Virology **272,** 218–224 (2000)
doi:10.1006/viro.2000.0374, available online at http://www.idealibrary.com on IDEAL®

Characterization of Φ8, a Bacteriophage Containing Three Double-Stranded RNA Genomic Segments and Distantly Related to Φ6

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Received February 28, 2000; returned to author for revision April 11, 2000; accepted April 21, 2000

The three double-stranded RNA genomic segments of bacteriophage Φ8 were copied as cDNA, and their nucleotide sequences were determined. Although the organization of the genome is similar to that of Φ6, there is no similarity in either the nucleotide sequences or the amino acid sequences, with the exception of the motifs characteristic of viral RNA polymerases that are found in the presumptive polymerase sequence. Several features of the viral proteins differ markedly from those of Φ6. Although both phages are covered by a lipid-containing membrane, the protein compositions are very different. The most striking difference is that protein P8, which constitutes a shell around the procapsid in Φ6, is part of the membrane in Φ8. The host attachment protein consists of two peptides rather than one and the phage attaches directly to the lipopolysaccharide of the host rather than to a type IV pilus. The host range of Φ8 includes rough strains of *Salmonella typhimurium* and of pseudomonads

Key Words: bacteriophage; double-stranded RNA; *Pseudomonas*.

INTRODUCTION

Bacteriophage Φ8 was isolated from the leaves of the snow pea plant (*Pisum sativum*) (Mindich *et al.,* 1999). It is similar in structure to bacteriophage Φ6, which contains a genome of three segments of double-stranded RNA (dsRNA) (Semancik *et al.,* 1973) packaged inside a procapsid that is covered by a shell of protein P8 and a lipid-containing membrane with additional viral proteins (Vidaver *et al.,* 1973). The genome of Φ6 has been cloned and sequenced, and the life cycle and structure of the phage have been the subject of considerable investigation (Butcher *et al.,* 1997; de Haas *et al.,* 1999; Mindich, 1999). The Φ6 infects pseudomonads by attaching to a type IV pilus that is retracted so the viral membrane can fuse with the outer membrane of the host. The nucleocapsid is then found in the periplasmic space. A viral muramidase digests the cell wall, and the nucleocapsid enters the cell, wherein it transcribes its genome. The viral procapsid composed of proteins P1, P2, P4, and P7 packages plus-strand transcripts in a precise and controlled process that ultimately results in three dsRNA genomic segments in each virion. The filled procapsid acquires a shell of protein P8 and then a lipid-containing membrane that is assembled within the cell. The procapsid of Φ6 has the ability to package RNA, to synthesize minus strands to make dsRNA, and then to transcribe the genome. All of the reactions can be carried out *in vitro* under defined conditions. Until now, Φ6 has been alone in the family Cystoviridae and alone in the genus *Cystovirus* (Murphy, 1995). It is now clear that this group is composed of many more phages: some very similar to Φ6 and some, like Φ8, rather distantly related. These are the only bacteriophages with genomes composed of dsRNA. Their structure and replicative strategies show many similarities to the Reoviridae, dsRNA viruses that infect eukaryotic cells.

We present here the cDNA cloning and sequencing of the genome of Φ8 and point out the similarities between this virus and Φ6 as well as the marked differences in both structure and sequence.

RESULTS

RNA sequence

The cDNA copies of the Φ8 genomic segments were sequenced and arranged into three groups corresponding to the three dsRNA genomic segments. The arrangement of the genes and the unique restriction sites of the cDNA copies are shown in Fig. 1. The sizes of the three segments were found to be 7051, 4742, and 3192 bp for segments L, M, and S, respectively. This compares with the corresponding sizes of 6374, 4063, and 2948 in Φ6 (Mindich, 1988). The base composition of the segments is 54.0%, 55.3%, and 54.4% GC, respectively, for L, M, and S compared with about 56% GC in Φ6 (Mindich, 1988). Although the genomic segments of Φ6 have an 18-base identity at the 5′ end, the Φ8 segments have an identity of only 7 bases and they are different from those of Φ6: G(G/U)AAAAAAACUUUAUAUA for Φ6 versus GAAAUUU

