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Genetics

Determination of capsid size by satellite bacteriophage P4

[helper–satellite virus interaction/morphogenesis/*sid* (*size d*etermination) mutant/capsidful of DNA/heterozygous diploid phage]

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ABSTRACT **Satellite bacteriophage P4 requires all morphogenic gene products provided by a helper phage, such as coliphage P2, to assemble its own capsid, which is one-third the volume of the larger helper capsid. We have isolated a satellite phage P4 *sid* (*size d*etermination) mutant that is unable to direct the assembly of the small wild-type-size P4 capsid. Instead, this mutant produces P4 plaque-forming units with large P2-size capsids which contain two or three copies of the P4 sid_1 genome.**

P4 sid_1 is evidently mutated in a protein that is specifically responsible for determining the precise size and symmetry of the structure into which the helper P2 gene products will assemble. In addition, we have found that the physical size of the genome does not appear to play an essential role in the proper assembly of the icosahedral capsid, since the majority of the P4 sid_1 plaque-forming units do not contain a complete capsidful of DNA.

Satellite phage P4 is a defective bacteriophage that alone can only replicate its DNA and lysogenize the bacterial host (1, 2) or maintain itself as an independent plasmid (D. Shore and R. Goldstein, unpublished results). For lytic growth it depends on all known morphogenic gene products of a helper, such as coliphage P2, for the production of its capsid and tail (1, 3). P2 can act as a helper during coinfection with P4 (1), though the P2 burst is drastically reduced due to "interference" with its lytic growth by the satellite (G. Dehò and R. Goldstein, unpublished results). P2 can also help P4 during infection of a P2 lysogen (1). In this latter case, P2 is derepressed by P4 and its DNA is replicated, though complete lytic development of the helper does not occur (B. Lindqvist and E. Six, personal communication; G. Dehò and R. Goldstein, unpublished results).

The only observed homology between the DNA of helper and satellite is in their 19-nucleotide-long cohesive ends, which appear to be identical (4). The molecular weight of the P4 genome (7×10^6) is approximately one-third that of the P2 helper (5), and P4 can "direct" the assembly of gene products provided by the helper to form an icosahedral capsid one-third the volume of the helper capsid (6). Determination of small capsid size by P4 appears to be a tightly controlled non-self-assembly process, as is the case for other icosahedral viruses (7). Because indirect evidence suggests that the physical size of the P4 genome is not responsible for capsid size determination (6, 8, 9), it seems likely that satellite P4 codes for a specific gene product(s) that somehow interacts with the products provided by the helper to direct the formation of the smaller size icosahedral satellite capsid (see Fig. 1).

We set out to isolate a *size d*etermination (*sid*) mutant of satellite P4 that had lost the ability to direct helper gene products into forming the small, satellite-size capsid. In seeking mutants conditionally defective in a normally required function, such as capsid size determination (7), it might be assumed that under nonpermissive conditions a mutant would produce no viable satellite-size capsid structures and therefore have a lethal phenotype. Yet *in vitro* DNA packaging studies demonstrating that two or three copies of P4 DNA could be packaged into the large helper capsid (9) suggested that such a mutant might be viable under nonpermissive conditions, and form plaque-forming units (PFU) by simply packaging several copies of its DNA into a helper-size capsid. On the basis of this latter hypothesis we isolated a P4 *size d*etermination mutant, called P4 sid_1, by utilizing the density shift in a CsCl equilibrium gradient expected for "heavy" P4 virions containing two and three copies of the P4 genome.

