Mutant *tsI* of Bacteriophage PM2 Is Defective in the Major Capsid Protein and Fails to Package Its DNA

GREGORY J. BREWER

Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine,
Springfield, Illinois 62708

Received 29 June 1982/Accepted 5 October 1982

Infection of *Alteromonas espejiana* at restrictive temperature with mutant *tsI* of bacteriophage PM2 resulted in the intracellular accumulation of virus-sized empty-appearing membrane vesicles. The DNA associated with purified vesicles was fully susceptible to digestion with DNase. Sedimentation analysis and electron microscopy suggested a full-length linear form of the normally circular viral genome. A pulse-chase-shift experiment suggested that [³H]thymidine-labeled DNA made under restrictive conditions is assembled into virions after shift to permissive temperature. A defective structural protein in the *tsI* virion appears to be the cause of a rapid rate of thermal inactivation of infectivity. Analysis of the proteins of *tsI* by isoelectric focusing indicated a more alkaline isoelectric mobility of the major capsid protein, sp27. Six spontaneous revertants of *tsI* showed reversion to the wild-type isoelectric form of sp27. These results identify sp27 as the defective gene product of *tsI*. Taken together, these results suggest that the membrane of PM2 is formed without the aid of an inner core or an outer scaffolding.

Mutant *tsI* of bacteriophage PM2 is interesting because it forms empty-appearing membrane vesicles inside the host bacterium infected at restrictive temperature (4). This observation alone raises important questions about membrane morphogenesis. Are these vesicles made de novo, or are they derived from the host cytoplasmic membrane as they accumulate inside the cell? How is their size and shape regulated? In the case of the enveloped animal viruses, membrane morphogenesis is controlled by the size and shape of the nucleocapsid, which serves as a mold (8). Another mechanism for membrane morphogenesis is the assembly of a scaffolding of protein around the outside of a membrane. Cytoplasmic vesicles of eucaryotes appear to be formed by clathrin in this manner (13). The finding that *tsI* vesicles of bacteriophage PM2 do not contain the outer major capsid protein argues against this mechanism for PM2 membrane formation (7). This paper presents evidence for the formation of a membrane before the DNA is packaged to serve as a core. It also identifies the genetic defect in *tsI* to be in the major capsid protein, which apparently fails to form an outer coat around the membrane under restrictive conditions.

MATERIALS AND METHODS

Cells and virus. Culturing of PM2 and its host, *Alteromonas espejiana*, have been described (4). Mu-

tant *tsI* of PM2 was isolated by its ability to replicate at 20°C but not at 29°C (5). *tsI* vesicles were purified by density gradient and velocity centrifugation from cells infected at restrictive temperature (6). Wild-type and *tsI* virus were purified by differential sucrose velocity gradient and CsCl equilibrium density gradient centrifugation (5).

Exposure of *tsI* vesicle DNA. *tsI* vesicles were purified from a 10-ml culture of cells labeled with ³²P (5 µCi/ml) beginning one generation before infection. This procedure maximally labels the DNA, which contains 70% of the virus phosphorus, and partially labels the membrane, which contains 30% of the virus phosphorus (10). To one-half of the vesicle preparation was added 0.01 volume of DNase to yield 10 µg/ml. This sample and the untreated control were incubated at 22°C for 30 min, followed by chilling to 4°C. The samples were placed on top of two 5 to 55% sucrose gradients made in VSM (5) and centrifuged for 21 h at 40,000 rpm in an SW40 rotor at 4°C. Samples to be analyzed by velocity floatation were prepared similarly, adjusted to 45% (wt/wt) with solid sucrose, and applied to the bottoms of two 5 to 40% sucrose-VSM gradients. Centrifugation proceeded for 24 h at 38,000 rpm in an SW40 rotor at 4°C.