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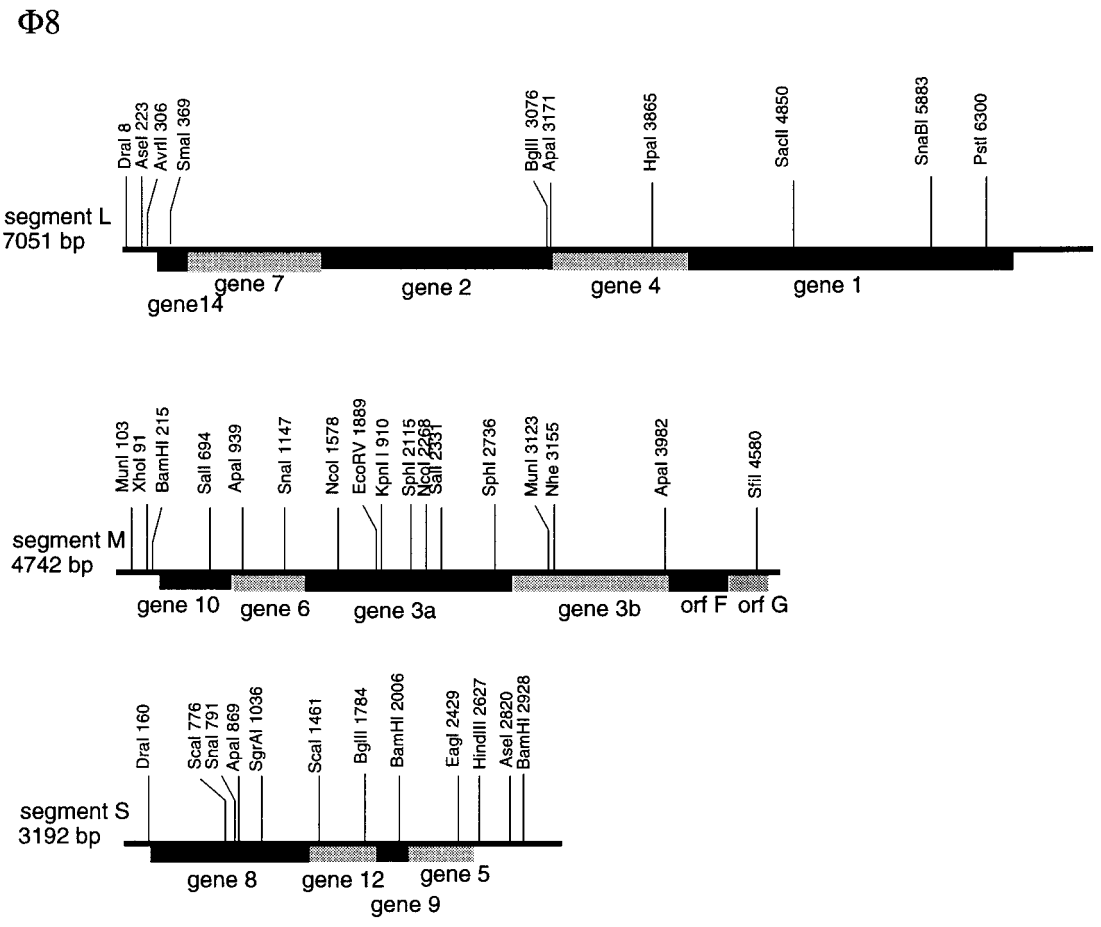

FIG. 1. Restriction map of the cDNA copies of the three genomic segments of Φ8. The genes are numbered so as to correspond to those of Φ6.

for Φ8. The sequences at the 3′ ends of the segments show common predicted secondary structures, but these are also less extensive than those for Φ6 and show less identity (Fig. 2).

The sequences of the first 300 nucleotides in each plus-strand transcript of the genome are necessary and sufficient for packaging in Φ6 (Gottlieb *et al.,* 1994). This region folds into a complex of stem-loop structures called the *pac* region (Mindich, 1999). The same regions in Φ8 can also be folded into a similar complex although with completely different sequence and structure. In Φ6, the *pac* sequences terminate about 50 nucleotides before the first *orf*. Because the *orf*s in Φ8 start earlier in each genomic segment (Table 1), we might expect the *pac* regions to be smaller than those found in Φ6.

