 (0)		
ptg191			~0.05 (1)	3.5 (178)
ptg19-2				<0.04 (0)

[a] The recombination value recorded is the percentage of wild-type recombinants multiplied by two. Numbers in parentheses indicate the number of wild-type plaques on which the estimate is based. See text for additional details.

dictable because, upon infection of a bacterium on the plate, an opportunty is again provided to form a wild-type recombinant particle which will in most cases be able to outgrow the petite genotypes (all of which make smaller than normal plaques) during growth of the plaque. The result would be a phenotypically wild-type plaque resulting from an originally nonrecombinant phage particle. The recombination values seen in Table 2 may therefore have been reduced because of unequal input multiplicities, but that effect is probably more than compensated by the presence of heterozygotes. In spite of these quantitative uncertainties, it seems tentatively reasonable to assume that the three new mutations are probably in the same gene as is the original mutation *ptE920g*.

The unusually high recombination value between *ptE920g* and *ptg19-80* is not understood and would not have been anticipated in view of the close linkage of *ptg19-80* with *ptg191*, which itself shows much less recombination with *ptE920g*. The cross in question has been repeated three times with similar results.

Infectivity of giant particles. Giant particles (Fig. 2) have not heretofore been described in connection with petite mutations, although Cummings et al. (5, 6) have been able to induce apparently similar particles (lollipops) by incubation of cells infected with T-even phages in the presence of inhibitory concentrations of L-canavanine. The giant particles produced by *ptg* mutants have been shown by two methods to be infectious. One method depends on a quantitative comparison of titer obtained from electron micrographs with titer obtained from plaque assays by using a preparation of giant particles purified by passage through zonal sucrose gradients. Such purified preparations yield ratios of plaque-forming titer to particle counts approaching unity, indicating that most of the giant particles are infectious.

The second method used to test for viability of giant phages involves the use of UV irradiation. It seemed plausible that, if giants are capable of injection and phage production, they might well inject more than a single phage-equivalent of DNA into the host cell. These multiple genomes should in that case be able to complement one another. If so, giant phages, irradiated with UV before mixing with an excess of sensitive plating bacteria, ought to display high resistance because of reactivation resulting from complementation and recombination among the multiple genomes. The result would be expected to mimic multiplicity reactivation which results from injection of a single genome by each of several UV-inactivated normal-size particles (23). This expectation is confirmed by the experiment described in Fig. 3. It is seen that giants are much more resistant to UV irradiation than are normal plaque-former particles (*r48*) and that the survival curve shows a downward curvature reminiscent of multiplicity reactivation curves (23). Clearly, giant particles are infectious, evidently injecting multiple copies of the viral genetic material.

Extrapolations of curves B, C, and D in Fig. 3 based on the shape of the survival curve A of pure giants intersect the zero-dose ordinate quite close to the fraction of giants originally present in the mixed suspensions. This suggests that UV-survival curves might be used to make rather precise estimates of the frequencies of giants in stocks of *ptg* mutants. Caution must, however, be exercised because considerable variability exists in giant length. The pure giant preparation used here was derived from sucrose gradient separation, which can be expected to exercise selection for longer and more uniform giants than those present in the original stock, short giants having been selectively discarded (Fig. 5b). Furthermore, giant size can vary considerably from mutant to mutant (compare *ptg191* giants with *ptg19-80* giants in Table 1). Such factors introduce variability which will seriously affect extrapolations. Until these factors are eliminated or evaluated, the method, though a useful qualitative tool, must be considered as only semiquantitative.

Time of intracellular appearance of giant particles. The observation has previously been