Size analysis of *tsI* vesicle DNA: velocity sedimentation. Wild-type PM2 was labeled with [³H]thymidine (6). *tsI* vesicles were labeled with ³²P, as above. To each preparation was added 200 µg of unlabeled wild-type PM2. The samples were centrifuged at 30,000 rpm for 40 min at 4°C. The pellets were resuspended in 0.2 ml of lysis buffer (20 mM Tris-chloride [pH 8.0], 1 mM EDTA, 100 mM NaCl) and 0.5% Sarkosyl detergent. DNA was extracted twice with 0.75 and 0.5 volumes of distilled phenol which had been saturated with lysis

buffer. The upper layer was dialyzed for 4 h at 4°C against buffer A (20 mM Tris-chlorine [pH 8.0], 0.1 mM EDTA, 200 mM KCl) and then overnight against buffer B (20 mM Tris-chlorine [pH 8.0], 0.1 mM EDTA, 50 mM KCl). To determine radioactive spill-over of ^{32}P into the ^3H counting channel, three samples were applied to three 5 to 20% sucrose gradients in 20 mM sodium phosphate (pH 7.0)–1 mM EDTA–100 mM NaCl. Sample 1 contained ^{32}P -labeled *tsI* DNA alone; sample 2 contained [^3H]thymidine-labeled wild-type DNA alone; sample 3 contained a mixture of these two labeled DNAs. After sedimentation at 22,500 rpm for 16 h at 15°C, 0.5-ml fractions were collected directly into scintillation vials and counted after the addition of scintillation fluid. The positions of radioactive peaks were converted to sedimentation values (19).

Electron microscopy of DNA. Samples were prepared for electron microscopy by the Kleinsmidt procedure by using a film of cytochrome *c* and sample in 55% formamide, floated onto a subphase containing 18% formamide (14). After rotary shadowing with platinum-palladium, samples were examined at 40,000 V with a Phillips 301 electron microscope. The negative magnification was 14,800, as determined by calibration with spacings from a carbon replica of a grating (E. F. Fullam, personal communication). Length measurements were made with a map measurer on tracings from 2.44× enlargements.

Fate of *tsI* DNA made under restrictive conditions. Three cultures were prepared as outlined in Table 1. A thymidine auxotroph was used to optimize incorporation of [^3H]thymidine (6). Cells were grown at 29°C overnight and diluted to an optical density at 440 nm of 0.25 in MTG (5) containing 10 µg of thymidine per ml and 0.1 mM each of a mixture of 19 amino acids (missing methionine). The cells were grown to an optical density at 440 nm of 0.40, collected by centrifugation, and resuspended in the original volume of MTG containing 1.4 µg of thymidine per ml, 0.1 mM each of a mixture of 19 amino acids, and 0.22 µg of

methionine per ml. [^3H]thymidine, 20 µCi/ml, was added to the appropriate cultures (Table 1). Time zero began after 20 min of incubation. [^{35}S]methionine was then added to 2 µCi/ml. Cells to be infected received *tsI* at a multiplicity of 10 PFU/cell. Cultures to be chased received thymidine at 70 µg/ml and methionine at 22 µg/ml. All cultures were shifted to 20°C. After 30 and 90 min, samples were chilled to 4°C, and virus was isolated and assayed for PFU and radioactivity.

Isoelectric focusing of virus proteins. Samples were prepared from ^{35}S -labeled gradient-purified virus by collection on filters (0.025-µm pore; VSWP; Millipore Corp., Bedford, Mass.) and dissolution in the sodium dodecyl sulfate (SDS)-containing buffer of Ames and Nikaido (1). A slab gel containing 7.5% acrylamide was formulated and run as described (18) for the first dimension only.

Isolation of *tsI* revertants. *tsI* virus (3×10^8 PFU) was plated for plaque formation and incubated at 29°C. Eight of the plaques were picked, replated for purity, and grown up to stocks containing about 10^{11} PFU. These stocks were used to infect 10-ml cultures containing [^{35}S]methionine at 10 µCi/ml and 1.5 µM methionine, and virus was purified (5). Seven of the eight stocks produced sufficient labeled virus for isoelectric focusing.