Identification of genes

The genes of segment L are arranged in a manner similar to those of Φ6. The assignments could be made on the basis of chromosomal position, size, and structural similarity with Φ6. However, amino acid similarity was absent in genes 1, 4, and 7 on the basis of a blastP 2.09 comparison (Tatusova and Madden, 1999). Only gene 2, which codes for the polymerase, showed some similarity to gene 2 of Φ6, but this involved only 20% identity in amino acids. The aspartate sequence GDD in motif VI (Koonin *et al.,* 1989) was present in the Φ8 gene but was SDD in Φ6. Gene 4 of Φ8 had a Walker motif A (Walker *et al.,* 1982) for ATP binding, which was GTAGGKT, as contrasted to GATGSGKS in Φ6. Protein P4 is the NTPase necessary for genomic packaging in Φ6 (Gottlieb *et al.,* 1992). Plasmids expressing genes 1, 2, 4, and 7 of Φ8 in *Escherichia coli*, *Salmonella typhimurium*, or *Pseudomonas phaseolicola* formed procapsids of the same general proportions as that found with Φ6 procapsids (unpublished results).

Genes in segments S and M were named for genes in the same position in Φ6; however, no sequence similarity was found, and the functions of the genes are not necessarily the same. The gene assignments could also be integrated with the results of gel analysis of protein samples of purified virions, detergent-extracted virions, and radioactively labeled infected cells (Fig. 3). Assignments were made on the basis of amino acid sequencing analysis of bands transferred from polyacrylamide gels, the location of nonsense mutations and their polar-

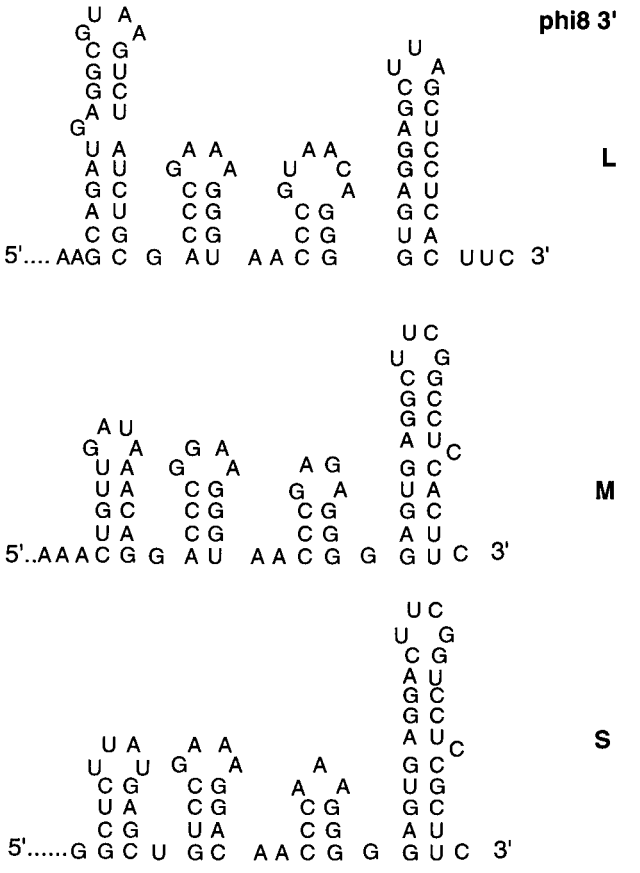

FIG. 2. Secondary structure predicted for the 3′ ends of the three plus strands of the Φ8 genome.

ity relationships (Fig. 4), and the results of deletions caused by recombination. All of the gene products of Φ8 segments S and M were found in the membrane of the virion with the exception of proteins PF and P12. The product of gene 6 was not found. The migration behavior of some of the membrane proteins (e.g., P9 and P10) was found to be anomalous. This is reminiscent of the results found with some Φ6 proteins (Mindich, 1988).

A major difference between Φ8 and Φ6 is that the Φ6 membrane requires only protein P9 as a structural protein. The other membrane proteins, P10, P6, P3, and P13, can be absent, and membrane will still be formed and located on the mature virion (Mindich *et al.,* 1976; Johnson and Mindich, 1994). In the case of Φ8, the absences of P10, P3a, P3b, and PF all result in the absence of membrane on the particle (unpublished results). We have not yet isolated mutants defective in P6 or P9. The procapsid of Φ6 is covered by a shell of protein P8 (Mindich *et al.,* 1976). In the case of Φ8, Triton X-100 removes protein P8 along with the other membrane proteins (Fig. 3).