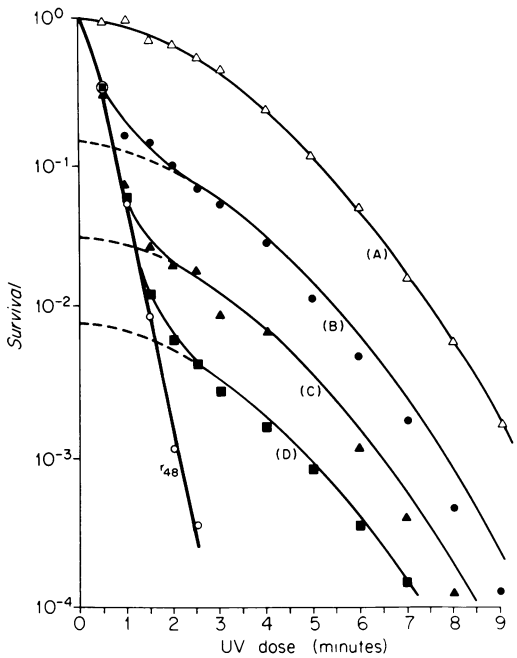

FIG. 3. *Survival of giant phages as a function of UV dose. A stock of pure (>90%) giants was prepared by zonal centrifugation and concentrated as described in Materials and Methods. The length distribution of this stock is shown in Fig. 5b. Samples were mixed with suspensions of normal-length phages carrying the mutation r48. The four suspensions irradiated contained (A) pure giants, (B) 15% giants mixed with 85% r48, (C) 3.3% giants with 96.7% r48, and (D) 0.8% giants with 99.2% r48. The ordinate gives survival on a logarithmic scale. Each point represents the total survival (giants + r48) at a particular dose which is given on the abscissa. The survival curve for r48 is the survival in sample (D), where r48 represents the majority of survivors up to and including the 90-s dose. Symbols: r48, O; (A), Δ; (B), ●; (C), ▲; (D), ■. Curves B, C, and D, which have been extrapolated through zero dose, have the same shape as curve A and were drawn to intercept zero dose at the calculated fraction of giants present in each sample.*

made (13) that the mutant *ptE920g* produces mainly normal-size particles during the early stages of the latent period, but that petite particles become more abundant as the latent period advances, and especially if conditions favoring lysis inhibition prevail. Mutant *ptg191* was used to see whether the appearance of giant particles follows a similar time course (Fig. 4). An exponentially growing culture of *E. coli* B at about 10^8 cells/ml was infected at 30 C with that mutant at an MOI of about 2.7 plaque-former phages plus approximately 6 petite particles/cell. The unadsorbed plaque formers amounted to less than 1 per 10 cells. Samples were removed at 23, 27, 29, 33, and 40 min after infection and were immediately shaken with chloroform to arrest development and induce prompt lysis.

The lysates thus obtained were diluted at least 100-fold in M9+ medium lacking glucose and Casamino Acids, and to each diluted lysate T4D*r48* particles were added to give approximately equal titers of *r48* and *ptg191* plaque-former particles. UV survival curves were made on each mixture, the *r48* with its readily distinguishable plaque morphology serving as a normal-phage survival control. Because giants have been shown to be UV resistant, comparison of survival curves of the lysates was expected to give a semiquantitative estimate of the frequencies of giant particles in the various samples.

It is evident that giant particles are rare even as late as 27 min after infection when an average of 26 plaque-former particles were present per cell (Fig. 4). Thereafter the relative frequency of giants increased continuously until at least 40

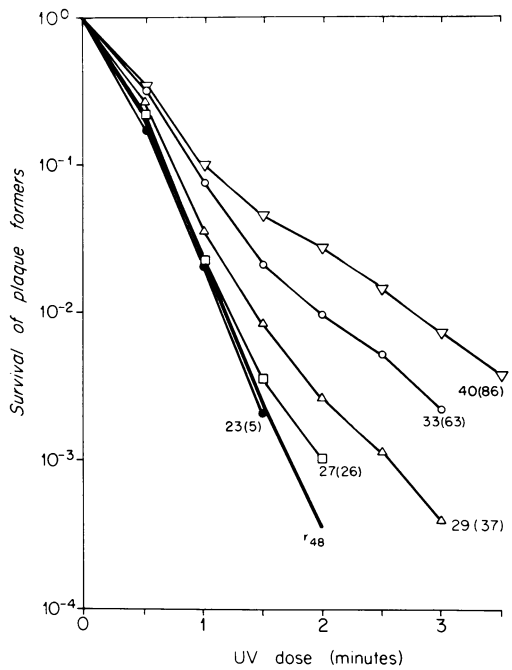

FIG. 4. *UV resistance used to reveal the time of first appearance of intracellular giant phage particles. Premature lysates, from a culture of E. coli B multiply infected with ptg191, were mixed with r48 and irradiated with UV. The survival of r48 (control for normal-size particles) is averaged from all samples at each dose and is shown as the heavy line in the figure. The first number given at each curve represents the time at which lysis was induced by addition of chloroform. It is given in minutes after mixing phage and bacteria. The numbers in parentheses indicate the number of plaque former particles produced per cell in each sample.*

min (86 plaque formers/cell) when the last sample was taken. It may be concluded that giants and petites have the similarity that both are rare early in the growth cycle but become increasingly frequent as intracellular phage growth continues.

