Effects of Genome Size on Bacteriophage φX174 DNA Packaging in Vitro*

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Effects of the size of template DNA on the DNA packaging reaction of bacteriophage φX174 were studied using plasmids of various sizes which contain the φX174 origin of DNA replication and the in vitro phage synthesizing system (Aoyama, A., Hamatake, R. K., and Hayashi, M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4195–4199). DNA between 78.5% and 101% of the length of φX174 DNA produced infectious particles efficiently. Packaging of DNA smaller or larger than this range produced uninfected defective particles. Although these particles contained circular single-stranded DNA, they suffered structural changes which altered the sedimentation properties or the ability to adsorb to the cells. Mutant phage were found from the packaging reaction of DNA larger than 101% of φX174 DNA. These mutants deleted the termination region of DNA, suggesting that they were produced by early termination of the phage synthesizing reaction.

Bacteriophage φX174 contains single-stranded circular DNA of 5386 bases in length in icosahedral capsid (1). The ssDNA*1 is packaged into phage capsid after three successive stages of DNA synthesis (for review, see Ref. 2). Stage I is conversion of the circular ssDNA to a double-stranded replicative form DNA. Stage II is semiconservative replication of the RF DNA. Stage III is the synthesis of the circular viral DNA using RF DNA as template. Packaging of viral DNA is coupled to DNA synthesis during stage III (for review, see Ref. 3). This process is initiated by φX174 gene A protein which introduces the specific endonucleolytic cleavage on the φX174 supercoiled RF DNA between nucleotides 4305 and 4306 of the viral strand (4–7). The synthesis of the viral DNA starts from this point (origin of viral DNA synthesis) and proceeds 5' to 3' by a rolling circle mechanism (8). The preformed φX174 capsid precursor (prohead) attaches to the replication fork and the displaced viral DNA is packaged into the prohead in the 5' to 3' direction as the replication fork moves (9–11). The φX174 gene A protein attaches covalently to the 5'-phosphate end of the viral strand at the replication fork and remains attached during viral DNA synthesis (12, 13). After one round of replication, a circular viral DNA is formed within the prohead by the nicking-closing activity of the φX174 gene A protein that attaches to the 5'-end of the viral strand between nucleotides 4305 and 4306 (14, 15). To understand the mechanism of the DNA packaging process during stage III, the size of the genome packagable to the phage capsid is one of the important morphogenetic parameters. This problem was studied in vivo in several laboratories using recombinant DNA technique (16–18). van der Ende et al. (16) cloned various parts of φX174 RF DNA with φX174 HaeIII-Z6B fragment which contains the origin of φX174 viral DNA synthesis into plasmid pACYC 177 to make recombinant plasmids with various sizes (16). When Escherichia coli cells that carry these recombinant plasmids were infected with φX174, phage particles that contain recombinant plasmid ssDNA are co-produced with φX174 phage. Using this assay method, they showed that the lower limit of the genome size is between 3.96 kb and 4.41 kb and the upper limit is between 5.58 kb and 5.70 kb. DNA smaller than this limit could be packaged but the resultant phage particles were uninfected. The upper limit of the genome size was studied by Müller and Wells (17) and Russell and Müller (18) in more detail. The mutants of φX174 that have the insertion of DNA fragments in the intercistronic region between genes J and F of the genome were used for this purpose. They found that the upper limit could be at least 6.09 kb. However, the phage particles with genomes larger than 5.55 kb were highly unstable. Thus, the size of genome packagable within the capsid has been established.

In this report, the packaging of DNA smaller or larger than the limit was studied using the in vitro phage synthesizing system developed in our laboratory (19). The system is composed of the purified E. coli and the viral enzymes and is capable of synthesizing infectious phage with the addition of φX174 RF DNA. Recombinant plasmids of various sizes were constructed by cloning φX174 HincII-3 DNA fragment, which contains the origin of φX174 viral DNA synthesis, into plasmid pBR322, pBR325, or their derivatives. These were used as template in the in vitro phage synthesizing system. During these experiments, we found mutants which had deleted DNA. Characterization of these deletion mutants as well as other phage particles synthesized in the in vitro system are described.