Polar relationships

Most genes in the Φ8 genome have recognizable Shine-Delgarno ribosome binding sequences. However, as in the case of Φ6, a number of genes do not have these motifs and are dependent on upstream genes for their ribosome loading and are consequently subject to polarity. In segment L, gene 2 translation depends on that of gene 7, whereas gene 7 is preceded by a gene that we call 14 in analogy to that found in Φ6. In Φ6, gene 14 is not indispensable but might play a role in the expression of gene 7. Gene 7 is polar on gene 2. As in the case of Φ6, both genes 4 and 1 have their own ribosome binding sites. The site for gene 4 is far from the consensus sequence (Table 2); however, the expression of the gene seems normal and shows no polarity. Gene 3a in segment M is polar on 3b (Fig. 4); however, there is a good SD motif in front of gene 3b. Gene 8 is polar on gene 12 as in the case of Φ6, but there is a ribosome binding motif in front of gene 12, although it is rather far upstream (Table 2).

DISCUSSION

It seems clear that Φ8 is related to Φ6, because both have three segments of dsRNA, a procapsid composed of four proteins of similar sizes, and a lipid-containing membrane as an outer envelope. They propagate on related host cells and have similar base compositions. There is, however, virtually no sequence similarity between the two viruses with the exception of the polymerase sequences. The consensus sequence at the 5′ end of the plus strands is very different, and the *pac* sequences are completely different. In addition, the two phages deal with membrane in very different ways: Φ6 attaches to host cells through type IV pili; whereas Φ8 attaches directly to the rough lipopolysaccharide (Mindich *et al.,* 1999). Consequently, the host range of Φ6

TABLE 1

List of *orf*s for Φ8

orf	Segment	Start	End	aa[a]	MW[b]	IEP[c]
P1	L	4083	6458	792	86.9	5.91
P2	L	1211	3118	636	71.6	8.84
P3a	M	1202	2806	535	59.4	6.72
P3b	M	2900	4045	382	41.2	5.43
P4	L	3121	4083	321	34.2	7.75
P5	S	2067	2573	169	18.9	9.76
P6	M	778	1209	144	14.5	6.82
P7	L	507	1208	234	25.4	10.27
P8	S	188	1285	366	39.2	5.14
P9	S	1885	2061	59	6.2	9.51
P10	M	263	775	171	17.6	4.37
P12	S	1288	1818	177	19.4	4.74
P14	L	253	507	83	9.2	6.12
PG	M	4384	4674	97	11.2	10.74
PF	M	4045	4371	109	12.3	10.92

[a] The number of amino acids in the *orf.*
[b] Molecular weight in kDa.
[c] Predicted isoelectric point of the protein.

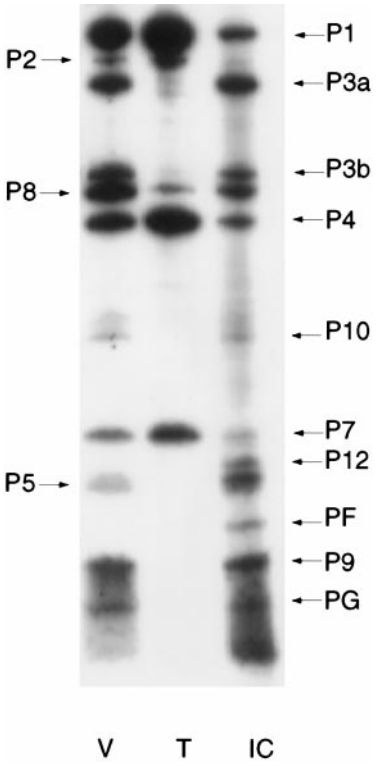

FIG. 3. Autoradiogram of PAGE analysis of whole virus (V), Triton X-100-treated virus (T), and cells infected with Φ8 in the presence of rifampicin (IC).