RESULTS

DNA of *tsI* vesicles. At restrictive temperature, infection of cells with mutant *tsI* of bacteriophage PM2 causes the synthesis of virus-sized membrane vesicles (4). Buoyant density measurements and radiolabeling with [^3H]thymidine or ^{32}P indicated the presence of DNA. If the DNA of these vesicles were already packaged inside the membrane, it would be resistant to digestion with DNase. Therefore, *tsI* vesicles were isolated from cells infected at restrictive temperature in the presence of ^{32}P to label the

TABLE 1. *tsI* DNA made under restrictive conditions: assembly into virions under permissive conditions

Expt	Positive control	Negative control
Load cells with [^3H]thymidine- [^{35}S]methionine		Load cells with [^3H]thymidine- [^{35}S]methionine
↓		↓
Infect with <i>tsI</i>	Incubate cells at 29°C for 60 min	↓
↓	↓	↓
Chase cells with [^1H]thymidine- [^{32}S]methionine	Load cells with [^3H]thymidine- [^{35}S]methionine	Chase cells with [^1H]thymidine- [^{32}S]methionine
↓	↓	↓
↓	Shift to 20°C	↓
↓	↓	↓
↓	Infect with <i>tsI</i>	Infect with <i>tsI</i>
↓	↓	↓
↓	Incubate at 20°C	↓
↓	↓	↓
↓	Isolate virus	↓
↓	↓	↓
	Measure cpm/PFU	

DNA and lipid. One sample of vesicles was treated with DNase. The other was not treated and served as a control. Both samples were analyzed by sedimentation through a sucrose velocity gradient (Fig. 1). In contrast to the band of untreated vesicles in the middle, vesicles treated with DNase barely entered the gradient. The DNA appears to have been digested, releasing nucleotides and much lighter vesicles. To discriminate digested DNA from a protein-membrane complex, another pair of samples was placed at the bottom of centrifuge tubes and analyzed by velocity floatation. The treated vesicles were observed to float up in a sucrose gradient in which the untreated vesicles remained at the bottom of the gradient (Fig. 1B). Digested nucleic acid remained at the bottom of the gradient. The extent of digestion and restriction mapping of the attachment region is being investigated. Thus, the DNA of *tsI* vesicles is accessible to DNase. It does not appear to be sealed inside the membrane.

Failure to package the DNA of *tsI* may be due to defective DNA processing. The DNA extract-

ed from fresh wild-type virus sediments at 28S as a double-stranded, circular, superhelical molecule (11). The DNA of older preparations sediments at 21S as a nicked, relaxed circle. The DNA extracted from *tsI* vesicles sedimented at 19S (Fig. 2). According to Opschoor et al. (19), a sedimentation coefficient of 18.7S is expected for a linear form of PM2 DNA (molecular weight, 6.3×10^6 [10]). The DNA of *tsI* vesicles appears to be a full-length linear form of the wild-type DNA. This was confirmed by electron microscopy (Fig. 3). In this preparation of *tsI* virus DNA, 84% of the molecules were supercoiled. The 11% of the molecules showing a circular topology had a mean contour length of $3.3 \pm 0.1 \mu\text{m}$. These results are similar to those for wild-type DNA. Based on $0.51 \mu\text{m}/\text{megadalton}$ (2), a full-length DNA molecule would be $3.2 \mu\text{m}$. After processing for electron microscopy, the DNA of *tsI* vesicles (Fig. 3B) was 96% linear molecules of a broad size distribution that varied with the position on the gradient from which they were collected. For example, one light fraction showed a mean contour length of $1.2 \pm 0.6 \mu\text{m}$, whereas a heavier fraction was $1.7 \pm 0.9 \mu\text{m}$. The distribution was not gaussian and showed a distinct grouping near the length of a full genome ($3.2 \pm 0.2 \mu\text{m}$).

Utilization of *tsI* DNA made under restrictive conditions. To be of biological relevance, the DNA-membrane complex made at restrictive temperature must be an intermediate of normal assembly which can be utilized to make infectious virus upon release to permissive temperature. The preformed DNA-membrane complex appears to be readily utilized because infectious virus is produced without a lag upon shift from restrictive to permissive temperature (7). Further demonstration of utilization of the *tsI* viral DNA was attempted by the pulse-chase-shift experiment outlined in Table 1. Virus was isolated at two times after shift to permissive temperature. At 30 min, the rate of virus assembly had reached an optimum. Ninety minutes is sufficient time for de novo virus assembly beginning at the time of the shift (7). With [^3H]thymidine as label, virus isolated 30 min after shift to permissive temperature had a specific activity (counts per minute per PFU) 17 times that of the negative control in which DNA synthesis occurred during the chase (Table 2). After 90 min, when some DNA had been synthesized under the permissive conditions during the chase, the specific activity of isolated virus was still five times that of the negative control. Thus, virus assembled after the shift contain DNA made before the shift. The fate of viral proteins synthesized under restrictive conditions was also examined by following the incorporation of [^{35}S]methionine into virus isolated after the shift to permis-