MATERIALS AND METHODS

Bacteria and Plasmids—Escherichia coli strains used were HF4704 (φX174) and C600 SFS (recBC Δjop11). Plasmid pKJB51, a derivative of plasmid pBR322 with a deletion in the DNA sequence between the BamHI site and the PvuII site (nucleotides 375–2069 of pBR322), was constructed by Dr. R. K. Buckley in our laboratory. Plasmid pAS976, also a derivative of plasmid pBR322 with a deletion in DNA sequence between the EcoRI site and the BamHI site, was a gift from Dr. A. Shafferman in Dr. D. R. Helinski's laboratory. Plasmid pAS976 was derived by digesting pBR322 with EcoRI, filling the 3'-recessed ends, ligation with BamHI linkers, and cleaving with BamHI and religation. Therefore, this plasmid has both an EcoRI site (restored by the end C nucleotide of the BamHI linker) and a BamHI site (20).

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1 The abbreviations used are: ss, single-stranded; ds, double-stranded; RF, replicative form; kb, kilobase pairs; SDS, sodium dodecyl sulfate; Amp, ampicillin.
Isolation of DNA and Enzymes—The RF I DNA of φX174 and plasmids were purified as described previously (21). The φX174 gene A protein, gene C protein, gene J protein, prohead, E. coli DNA polymerase III holoenzyme, rep protein, and dUTPase were purified as described previously (19).

On a 15-20% sucrose gradient, the size of 6x174 RFI DNA synthesis or the packaging reaction, acid-insoluble radioactivities or DNase-resistant, acid-insoluble radioactivities of the 7-pl diluted samples from the reaction mixture as previously described and carries Amp resistance, but varies in size from 60% to 123% of the original number of uninfected cells. The three 1.5 ml samples were mixed with 1/10 volume of cold 80% trichloroacetic acid and incubated at 0°C for 1 h. The precipitate was collected by filtration through a Whatman glass fiber disc, rinsed with cold 6% trichloroacetic acid, dried, and counted.

RESULTS

Stage III Reactions Using Recombinant Plasmid DNA as Template—The in vitro stage III system contains purified φX174 gene A protein, gene C protein, gene J protein, prohead, E. coli rep protein, DNA polymerase III holoenzyme, and dUTPase (19). The system synthesizes and packages φX174 viral strand DNA when φX174 RF I DNA is added as template (φX174 system). The final product of this reaction is infectious φX174 phage particles which contain φX174 circular viral strand DNA. Previously we showed that the φX174 HincII-3 DNA fragment, which contains the origin region of φX174 DNA replication, cloned into the plasmid pACYC184 supports the stage III reaction when added to the stage III system as template (21). The final product of this reaction was the φX174 phage particles that contained either strand of the recombinant plasmid DNA depending on the orientation of the φX174 HincII-3 DNA fragment in the template DNA. The chimeric phage particles were infectious. The recombinant plasmid ssDNA was transferred to the host cell upon infection and converted to dsDNA in the cell. This process yielded the Amp-resistant transformants. To study the effects of the size of the template DNA in the stage III reaction, recombinant DNA with various sizes were constructed by cloning the φX174 HincII-3 DNA fragment into the EcoRI site of plasmid pBR322, pBR325, or their derivatives. Eleven recombinant plasmids that have φX174 HincII-3 DNA fragment in the same orientation and carries Amp resistance, but varies in size from 60% to 123% of the size of φX174 RFI DNA.

In Vitro Stage III Reaction—The complete reaction mixture (25 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 20 mM MgCl₂, 0.09 mM concentration each of dATP, dGTP, dCTP, and [3H]dTTP (400-1600 cpm/pmol of deoxyribonucleotides), 0.8 mM rATP, 0.1 mg/ml bovine serum albumin, 0.1 pmol of template DNA, 280 ng of φX174 gene A protein, 75 ng of φX174 gene C protein, 48 ng of φX174 gene J protein, 20 μg of the prohead, 120 ng of E. coli DNA polymerase III holoenzyme, 7 ng of E. coli rep protein, and 4 units of E. coli dUTPase. The reaction mixture was incubated at 30°C for 30 min and cooled on ice. To examine the DNA synthesis or the packaging reaction, acid-insoluble radioactivities of DNA or DNase-resistant, acid-insoluble radioactivities of the reaction mixture were determined as described previously (19). The infectivity of the product was determined by the titration of the diluted samples from the reaction mixture as previously described (21).