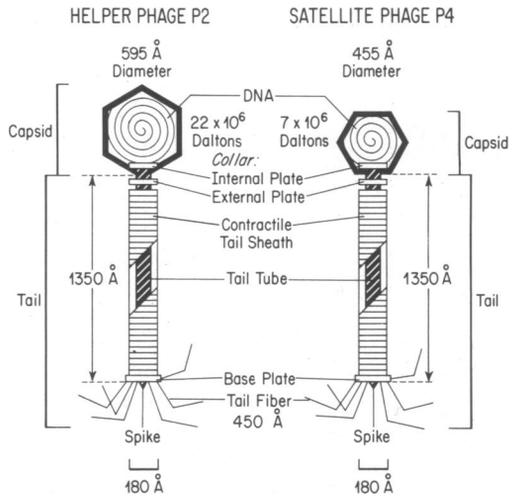

FIG. 1. Schematic diagram of P2 and P4 PFU based on electron microscopic analyses of the phage. It should be noted that the capsid alignments and sizes of P2 and P4 presented previously (6) have been corrected as a result of electron microscopic experiments described in this paper.

These results demonstrate a useful genetic approach towards understanding the mechanism by which satellite P4 determines its small capsid size. Unexpectedly, our results, in contrast to *in vitro* studies (9), also demonstrate that a complete capsidful of DNA is not required or even advantageous in the assembly process. Such a finding strongly suggests that the absolute physical size of a viral genome plays no essential role in the proper assembly of an icosahedral virion.

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Abbreviations: *sid*, *size d*etermination; PFU, plaque-forming units; MOI, multiplicity of infection.

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MATERIALS AND METHODS

Bacterial Strains. All bacterial strains are derivatives of *Escherichia coli* strain C-1a (10). The nonsuppressor strain C-339, a P2 *lg cc* lysogen of strain C-1a, was used for liquid growth of phage P4. The *lg* mutation corrects for a defect in particle assembly, thereby allowing for a larger burst size (11). Derivatives of strain C-1a containing the *supD* and *supF* amber suppressors are described by Sunshine *et al.* (12). The streptomycin-resistant strain C-1758 is described by Gibbs *et al.* (8). Either C-339 or C-1197 (8) was used as a plating indicator for phage P4.

Bacteriophage Strains. P2 *wt* (10) or P2 *lg cc* (mentioned above) were used as helper prophages. P4 *wt* was isolated from a bacterial strain that also released helper bacteriophage (1). P4 vir_1 is an immunity-insensitive clear-plaque mutant that is a derivative of P4 *wt* (1). L.B. broth described by Bertani (13), supplemented with 0.1% glucose, was used for growth of all indicator strains of bacteria.

Growth and Purification of Phage Stocks. Ten milliliters of an overnight culture was inoculated into 400 ml of modified L.B. broth (14) and grown to an OD_{600} of 0.1 at 37° with aeration. At this time the culture was inoculated with the appropriate phage at a multiplicity of infection (MOI) of 0.05. The OD_{600} was monitored periodically, and Na_2EDTA was added to 2 mM when growth ceased being logarithmic. Aeration was continued until lysis was complete. This protocol allows for two rounds of P4 growth. Upon lysis the culture was brought to 3% NaCl and 5% (wt/vol) polyethylene glycol and centrifuged for 40 min at 10,000 × *g*. The phage pellet was resuspended in 4 ml of P4 buffer (8). One milliliter of chloroform was then added, and the mixture was briefly vortex mixed and subsequently spun in a clinical centrifuge for 15 min. Under these conditions the phage separate from the cellular debris and may be drawn off with a pasteur pipette.

Mutagenesis. Samples containing 10^8 P4 vir_1 were UV irradiated to a survival frequency of 10^{-6} and a P2 lysogenic suppressor-containing host was irradiated to give a surviving fraction of 5×10^{-2}. These cells were infected with irradiated phage at low MOI (0.05) so that the cells were singly infected. Mutants were allowed to segregate by growing through 1 cycle at 30°.

One-Cycle Phage Growth Experiments. An overnight culture of the host bacterium was diluted 1:100 in modified L.B. medium and grown at 37° with shaking until the cell density reached 1×10^8/ml. Cells were then pelleted in a Sorvall RC2-B centrifuge for 20 min at 10,000 × *g* and resuspended in an equal volume of fresh medium. Phage were then added at an MOI of 5–10 and allowed to adsorb, without shaking, at 37° for 10 min. At this time infectious centers and free phage were measured. (Free phage were measured by plating an aliquot of the infected culture on a plate containing streptomycin and a lawn of streptomycin-resistant indicator cells.) Anti-phage serum was then added to the culture at a $K = 5$. Five minutes later free phage were again measured and the inoculum was diluted 1000-fold into fresh, prewarmed media lacking $CaCl_2$ to prevent any possible readsorption of the phage burst. After 90 min the progeny phage were assayed.