A brief discussion of preliminary observations on thin section of cells infected with *ptg191* is presented here because they, too, show a striking difference, with time, in the number of giant phage per cell. (These data will be discussed in detail in a future paper.) The conditions of infection differed from the UV experiment. Bacteria at a titer of 4×10^8 cells/ml were infected with three plaque-former and 5 petite particles per bacterium. Lysis inhibition was induced by an identical superinfection 8 min after the primary infection. Samples fixed 30 min after the primary infection showed very few giants, whereas they were numerous by 60 min after infection. The later samples also showed giant phages in most cells of the population, showing that their frequency as seen in lysates is the result of most cells producing a few giants. rather than a few cells producing most of the giants. In addition to normal and petite phages, large numbers of aberrant structures (lumps, polyheads, and tau-particles) were seen in cells infected with each of the mutants listed in Table 1. Since these structures are made from uncleaved P23 (21, 22), there is probably a reduced amount of head protein available, which may be related to the reduced burst size which is observed with these mutants.

Length distribution. Giant phage found in lysates of mutants *ptg19-2*, *ptg191*, and *ptg19-80* have a heterogeneous head length distribution, from slightly longer than normal T4 particles to more than 10 times the normal length. The length distribution is itself somewhat variable under different, but as yet undefined, growth conditions (such as medium, temperature, and/or physiological state of the host bacteria). Figure 5a shows the distribution of longer-than-normal particles from a lysate of *ptg191* prepared for EM by the quantitative agar-filtration method. Among those phages of greater-than-normal head length, the modal class is composed of capsids which are two to three times the normal length when all giant particles in the lysate are measured. Purification by sucrose gradient centrifugation selectively alters the distribution, longer phages being much more frequent (Fig. 5b).

Morphology of giant phage. The most striking feature of the giant phages is their increased length, but in many micrographs the phages appear much wider than normal, that being the usual appearance by common negative staining

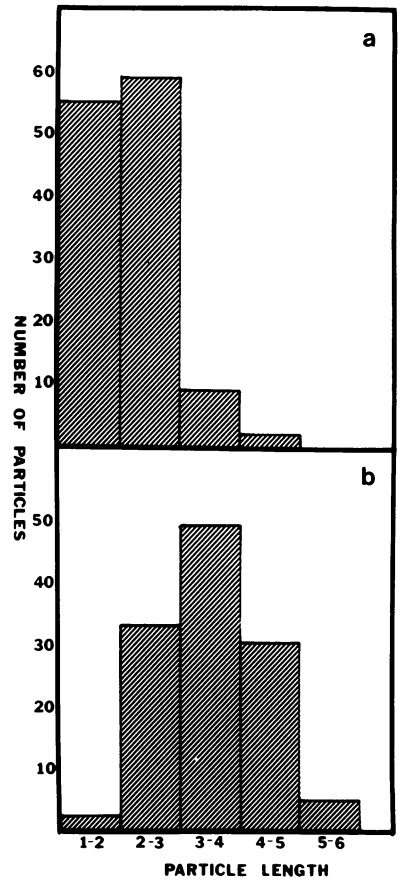

FIG. 5. *Length distribution of giant phage heads as measured by electron microscopy. a, Giants measured from an unfractionated lysate prepared by agar filtration; b, giants obtained from the rapidly sedimenting band in a sucrose gradient. Particle length is expressed in equivalents of a normal phage head length.*

procedures. The following observations, however, indicate that the excessive width is an effect of flattening of the phage head during preparation of the specimen. (i) Intracellular giant phages seen by thin sectioning after fixation have the same diameter as the normal phages in the same cell. (ii) Giant phages fixed in formaldehyde and metal shadowed in the agar-filtration procedure have the same diameter as normal phages. (iii) Preparations of giant phages taken from CsCl equilibrium density gradients after more than 24 h, as well as (iv) giant phages after treatment with saturated uranyl acetate for 30 min, show many giants with the same diameter as normal phage. Presumably, all these treatments protect the giant phages against flattening deformations, which may be related to the large distance between the ends or caps of the phage head. These caps usually resist the flattening seen in air-dried,

metal-shadowed preparations of unfixed giant phages.