In Vitro Stage II (+) Reaction—The complete reaction mixture (25 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 0.08 mM concentration each of dGTP, dCTP, dTTP, and [3H]dTTP, 0.8 mM rATP, 0.1 mg/ml bovine serum albumin, 0.1 pmol of template DNA, 280 ng of φX174 gene A protein, 120 ng of E. coli DNA polymerase III holoenzyme, 7 ng of E. coli rep protein, and 1.4 μg of E. coli single-stranded DNA binding protein. The reaction was at 30°C for 20 min.

Analysis of the Stage III Products by Sucrose Density Gradient Sedimentation—Stage III reaction products were prepared using either the recombinant DNA or φX174 RF I DNA as template under the standard condition except that [3P]dATP was used instead of [3H]dTTP as a radioactive precursor and the reaction mixture was four times larger than the standard reaction mixture. After incubating at 30°C for 30 min, the reaction mixture was cooled on ice and loaded onto a 15-30% sucrose gradient with a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 0.1 mM NaCl with the cushion of 50% sucrose made in the same buffer. The sample was centrifuged at 49,000 rpm for 70 min in a Beckman SW 50.1 rotor at 4°C. The sample was fractionated into glass tubes (0.13 ml/tube). The obtained radioactive and soluble radioactivities and the infectivities of the fractions were determined as described previously (19).

Adsorption Experiment—The procedure was the modified method of Newbold and Sinshmeer (23). E. coli Hφ4704 cells were grown in a tryptone/KCl (1% and 0.5%, respectively) medium containing 10 mM sodium phosphate (pH 7.2) and 20 μg/ml thymidine to an A₆₀₀ nm = 0.8 (4.5 × 10⁹ cells/ml) at 37°C. The 32P-labeled stage III products were added in less than a 100-μl volume to 3 ml of the cell culture in a centrifuge tube at 37°C. The mixture was brought to 10 mM MgCl₂ and 5 mM CaCl₂ and incubated at 37°C for 10 min. A 1.5-ml portion was removed to assay total radioactivity and the remaining 1.5 ml was added to 80% trichloroacetic acid, and incubated at 0°C for 1 h. The precipitate was collected by filtration through a Whatman glass fiber disc, rinsed with cold 6% trichloroacetic acid, dried, and counted.

Characterization of Products of Stage III Reaction Using Recombinant Plasmid DNA as Template—The recombinant plasmid DNA were classified into three groups according to the size (Fig. 1) and the ability to support the production of infectious phage (Fig. 2): group A includes pφX3250, 3350, and 3650; group B includes pφX4200, 4500, 4850, and 5400; group C includes pφX5660, 5950, 6450, and 6600. The recombinant plasmids from group A (pφX3250), group B (pφX4200, 4850, 5400), and group C (pφX6600) were used to examine the structure of the products. As previously shown (19), the product of the φX174 system contained materials sedimenting...
Genome Size of $\phi X 174$

![Diagram of recombinant plasmids](image)

**Fig. 1.** Construction of various sizes of recombinant plasmids containing origin of $\phi X 174$ viral DNA synthesis. The recombinant plasmids were constructed as described under "Materials and Methods." The size of plasmid DNA was determined by agarose gel electrophoresis of EcoRI-treated samples.

with the $s$ value of 114 S and 90 S (Fig. 3a). The 114 S materials (fractions 6 to 9) were associated with infectivity as in vivo phage. The 90 S materials (fractions 15 to 19) were defective particles with no infectivity. The plasmids from group B produced a similar sedimentation profile (Fig. 3, c, d, and e). However, the plasmids from group A or C produced only one major peak that showed no infectivity (Fig. 3, b or f). The infectious phage was produced with low efficiency in these systems and sedimented faster than the major peak of defective phage particles. The DNA extracted from the defective particles of group A or C was susceptible to DNase I, whereas the DNA in the infectious particles of group B was not (Fig. 4). The degradation products from the DNase I treatment contained a mixture of relatively long DNA fragments and short DNA fragments. The DNA in the defective particles in group A was resistant to DNase I.