RESULTS

Isolation and Gradient Purification of the P4 *sid* Mutant. We wished to isolate a mutant of satellite phage P4 with a conditionally expressed alteration in the function of small capsid size determination. Indirect evidence from *in vitro* DNA packaging studies (9) suggested that under nonpermissive conditions such a mutant might produce a burst of diploid and triploid progeny of large helper capsid size, rather than no phage at all. If so, a mutant of this type would be overlooked in standard selections for conditional lethal mutants in capsid size direction, because PFU would result under both permissive and nonpermissive conditions. With this in mind, we made two further assumptions: (*i*) A capsid size determination mutant, under nonpermissive conditions, would be viable but most likely enfeebled, because it would be wasting several copies of its genome to fill the large helper size capsid. The result would be the formation of small plaques and/or a low burst under nonpermissive conditions. (*ii*) Because such a size direction mutant would fill the large helper size capsids with several copies of satellite DNA, it would be of greater density than *wt* P4.

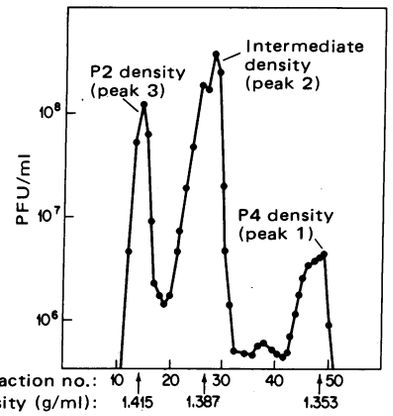

FIG. 2. CsCl equilibrium density gradient profile of P4 sid_1 PFU. Five milliliters of phage was adjusted to the appropriate density by addition of solid CsCl. Centrifugation was carried out in a Beckman L5-50 using an SW 50.1 rotor. Optimal separation of particles was obtained at a speed of 24,000 rpm for 72 hr at 4°.

Mutagenized P4 phage were examined to determine if they met the first assumption. Several independently isolated mutants gave tiny plaques and low bursts under nonpermissive conditions (nonsuppressor or 42°). Progeny of the individual mutants cycled under nonpermissive conditions were then examined for the presence of non-P4-size particles as indicated by PFU density profiles on a CsCl equilibrium gradient.

In a CsCl equilibrium density gradient one such new mutant, P4 sid_1, yielded the density profile of PFU shown in Fig. 2. The data show that the PFU are found divided into three peaks. The majority (80%) of the plaque-forming virions produced are of a density intermediate between that of P2 *wt* and P4 *wt*, while 19% are of the same density as the P2 helper, and less than 1% are of normal P4 *wt* density.

Our data also indicate that the P4 sid_1 phenotype is expressed at all temperatures tested (30°, 37°, and 42°) and in the presence and absence of the *supD* and *supF* (12) amber suppressors (data not shown).

Electron Microscopy. The three peaks of satellite P4 sid_1 PFU shown in Fig. 2 were examined in the electron microscope in order to determine their capsid size. Phage λ was included on each grid as an internal size standard. A value of 640 Å, obtained from low angle x-ray scattering, was assigned to the diameter of the λ capsid (S. C. Harrison, personal communication). Electron micrographs of the three classes of P4 sid_1 PFU are shown in Fig. 3, along with P2 *wt*, P4 *wt*, and λ. The relatively uneven and incomplete negative staining seen in the capsids of the PFU from the intermediate density peak indicates