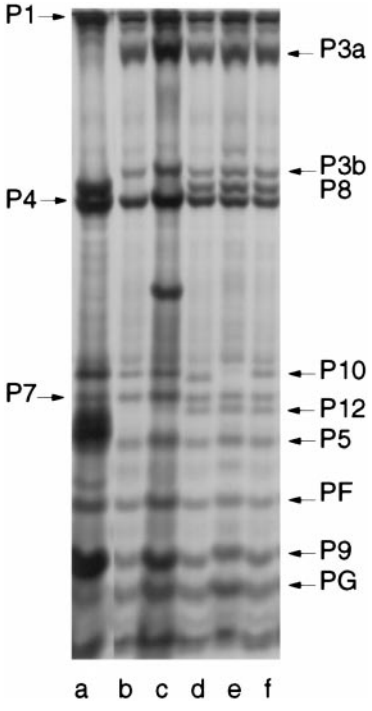

FIG. 4. Autoradiogram of PAGE analysis of cells infected with nonsense mutants of Φ8 in the presence of rifampicin: gene 3a (a), gene 8 (b and c), gene 10 (d and e), and wild type (f).

is limited to bacteria producing similar pili, whereas the host range of Φ8 extends to rough lipopolysaccharide strains of many genera when the establishment of a carrier state is included. It appears that plaque formation depends on more parameters than those involved in entry; consequently, the host range of Φ8 is extended only to some strains of *Salmonella* when plaque formation is considered.

The compositions of the membranes of the two virions show considerable difference. Protein P3 in Φ6 carries the host attachment specificity and is anchored by protein P6. Membrane assembles and covers the nucleocapsid in the absence of these proteins, but the virion cannot attach to host cells. In Φ8, this protein is split into two peptides, 3a and 3b, and membrane does not form in their absence. Triton X-100 removes the membrane from Φ6, leaving behind the nucleocapsid composed of the procapsid with proteins P1, P2, P4, and P7, which is covered by a shell of P8. Triton X-100 removes the membrane of Φ8, including protein P8. There is no indication that P8 forms a shell in Φ8 as it does in Φ6. The amount of P8 in the Φ8 virion is much less than the amount found in Φ6.

The comparison of the sequences of Φ8 and Φ6 is informative at several levels. In Φ6 and its close relatives, the sequence at the 5′-terminus of segment L is different from that of segments S and M (Mindich *et al.,* 1999). L starts with GU, whereas the other two segments start with GG. This difference leads to different behavior in *in vitro* transcription; the transcripts that start with GG are expressed to a greater extent. It has been proposed that this difference is responsible for differential transcription *in vivo* (Dijk *et al.,* 1995). In the case of Φ8, we find that the sequence at the terminus of the three seg-

TABLE 2

Ribosome Binding Sites for Φ8 Genes

UAAGGAGGUGAUC	Rev comp of 16S RNA
ACAUUGAAGGAGUUGACAAAUGAGU	P1
CCAUCAUCGAGCCGUACUGAUGGCA	P2
ACUGUUAAAGGAGCUUCAAAUGCUG	P3a
CGCGCGAAGGAGGCCGCAAAUGGCU	P3b
AAUUUGGAUUCGUCAAAUAAUGGCU	P4
AUAUUCUGGGCUUCUGAUCAUGACC	P5
UUGCCAUAGGAGCCAGCUAAUGGGU	P6
AGCAGGGAGCAUCGGAGUUAUGAAG	P7
GAGAUUGAGGAUAAAGACAAUGGGU	P8
AAACAUAAGGAUUUACCUCAUGAAU	P9
UCCUAACAAGGAGAUGCACAUGGGC	P10
CGAAGGAGAAGCGUUCGUGAUGCUU	P12
CGGCGACUAAGGAUGUAACAUGAUG	PG
CUGGCAAGACCUUCGUCGCAUGAGG	PF
AUAAUAGGAGUUAUGACUCAUGUCC	P14

Note. The nucleotide sequences around the initiating codons for each of the Φ8 proteins. The initiating codons AUG and the presumed binding sequences are underlined.

ments is GA, and the *in vitro* transcription of the three segments is approximately equal and robust (unpublished results). There does not seem to be a requirement for differentiation in structure or transcription activity. The RNA at the 3′-terminus folds into a series of stem-loop structures in Φ6. These structures of about 75 nucleotides play a role in polymerase recognition and *in vivo* stability (Mindich *et al.,* 1994). The terminal structures of L, M, and S are not identical; however, they can be exchanged with no prejudice (Mindich, 1996). The 3′-termini of the Φ8 RNA plus strands show similar, but more limited, secondary structure.