Capsomer structure is discernible on both full and empty giant phages by using negative staining and metal shadowing, but only when electron micrographs are examined by optical diffractometry. The capsomer arrangement closely resembles that described by Yanagida, DeRosier, and Klug (31) for certain classes of polyhead and will be the subject of a future paper. The important point here is that the capsomer structure is now visible in considerable detail (2.5–3.0 nm resolution) on heads of infectious T4 phage. Capsomers were described earlier at lower resolution on T2 by Bayer and Remsen (1). The tail and fiber structure appears identical to that of normal T4.

DNA content and buoyant density of giant phage. Because giant phage were shown to be viable and to carry and inject multiple copies of the phage genes, they must contain more DNA than do normal phage. This was shown directly by using the formamide-DNA-spreading method of Davis et al. (7) for EM examination of the DNA of giant phages from the purified preparation described by Fig. 5b. The results presented in Table 3 show that continuous bihelical DNA molecules of greater-than-unit length are released from giant phage particles. Close examination of the ends of 12 such molecules revealed that 8 of the 24 ends were apparently single stranded for lengths in the range of 1 μm or less. Resolution was inadequate to reveal terminal single-stranded regions much shorter than 0.5 μm. Additional experiments are contemplated to determine with higher resolution the size distribution and also to investigate whether one or both ends are uniformly single stranded. For the present it is difficult to assess the biological significance of these regions. Single-stranded ends with lengths similar to those described here have been observed by Huber-

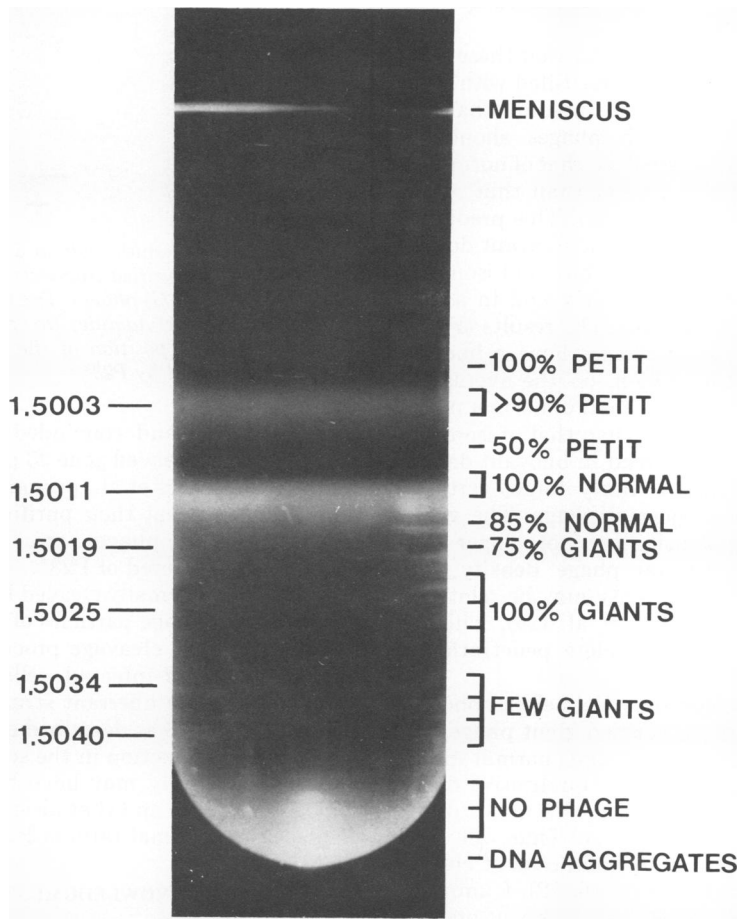

FIG. 6. *Photograph of a CsCl gradient containing a mixture of purified isometric petite, normal, and giant phages. Samples were removed and examined by electron microscopy and for refractive index. Results of microscope counts are given to the right of the photograph, and density is indicated at the left.*