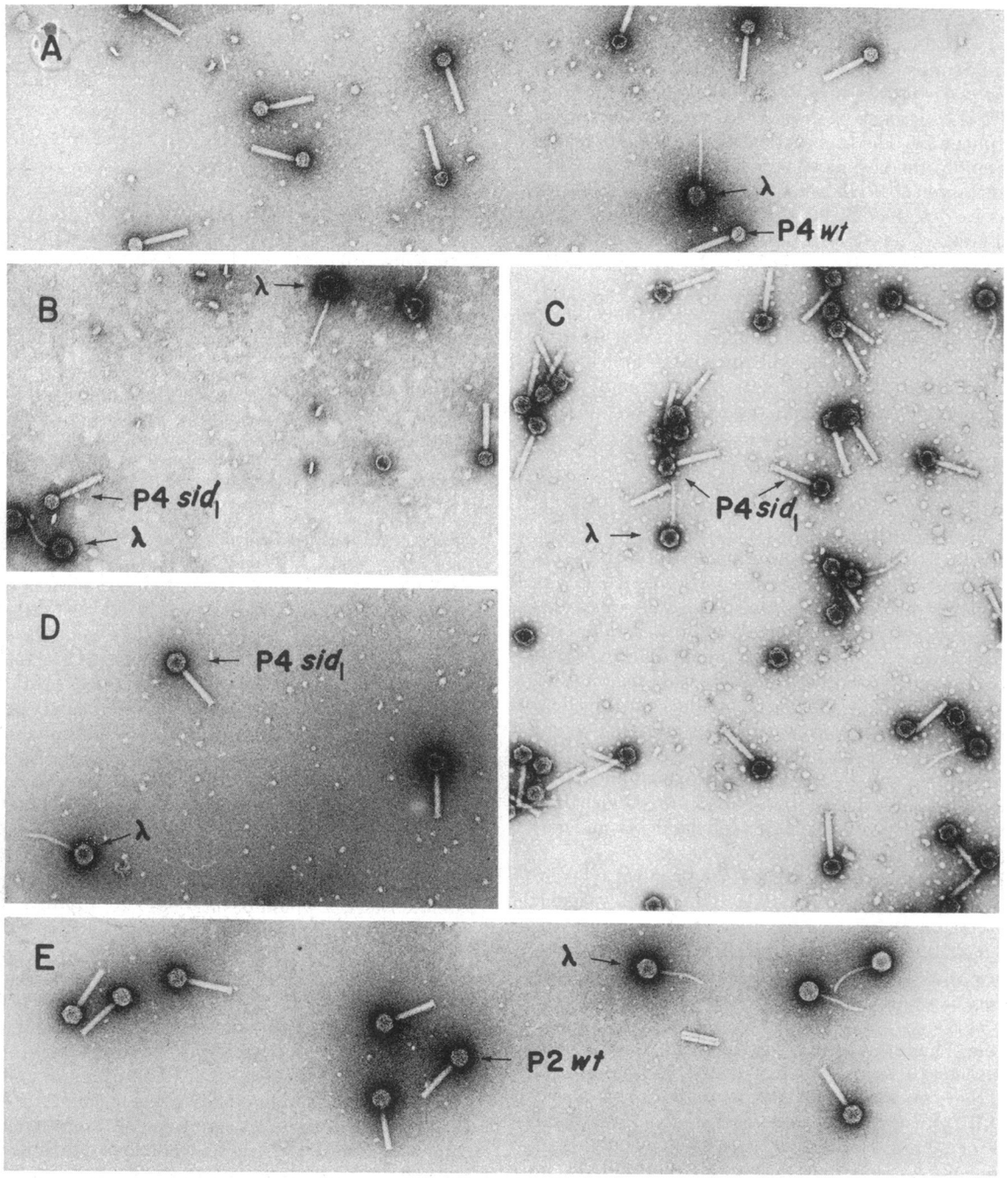

FIG. 3. Electron micrographs (approximately ×42,500) of P2 *wt*, P4 *wt*, and the three peaks of PFU produced by the P4 size direction mutant P4 sid_1 as purified and isolated from the CsCl equilibrium density gradient shown in Fig. 2. (*A*) P4 *wt* and λ; (*B*) P4 sid_1 of P4 density (peak 1) and λ; (*C*) P4 sid_1 of intermediate density (peak 2) and λ; (*D*) P4 sid_1 of helper P2 density (peak 3) and λ; and (*E*) P2 *wt* and λ. Electron microscopy was performed using a JEOL JEM 100B microscope and copper 400-mesh grids coated with a film of 1% parlodion and a thin film of carbon. One drop of sample to be examined was placed on the grid, after which excess sample not adsorbed was removed by rinsing with several drops of P4 buffer. One drop of a 2% uranyl acetate staining solution was next added to the grid and removed by blotting.

that the intermediate density peak P4 sid_1 capsids are incompletely filled with sid_1 DNA.

Measurement of capsid diameters of the three peaks of P4 sid_1 revealed that over 99% (peak 2 plus peak 3 in Fig. 2) of the PFU produced by the capsid size determination mutant are identical in size to the larger helper P2 capsid. In the histograms presented in Fig. 4 the capsid diameters of P4 sid_1 PFU from the intermediate density peak 2, of Fig. 2, are shown and compared to the diameters of P4 *wt* and P2 *wt*.

The capsid size histogram of P4 sid_1 of helper density, representing 19% of the total P4 sid_1 PFU, was identical with the histogram of the intermediate density P4 sid_1 peak in Fig. 4*B*. The histogram of the satellite P4 density sid_1 PFU (less than 1% of the total sid_1 PFU) was identical with the P4 *wt* capsid size distribution shown in Fig. 4A.

Kinetics of UV Inactivation of P4 sid_1 Phage. Kinetics of inactivation by ultraviolet light has been used to demonstrate the dimeric and trimeric genome content of P4 phage produced when exogenously added P4 DNA was packaged *in vitro* into large P2 capsids (6, 9). We carried out a similar series of experiments with P4 sid_1 phage of P4 density, intermediate density, and helper P2 density. Fig. 5 shows that the PFU of