The exploration of the infection, assembly, and replication strategies benefits markedly from the comparison of related viruses. Mutational analysis of a single virus type is considerably slower and more limiting. Preliminary observations indicate that Φ8 and Φ6 differ in additional important ways. It appears that the genomic packaging in Φ8 is less stringent than in Φ6 and that homologous recombination occurs in Φ8, whereas Φ6 has only heterologous recombination (unpublished observations).

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids

LM2489 is a rough derivative of *P. syringae pv. phaseolicola* HB10Y (HB) (Vidaver *et al.,* 1973) and was used as the primary host for plating Φ8 and Φ6. LM2509 is a derivative of LM2489 that lacks pili and is resistant to Φ6 but sensitive to Φ8. Strain ERA is an isolate of *Pseudomonas pseudoalcaligenes*. S4 is a derivative of ERA that contains a nonsense suppressor mutation (Mindich *et al.,* 1976).

Plasmid pLM1454 is a derivative of the cloning vector pT7T3 19U (Pharmacia). It was used for the cloning of cDNA copies of phage DNA produced by RT–PCR.

Media

The media used were LC and M8 (Sinclair *et al.,* 1976). Ampicillin plates contained 200 mg ampicillin/ml in LC agar.

Enzymes and chemicals

All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, Klenow enzyme, and Exonuclease BAL-31 were purchased from Promega (Madison, WI), New England Biolabs (Beverly, MA), and Boehringer-Mannheim (Indianapolis, IN).

Preparation of pure virions of Φ8

The Φ8 is not as stable as Φ6 in LC broth, so all liquid cultures were grown in minimal M8 medium (Sinclair *et al.,* 1975). Then 300 ml of fresh lysate was used to infect 1–3 liters of fresh LM2489 culture with a density of 2×10^8 cells/ml at a multiplicity of 50. After lysis, the culture was spun at 7000 rpm for 10 min at 4°C. NaCl (0.5 M) and 10% PEG-6000 were added to precipitate the phage. The suspension was centrifuged, and the pellet was resuspended in 20–30 ml of buffer B overnight at 4°C. Buffer B is composed of 10 mM $KHPO_4$, 1 mM $MgCl_2$, and 200 mM NaCl, pH 7.5.

The resuspended Φ8 was treated with DNase for 10 min at room temperature to make the suspension less viscous. It was then spun at 23,000 rpm for 90 min in a zone gradient of 10–30% sucrose in buffer B. The phage band was isolated and treated with PEG to precipitate the virions. The pellet was resuspended in buffer B, applied to a gradient of 40–60% sucrose in buffer B, and spun at 23,000 rpm overnight at 20°C in the SW41 rotor. The phage band was isolated and concentrated.

Isolation of the Φ8 dsRNA

RNA was isolated from the virus by phenol–chloroform (1:1) extractions. The RNA was then precipitated with 10% of 7.5 M NH_4Ac and 2.5 volumes of ethanol and resuspended in 50 μl of TE buffer. Finally, the RNA was treated with 200 μg/ml Proteinase K in 0.1% SDS at 37°C overnight. The RNA was then treated with phenol–chloroform, precipitated with ethanol, and resuspended in 50 μl of TE buffer. The purified RNA was separated on an 0.8% agarose gel, and the three segments were recovered by electroelution.

Preparation of cDNA (poly)A^+ tailing

The RNA was denatured by boiling for 5 min and rapidly cooling with dry ice–ethanol. We added 5× poly$(A)^+$ polymerase buffer to the RNA along with ATP and yeast (poly)A^+ polymerase (Amersham, Arlington Heights, IL). The mixture was incubated at 30°C for 1 min and transferred to ice, and the reaction was stopped with EDTA. The poly$(A)^+$ RNA was then extracted with phenol–chloroform, precipitated, and resuspended in water.

First-strand synthesis

Phosphorylated oligo(dT) (1 μl) was added to 10 μl of poly$(A)^+$ RNA. After 5 min at 70°C, the sample was cooled on ice for 5 min. Then 4 μl of 5× first-strand buffer, 3 μl of H_2O, 40 U of RNase inhibitor (RNasin), and 30 U of AMV reverse transcriptase were added and incubated at 42°C for 1 h. All products required for the first-and second-strand synthesis were provided with the Promega cDNA kit (Universal Riboclone cDNA Synthesis System). The reaction products were stored at −70°C overnight.