TABLE 3. *Lengths of nine DNA molecules from giant phages*

Molecule number	Length (μm)	Multiples of normal (50 μm)
1 (195-24)	76	1-2
2 (195-28)	79	
3 (195-33)	99	
4 (195-23)	102	2-3
5 (195-16)	115	
6 (195-26)	127	
7 (194-33)	141	
8 (194-20)	176	3-4
9 (195-12)	185	

man (17) and Broker (Ph.D. thesis, Stanford University, Stanford, Calif., 1972), who examined replication complexes extracted from infected bacteria. A possible relationship of single-stranded ends to the events in replication or recombination have been discussed by Watson (30) and by Broker.

The results described suggest that these giant phage heads are as completely filled with DNA as are normal heads and predict that the buoyant density of such phages should be greater, or at least as great as, that of normal T4 particles, and surely greater than that of isometric petite particles (13, 26). This prediction was tested by determining the buoyant density in CsCl of purified normal, giant, and isometric petite particles both separately and in a mixture of the purified phages. The results (Fig. 6) show that these giant phages have a buoyant density distribution which, on the average, is higher than, but overlaps that, of normal phage. It is considerably greater than that of isometric petite particles. The spread in buoyant density of the giant phages is fairly large, certainly larger than that of normal phage. The giants found at higher density are not longer than those nearer to normal phage density. The reason for this heterogeneity may be related to that described by Mosig et al. (26), which is presumably due to incomplete penetration of the heads by CsCl.

Protein composition. Gel electrophoresis was used to compare purified giant phage with purified isometric petite and normal phage (from a wild-type stock) for qualitative differences in protein composition. No major differences were detected (Fig. 7). Thus, all three types of phage capsid are composed of cleaved gene 23 product (P23*) (8, 16, 22). Cummings (4) compared the molecular weights of proteins from polyheads induced by L-canavanine with those from polyheads produced by gene 20

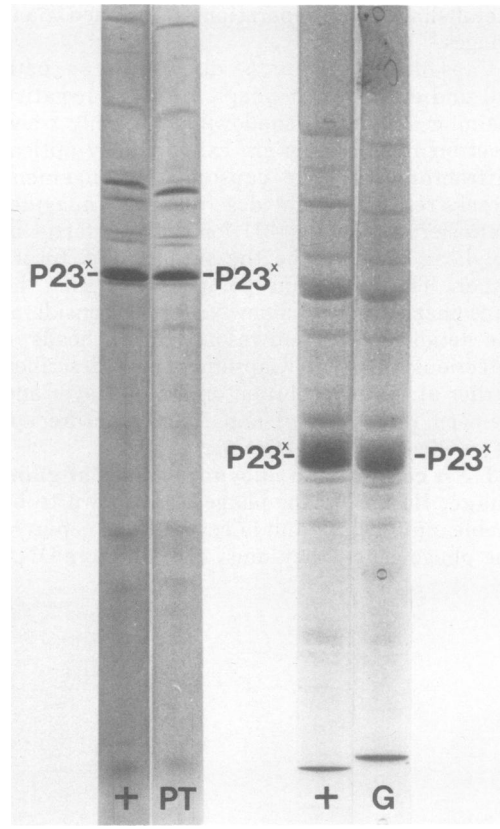

FIG. 7. *Polyacrylamide-sodium dodecyl sulfate-gel electrophoresis of purified isometric petite (PT), normal (+), and giant (G) phages. The pair of gels on the left contain 10% acrylamide; the pair on the right contain 8.5%. The position of the cleaved gene 23 product is indicated by P23*.*

amber mutants and concluded that both are composed of uncleaved gene 23 product. Recent results (Cummings et al., personal communication) indicated that their purified canavanine-induced lollipop phages are, like our giant particles, constructed of P23*.

Apparently, normally cleaved P23* is found in purified giant phage particles of mutant origin. Nevertheless, the cleavage process may not be normal in *ptg191*-infected cells, because the latter accumulate aberrant structures containing uncleaved P23 as described earlier. Alternatively, another reaction in the sequence leading to capsid assembly may have been altered by the *ptg* mutation, and that alteration may have displaced the normal ratio of P23 to P23*.

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