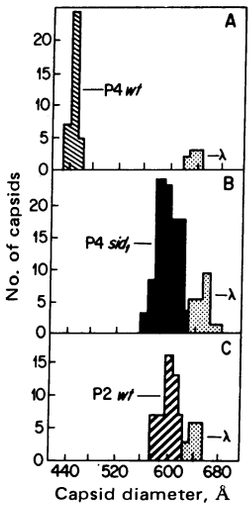

FIG. 4. Capsid size histograms of (*A*) P4 *wt*, (*B*) P4 sid_1 of intermediate density (peak 2 in Fig. 2), and (*C*) P2 *wt*. In each case, the phage samples were first mixed with a phage λ internal size control marker, whose capsid size distribution is also displayed.

helper density exhibit three-hit inactivation kinetics, suggesting that each particle of this density contains three copies of the information necessary to produce a P4 plaque. In contrast, the intermediate density phage exhibit two-hit kinetics, indicating two copies of the genome, and the P4 density phage exhibit one-hit kinetics, as does the control P4 vir_1.

We believe that the unusual density of the P4 sid_1 phage can thus be accounted for by the packaging of either two or three copies of the P4 sid_1 genome into a large helper-size capsid. Table 1 summarizes these results.

Formation of Heterozygous Phage by Packaging of Multiple Copies of P4 DNA. Two different genetically marked P4 genomes might be packaged into a single large P4 sid_1 capsid if the intermediate and helper density P4 sid_1 peaks contained, respectively, two and three copies of P4 DNA. The existence of such a heterozygote, we reasoned, would be consistent with the UV inactivation studies, which suggested that most P4 sid_1 PFU contain multiple copies of the sid_1 genome.

To test this hypothesis, P4 vir_1, a clear-plaque-forming virulent phage (1) that makes the normal small size P4 capsid, was used in coinfection with P4 sid_1. The P4 vir_1 plaque is much larger than the "tiny" P4 sid_1 plaque formed by any of the three density classes of P4 sid_1 phage. Our rationale was to first look for large plaques at intermediate density, because we hypothesized that they would be indicative of the packaging of vir_1 DNA into the large P4 sid_1 capsid. The segregation pattern of such plaques would then be determined to reveal any P4 sid_1 vir_1 diploid heterozygotes.