The phage made in the group B system (Fig. 5, fractions 7 to 10) adsorbed to the cells as efficiently as $\phi X 174$ 114 S phage (Fig. 5a, fractions 6 to 9). The products of the group A system showed little ability to adsorb to the cells (Fig. 5b, fractions 11 to 16). The slow sedimenting defective particles observed in the $\phi X 174$ system (Fig. 5a, fractions 15 to 18), group B system (Fig. 5c, fractions 13 to 16), or group C system (Fig. 5d, fractions 13 to 16) showed lower adsorption to the cells when compared to the infectious phage.

**Deletion Mutants Made in Group C System**—To study the nature of infectious phage made in low efficiency in group A or C system, the DNA were isolated from cells infected with these products and analyzed by gel electrophoresis (Fig. 6).

**Table I**

| Template DNA | $M_r$ | DNA synthesis |
|--------------|------|---------------|
|              | $kb$ | Total         | DNase-resistant |
| $pX3250$     | 3.25 | 200           | 155             |
| $pX3350$     | 3.35 | 197           | 110             |
| $pX3650$     | 3.65 | 207           | 133             |
| $pX4200$     | 4.20 | 239           | 145             |
| $pX4500$     | 4.50 | 259           | 132             |
| $pX5400$     | 5.40 | 275           | 124             |
| $pX5650$     | 5.65 | 269           | 126             |
| $pX6350$     | 5.95 | 272           | 99              |
| $pX6450$     | 6.45 | 235           | 107             |
| $pX6600$     | 6.60 | 275           | 97              |
| $\phi X 174$ | 5.35 | 261           | 153             |

The DNA isolated from the cells infected with group A products co-migrated with the respective template DNA as did those of group B products (Fig. 6a, 1 to 7). However, the
DNA from the cells infected with the group C products migrated faster than the respective template DNA (Fig. 6a, 8 to 11). In order to examine the difference between in vivo DNA and the template DNA used for the reaction mixture, the 6x174 HincII-3 DNA fragments (Fig. 6b, 1 to 7) and the 6x174 RF I DNA. The packagable size of the genome has been thus established both in in vivo and in vitro DNA packaging systems.

Although only group B DNA could produce infectious phage efficiently, group A, whose genome sizes are between 3.25 kb and 3.65 kb, or group C, whose genome sizes are between 5.65 kb and 6.60 kb, could also produce phage particles. However these particles were not infectious. This may be due to the

**DISCUSSION**

Effects of the size of template DNA on the DNA packaging of φX174 has been studied. The summary of the results are

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1. **Fig. 3.** Sedimentation profiles of stage III products. The 4x reaction mixtures containing various template DNA were incubated and analyzed by sucrose density gradient centrifugation as described under "Materials and Methods." The trichloroacetic acid-insoluble radioactivities (●) and the infectivities (○) of aliquots were determined as described previously. The infectivity were represented as described in the legend to Fig. 2. The template DNA used were: a, φX174; b, peX3250; c, pφX4200; d, peX4850; e, pφX5400; and f, peX6600.

2. **Fig. 4.** DNase sensitivity of the DNA packaged into phage particles. The products shown in Fig. 3 were digested with 10 µg/ml DNase I at 30 °C for 20 min. The DNA was extracted by incubating the sample with 0.5 mg/ml of pronase in 0.1% sodium dodecyl sulfate solution at 25 °C for 1 h. The samples were run through 1% agarose gel as described previously (21). The gel was dried under vacuum and the autoradiography was carried out. The markers were prepared by extracting DNA from the in vitro stage II(+) reaction mixtures (see "Materials and Methods") containing various template DNA. The in vitro stage II(+) system is capable of synthesizing circular ssDNA when φX174 RF I DNA (25) or the recombinant plasmid DNA which carries φX174 HincII-3 DNA fragment was added as template. The DNA sequences of the initiation/termination region of the φX174 capsid was previously determined by in vivo studies by van der Ende et al. and Müller and co-workers (16–18). The lower limit is between 3.96 kb and 4.41 kb (74% and 82% of φX174 DNA) and the upper limit between 5.50 kb and 5.70 kb (102% and 106%). A similar result has been obtained in the in vitro phage synthesizing system. Only group B plasmid, whose genome sizes are between 4.20 kb and 5.40 kb (78.5% and 101%), could produce infectious phage as efficiently as φX174 RF I DNA. The packagable size of the genome has been thus established both in in vivo and in vitro DNA packaging systems.