FIG. 1. Susceptibility to DNase of *tsI* vesicles. (A) Analysis by velocity sedimentation. One-half of a preparation ^{32}P -labeled *tsI* vesicles was treated with DNase. After incubation, this sample and the untreated control were placed on top of two sucrose gradients. After centrifugation, fractions were collected and analyzed for radioactivity by scintillation counting. The results from two gradients are superimposed: ●, treated with DNase; ○, untreated. Sedimentation is from left to right. (B) Analysis by velocity floatation. Samples prepared and treated similarly to those described above were made dense by the dissolution of solid sucrose to 45% (wt/wt). The samples were applied to the bottom of two sucrose gradients. The results from two gradients are superimposed: ●, treated with DNase; ○, untreated.

FIG. 2. Velocity cosedimentation of phenol-extracted ^{32}P -labeled *ts1* vesicle DNA (○) and [^3H]thymidine-labeled wild-type PM2 DNA (×). Wild-type PM2 DNA was isolated from virus grown in the presence of [^3H]thymidine and mixed with DNA isolated from *ts1* vesicles grown in the presence of ^{32}P . The combined DNA preparation was sedimented through a 5 to 20% sucrose gradient and analyzed for radioactivity.

FIG. 3. Supercoiled and circular forms of *ts1* virus DNA (A) and the predominant linear forms of *ts1* vesicle DNA (B). Bar, 1 μm. Preparations of *ts1* virus and *ts1* vesicles were separately prepared for electron microscopy by the Kleinsmidt procedure.

TABLE 2. Specific activities of *tsI* virus after shift to permissive conditions^a

Protocol ^b	Min after shift ^c	PFU ($\times 10^{-9}$)	³⁵ S (cpm $\times 10^{-3}$)	³⁵ S (cpm/PFU $\times 10^{-7}$)	³ H (cpm $\times 10^{-3}$)	³ H (cpm/PFU $\times 10^{-7}$)
Experiment	30	1.8	13	74	19	104
Experiment	90	30	57	19	89	30
Positive control	90	4.5	105	233	66	147
Negative control	90	14	23	17	8	6

^a See text and Table 1. Quotients may appear slightly in error due to rounding.

^b See Table 1.

^c Time after shift at which virus was isolated.

sive temperature. Thirty minutes after the shift and chase, the protein specific activity was four times that of the negative control (Table 2). By 90 min, it was equal to that of the negative control. Presynthesized protein appears to be utilized in assembly of virions, but to a lesser extent than presynthesized DNA.

Defective protein of *tsI*. The mutant protein responsible for the defect in *tsI* could function catalytically or structurally. If the defect were in a structural protein, the sensitivity to thermal inactivation of the mutant would be greater than that of the wild type. Figure 4 shows that at 50°C, *tsI* virus was inactivated with first-order kinetics at a rate 12 times that of the wild type.

FIG. 4. Thermal inactivation of wild type (●) and *tsI* virus (×) at 50°C. Virus preparations were placed at 50°C and assayed immediately for PFU. Subsequent samples were removed at the indicated times, diluted at room temperature, and assayed.

Often the mutation in a protein is due to substitution of an amino acid that results in an altered charge. This was tested by isoelectric focusing of purified virus (Fig. 5, lanes WT and *tsI*). In the presence of nonionic detergent and 8 M urea, the only virion proteins solubilized are sp43 and sp27. The apparent isoelectric point of the spike protein, sp43, is 2.6 for both *tsI* and wild-type virus. Wild-type sp27, the major capsid protein, shows multiple isoelectric forms at pH 5.5 and 5.0. The isoelectric form of sp27 of *tsI* was strikingly different at 7.0. Two-dimensional gels comparing total proteins from cells infected with *tsI* with wild-type PM2 showed no differences in the 11 nonstructural proteins whose synthesis is stimulated by infection (results not shown).