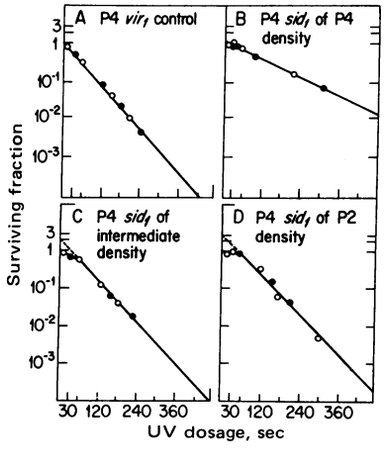

FIG. 5. Ultraviolet light inactivation of a P4 vir_1 control plus the three density classes of P4 sid_1 PFU shown in Fig. 2. The data represent two identical experiments, indicated by empty and filled circles. UV killing was performed according to Pruss *et al.* (9).

Table 1. Physical characterization of P4 sid_1 PFU

% of total PFU	Capsid diameter, Å*	Density†	Genome content‡
19	595	Helper P2	3
80	595	Intermediate	2
<1	455	Satellite P4	1

* From electron microscopy.
† From CsCl equilibrium density gradient analysis.
‡ From UV inactivation kinetics and since confirmed by electron microscopy (R. Goldstein, unpublished results).

We therefore coinfected exponentially growing C-339 cells (lysogenic for helper P2) with both P4 vir_1 and P4 sid_1, each at an MOI of 10. Progeny were harvested after lysis and fractionated in a CsCl equilibrium density gradient. The intermediate density PFU, i.e., those particles containing two P4 genomes, were then analyzed for the presence of the two parental types. Both large plaques (vir_1 plaque size morphology) and tiny plaques (sid_1 plaque size morphology) were found. Each type of plaque was subsequently picked and then streaked on agar plates, which were finally overlaid with a lawn of sensitive bacteria.

We found that 100% (50 out of 50) of the small sid_1 phenotype plaques when so replated yielded only small plaques. These particles breed true and evidently contain only two copies of sid_1 DNA. In contrast, only 22% (11 out of 50) of the large vir_1 phenotype plaques yielded only large plaques, while the remaining 78% segregated so as to give a mixture of small and large plaques. Most of these large plaques, then, do not breed true and evidently contain both the sid_1 and vir_1 genome. The simplest explanation is that the virions of intermediate density contain two copies of the P4 genome in any of three possible combinations of the sid^+ and sid^- genomes.

DISCUSSION

Central to an understanding of viral morphogenesis is the elucidation of the mechanism by which the proper size and symmetry of an icosahedral capsid is determined. During the past 15 years the static model for self-assembly of capsids (15) has been replaced by growing evidence for a tightly controlled dynamic assembly process involving the processing of subunit protein building blocks concomitant with lattice rearrangements of the subunit proteins (7, 16–18).

The P2–P4 system presents a novel opportunity to directly study and dissect the process of capsid size determination. Satellite P4 requires all of the 17 known helper P2 late gene products involved in P2 morphogenesis, yet P4 effects their assembly into an icosahedral capsid approximately one-third the overall volume of the capsid normally formed by bacteriophage P2 (6). In the absence of satellite P4, the P2 helper never assembles small satellite-size capsids from these same 17 late gene products. These differing assembly patterns suggest that the satellite P4 codes for a specific gene product(s) involved in directing the assembly of helper gene products to form the small satellite capsid (6, 14). Determination of the identity and role of the satellite-specific sid^+ (*size determination*) gene

product should provide a new approach to a central question in viral morphogenesis.