Second-strand synthesis

After thawing, the reverse-transcribed RNA, 40 μl of 2.5× second-strand buffer, 37.6 μl of H_2O, 0.8 U of RNase

H, and 23 U of *E. coli* DNA polymerase I were added. After the second-strand synthesis proceeded for 3 h at 16°C, the *E. coli* DNA polymerase I was inactivated at 70°C for 10 min. Then T4 DNA polymerase was added for 10 min at 37°C to blunt the ends of the cDNA. The sample was then treated with phenol–chloroform, ethanol precipitated, and resuspended in 2.5 μl of dH_2O.

Preparation of the vector used for cloning

pLM1454 was cut with *Hin*cII, dephosphorylated with shrimp alkaline phosphatase and then purified by electrophoresis, electroeluted, precipitated, and resuspended in 20 μl of TE buffer. The ligation mixture was composed of 2.5 μl of Φ8 cDNA, 0.5 μl of vector, 0.5 μl of 10× ligation buffer, 0.5 μl of 10 mM ATP, and 2.5 U of T4 DNA ligase. All products are provided with the Promega cDNA kit. Incubation was overnight at 16°C. The ligation mixture was used to transform supercompetent Epicuran *E. coli* (Stratagene, La Jolla, CA). The cells were resuspended in 100 μl of SOC medium and plated onto LC plates with 40 μg/ml X-gal and 200 μg/ml ampicillin. White colonies were picked, and small DNA preparations were made. The plasmids were cut with restriction enzyme *Pvu*II, and promising candidates were sequenced first with M13 primers and then with oligonucleotides prepared on the basis of the sequence found. At the point at which it seemed that the ends of the segments were identified, we prepared cDNA copies by using RT–PCR with oligonucleotides that had sequences found in the first copies found. Sequencing was performed at the New York University Medical Center Sequencing Facility. The sequences were assembled using the GCG program GelStart. The sequence of the Φ8 genome was submitted to GenBank, and the accession numbers for segments L, M, and S are AF226851, AF226852, and AF226853, respectively.

Preparation of complete cDNA plasmids

The cDNA pieces were assembled to form complete copies of the three genomic segments. In many cases, the connections could be made by using unique restriction sites made evident by the sequencing project. The ends of segments were prepared by using oligonucleotides with convenient restriction sites as primers for PCRs. Three plasmids were prepared: pLM2424, pLM2445, and pLM2452. They contain exact complete copies of genomic segments L, M, and S, respectively, in plasmid pT7T3 19U. The sequences start at the first nucleotide of the T7 RNA polymerase transcript .

In vitro protein synthesis

Plasmids were cut with various restriction enzymes and then transcribed with T7 RNA polymerase. The transcripts were then added to lysates of *E. coli* prepared for *in vitro* protein synthesis in the presence of ^{35}S-methionine (Promega). The samples were precipitated with acetone, resuspended in sample cracking buffer, and analyzed on 18% acrylamide gels. The gels were soaked in EnHance (New England Nuclear Research Products, Boston, MA), dried, and incubated with film to produce autoradiograms.

Preparation of radioactively labeled phage

LM2489 was infected with Φ8 at a multiplicity of infection of 20 in M8 medium supplemented with amino acids, metal ions, and glucose. Then 10 μCi/ml ^{35}S-methionine was added, and the culture was allowed to proceed to lysis. The phages were purified as described above except that only the zone sedimentation was performed. Triton X-100 at a 2% concentration was used to remove the viral membrane before fractionation in the ultracentrifuge. In other experiments, virions were purified by equilibrium centrifugation before Triton X-100 treatment and zone centrifugation, with similar results.

Isolation of nonsense mutants

High titer phage stocks in LB medium were diluted 10-fold into Tris-maleate buffer with 800 μg/ml nitrosoguanidine and incubated at room temperature for 3 days (Lehman and Mindich, 1979). Surviving phages were plated onto lawns of S4, and plaques were gridded onto lawns of S4 and LM2489. Virus that grew on S4, but not on LM2489, was purified, and then infected cells were labeled with ^{35}S-methionine in the presence of rifampicin.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health Grant GM31709.

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