The *tsI* phenotype of a high isoelectric point of sp27 could be due to temperature-sensitive processing or temperature-sensitive folding of an altered primary sequence. To test these hypotheses, the isoelectric point of sp27 was analyzed from cells infected at either restrictive or permissive temperature. The isoelectric point of sp27 was unaffected by temperature in both the wild-type and *tsI* infections. These results suggest a point mutation in the viral gene coding for sp27. The reversion frequency of *tsI* is 3×10^{-7} (5). Six of seven spontaneous revertants to growth at restrictive temperature showed reversion of the isoelectric point of sp27 to that of wild-type sp27 (Fig. 5, lanes R1 to R6). A seventh revertant had an isoelectric point similar to that of the lower isoelectric form of wild-type sp27.

DISCUSSION

Mutant *tsI* of bacteriophage PM2 appears to bear a mutation in the viral gene coding for the major capsid protein, sp27. The apparent isoelectric point of the mutant sp27 is at least 0.5 pH units higher than that of the wild type. Based on the amino acid composition of sp27 (protein II [12]), such a shift is unlikely to be explained by a maximum charge shift substitution of one acidic amino acid for a single basic residue

FIG. 5. Isoelectric focusing slab gel electrophoresis of purified wild type (WT), *ts1*, and seven *ts1* virus revertants (R1 to R7). Wild type, *ts1* virus, and seven *ts1* revertants were prepared by growth in the presence of [35 S]methionine. The virus was purified, dissolved in SDS, and applied to an isoelectric focusing slab gel. An unused lane was removed for determination of the pH gradient (right). Proteins were located by autoradiography of the dried gel.

among the 30 lysines and arginines (calculated shift, 0.08 pH). The possibility of a charge shift due to processing of the primary transcript appears unlikely for two reasons. First, the isoelectric point of sp27 of *ts1*, as reported here, is independent of the temperature of growth. Second, the apparent molecular weight on SDS gels of *ts1* sp27 is the same as that of the wild type (5). Of course, a small change of less than 10 amino acids may not be detectable. One possible explanation for the large charge shift is binding of more SDS by the mutant sp27 than by the wild type. Point mutations (17) and changes in charge (20) have been shown to alter mobility on SDS gels of proteins of the same molecular weight. Isoelectric focusing of SDS-protein complexes would be even more sensitive to such changes.

The DNA of *ts1* vesicles appears to be exposed, exterior to the membrane, full length, linear, and attached to the viral membrane. The linear conformation of the DNA could be due to an endonucleolytic cleavage of an originally circular molecule. An endonuclease has been reported to be present in purified virions (15). The exposure of the DNA could be due to failure to package the genome in the mutant under restrictive conditions. In electron micrographs of cells infected with *ts1*, the empty appearance of the vesicles suggests that the DNA was never packaged. The results of the pulse-chase-shift experiment suggest that the viral DNA made under restrictive conditions is utilized in the final assembly steps, which are permitted at lower temperature. It is interesting that, in relation to the controls, the specific activities of the protein label are less than those of the DNA label. This may reflect a preferential use in virus assembly of protein synthesized and folded under permissive conditions, especially sp27, which accounts for about one-half of the methionine in virions. In *ts1* vesicles made at restrictive temperature, the presence of only sp6.6 (7) would carry less than one-quarter of the methionine in the virus. As discussed previously (7), similar studies to follow the fate of the vesicle by using membrane labels are difficult to interpret due to the large pool consisting of the cell membrane. Alternatively, the DNA may have been packaged, but upon manipulation or isolation of the vesicles, it was released from inside the membrane. Although precedent exists in the case of 10^- and 26^- mutants of bacteriophage P22 (3), this explanation seems less likely, since the vesicles of sectioned cells appear empty (4). The intermediate state of a linear molecule could be tested by exposing cell-free extracts of infected cells (16) to purified wild-type sp27. These experiments on complementation of DNA packaging are in progress.