We have devised a system for the isolation of *sid* mutants, and have preliminarily characterized one such mutant, P4 sid_1. Isolation of this mutant presents direct genetic evidence that satellite virus P4 codes for a function which influences assembly of helper gene products by determining the production of the small satellite capsid. Biochemical and genetic analyses of *sid* mutants should lead to an understanding of the mechanism(s) involved in capsid size determination and the structural, catalytic, or regulatory nature of the sid^+ protein.

In the case of satellite P4, the sid^+ function is unusual in that it is not essential in the assembly process, due to the availability of large but functional helper-size capsids. This contrasts with phage such as T4, P22, λ, and P2, for which all mutations identified as involved in key assembly steps have proven essential and therefore conditionally lethal (7). Another unusual property of the P4 sid_1 mutant is that it produces a burst of diploid and triploid homozygous PFU. Furthermore, heterozygous progeny can be obtained at high frequency from coinfection of P4 sid_1 with other P4 mutants. During our preliminary characterization of the sid_1 mutant we have been unable to find conditions that suppress the large capsid phenotype. Hence, sid_1 may be a deletion or a missense mutant.

Because of the lack of an obvious conditional phenotype for the sid_1 mutation, we have not been able to determine positively that it is affected in a P4-coded protein rather than a site on the P4 DNA that might be directly involved in determining small capsid size. We have evidence for the former hypothesis. First, in complementation experiments with P4 vir_1 (i.e., sid^+), we have found sid^- and sid^+ to be codominant because approximately the same percentage of large and small capsids was found when the input MOI of each was equal (D. Shore, C. Diana, and R. Goldstein, unpublished results). Also of significance in these complementation experiments was the production of diploid homozygous P4 $vir_1 sid^+$ progeny with the large sid^--size capsid. This result suggests that the P4 sid_1 mutant can act in *trans* to bring about the production of large P2-sized capsids. It seems extremely unlikely that the above results could be explained by the P4 sid_1 DNA itself acting structurally to effect the formation of the large capsid after which it is released to be recycled and then replaced by the two $vir_1 sid^+$ genomes. Instead, we believe the above results more reasonably support the idea that P4 *wt* produces a diffusible product normally involved in determining small capsid size, but in its defective form causes the production of large P2-size capsids. Second, preliminary sodium dodecyl sulfate/polyacrylamide gel electrophoresis experiments lend additional support to this view, because they show the P4 sid_1 capsid to be different from both P2 *wt* and P4 *wt* with respect to the species and ratios of cleavage products that arise during cleavage and processing of the major capsid protein (D. Shore and R. Goldstein, unpublished results). This result raises the possibility that P4 sid_1 is affected in the actual cleavage of the major capsid protein of helper P2 or in the control of this process, events shown to be associated with the assembly of both P2 and P4 (6, 19). On the basis of our present knowledge of protein processing in bacteriophage capsids (6, 7, 17–19), such a defect is likely to be the result of a defective protein.

Previous *in vitro* DNA packaging studies using helper-P2-infected cellular extracts plus exogenously added P4 DNA have shown that the majority of the satellite P4 DNA packaged into large size helper P2 capsids is trimeric (9). For this reason it came as a surprise to us that the majority of the P4 sid_1 PFU formed in the cell contain dimers. In contrast to *in vitro* studies (9), our new results suggest that a complete capsidful of DNA is not necessarily advantageous for or favored by the assembly process. Replication or processing of dimeric in preference to trimeric forms of P4 sid_1 DNA most likely cannot account for the result, because we have recently obtained data showing that the mutant produces dimeric and trimeric closed circular DNA molecules in equal amounts as packaging substrates (C. Diana and R. Goldstein, unpublished results). Overall stability, in terms of DNA condensation and packaging arrangements for dimers versus trimers, may play some important role in the selection mechanism. Low angle x-ray diffraction studies may provide an insight into the question of the DNA packing arrangements found in the dimer and trimer PFU. Yet, far and away the most interesting implication of the incomplete filling of capsids by P4 sid_1 can be suggested at this time, and is consistent with many less direct observations from other icosahedral capsid systems: the absolute physical size of the viral genome plays no critical role in determining capsid size and symmetry in the assembly process.

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