The phenotype of mutant *ts1* aids in understanding the assembly of PM2 (8). Current data support the following working hypothesis. Before assembly of the viral capsid, a phospholipid bilayer membrane vesicle appears to be formed from the host cytoplasmic membrane. Associated with this membrane is viral DNA. Membrane-associated DNA is packaged along with encapsidation by sp27. sp27 may stimulate encapsidation or stabilize a membrane vesicle filled with DNA or both. One new prediction arises from the observation of DNA tethered to the viral membrane vesicle. An attachment protein may be present in low abundance at one site on the membrane (see model in reference 8). Preliminary results have identified a protein with a molecular weight of 15,000 as a candidate for this function.

ACKNOWLEDGMENTS

I am grateful for the technical assistance of James Webb and Carol Schacht and the typing of Connie Burton.

This work was supported by Public Health Service grant AI17679 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Ames, G., and K. Nikaïdo. 1976. Two-dimensional gel electrophoresis of membrane proteins. *Biochemistry* 15:616-623.
2. Bode, N. R., and H. J. Morowitz. 1967. Size and structure of the *Mycoplasma hominis* H39 chromosome. *J. Mol. Biol.* 23:191-199.
3. Botstein, D., C. H. Waddell, and J. King. 1973. Mechanism of head assembly and DNA encapsulation in *Salmonella* phage P22. I. Genes, proteins, structures and DNA maturation. *J. Mol. Biol.* 80:669-695.
4. Brewer, G. J. 1976. Control of membrane morphogenesis in bacteriophage PM2. *J. Supramol. Struct.* 5:73-79.
5. Brewer, G. J. 1978. Characterization of temperature sensitive mutants of bacteriophage PM2: membrane mutants. *Mol. Gen. Genet.* 167:64-74.
6. Brewer, G. J. 1978. Membrane-localized replication of bacteriophage PM2. *Virology* 84:242-245.
7. Brewer, G. J. 1979. In vivo assembly of a biological membrane of defined size, shape, and lipid composition. *J. Virol.* 30:875-882.
8. Brewer, G. J. 1980. Control of membrane morphogenesis in bacteriophage. *Int. Rev. Cytol.* 68:53-96.
9. Brewer, G. J. 1982. Kinetics and characterization of the proteins stimulated by infection with bacteriophage PM2. *J. Gen. Virol.* 60:135-146.
10. Camerini-Otero, R. D., and R. M. Franklin. 1975. Structure and synthesis of a lipid-containing bacteriophage. The molecular weight and other physical properties of bacteriophage PM2. *Eur. J. Biochem.* 53:343-348.
11. Espejo, R. T., E. S. Canelo, and R. L. Sinsheimer. 1971. Replication of bacteriophage PM2 deoxyribonucleic acid: a closed circular double-stranded molecule. *J. Mol. Biol.* 56:597-621.
12. Hinnen, R., R. Chassin, R. Schafer, R. M. Franklin, H. Hitz, and D. Schafer. 1976. Structure and synthesis of a lipid-containing bacteriophage. Purification, chemical composition and partial sequences of the structural proteins. *Eur. J. Biochem.* 68:139-152.
13. Keen, J. H., M. C. Willingham, and I. H. Pastan. 1979. Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. *Cell* 16:303-312.
14. Kleinschmidt, A. K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. *Methods Enzymol.* 12B:361-377.
15. Laval, F. 1974. Endonuclease activity associated with purified PM2 bacteriophages. *Proc. Natl. Acad. Sci. U.S.A.* 71:4965-4969.
16. Murialdo, H., and A. Becker. 1977. Assembly of biologically active proheads of bacteriophage lambda *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 74:906-910.
17. Noel, D., K. Nikaïdo, and G. F.-L. Ames. 1979. A single amino acid substitution in a histidine-transport protein drastically alters its mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry* 18:4159-4165.
18. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
19. Opschoor, A., P. H. Pouwels, C. M. Knijnenburg, and J. B. T. Aten. 1968. Viscosity and sedimentation of circular native deoxyribonucleic acid. *J. Mol. Biol.* 37:13-20.
20. Tung, J.-S., and C. A. Knight. 1971. Effect of charge on the determination of molecular weight of proteins by gel electrophoresis in SDS. *Biochem. Biophys. Res. Commun.* 42:1117-1121.