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A. Aoyama and M. Hayashi, unpublished results.
particles added are shown. Only the first 20 fractions of the sucrose
gradients are shown. Adsorption experiments were performed as described under "Materials and Methods."
The percentages of the absorbed phage particles to the total phage particles added are shown. Only the first 20 fractions of the sucrose gradients are shown. a, radioactivity; b, adsorption.

The products of stage III reactions directed by φX174 (a), pBR325 (b), pBR325 DNA (c), and pBR322 HindIII site 16-mer primer (counter clock, New England BioLabs). The DNA sequences of the origin (Ori) region are shown. E. coli HF4704 cells were infected with the stage III products directed by the recombinant plasmid pBR325 DNA. Four colonies with Amp-resistant phenotype were randomly selected. The strains were designated as D-4, D-14, D-34, and D-36. The DNA was isolated by an alkaline-SDS method and purified by the methylated albumin column chromatography (27). Each DNA was digested with BamHI. DNA sequence was determined by the method of Sanger et al. (28) using pBR322 HindIII site 16-mer primer (counter clock, New England BioLabs). The DNA sequences of the origin (Ori) region are shown. The nucleotide of pBR322 DNA sequence at the junction of pBR325 DNA and φX174 DNA in D-4, D-14, D-34, and D-36 were 5893, 5971, 5814, and 5866, respectively (29). The nucleotide of φX174 DNA sequence at the junction was δX174 4306 in all cases.

![DNA synthesis](image)

**Fig. 7. DNA sequences of the deletion products.** E. coli HF4704 cells were infected with the stage III products directed by the recombinant plasmid pBR325 DNA. Four colonies with Amp-resistant phenotype were randomly selected. The strains were designated as D-4, D-14, D-34, and D-36. The DNA was isolated by an alkaline-SDS method and purified by the methylated albumin column chromatography (27). Each DNA was digested with BamHI. DNA sequence was determined by the method of Sanger et al. (28) using pBR322 HindIII site 16-mer primer (counter clock, New England BioLabs). The DNA sequences of the origin (Ori) region are shown. The nucleotide of pBR322 DNA sequence at the junction of pBR325 DNA and φX174 DNA in D-4, D-14, D-34, and D-36 were 5893, 5971, 5814, and 5866, respectively (29). The nucleotide of φX174 DNA sequence at the junction was δX174 4306 in all cases.

| Template DNA | DNA synthesis | Infectivity |
|--------------|---------------|-------------|
| φX174        | 225           | 104         |
| D-4          | 14            | <10⁶         |
| D-14         | 28            | 7           |
| D-34         | 32            | 10          |
| D-36         | 41            | 15          |

**Table II**

**Template activities of the DNA with deletion**

Assay conditions were as described under "Materials and Methods."

Structural changes that affected the sedimentation properties (Fig. 3). Understanding the structure of these defective particles may be helpful to elucidate both the structure of phage and the mechanism of the DNA packaging. Since the prohead contains φX174 gene F, G, H, B, and D proteins (3), while the mature phage contains gene F, G, H, and J proteins, gene B and D proteins must be removed from the prohead structure, and gene J protein must be added to the phage particle during or after DNA packaging. Some of these processes may be affected by packaging DNA of group A or C, producing defective particles. In these particles, gene B or D proteins could remain, or the amount of gene J protein could be altered. Further studies should clarify this problem. Defective particles similar to those in group C system were observed in group B system (Fig. 3). DNA in these particles were susceptible to the action of DNase I (Fig. 4), indicating that DNA is partially exposed to the outside of the particles. Similar defective particles were observed in gene H mutant infected cells (24). Therefore, these deficient particles could have a similar structure to the particles made in gene H mutant infected cells. During the studies of the DNA isolated from the chimeric phage infected cells, we found that the group C system produced deletion mutants. All infectious particles tested in group C contained DNA smaller than the template plasmid.

A. Aoyama and M. Hayashi, unpublished data.
DNA (Fig. 6). DNA sequence analyses showed that the deletion started at various points on the plasmid portion of the recombinant plasmid and ended at the initiation/termination point of φX174 viral DNA synthesis (nucleotide 4306) (Fig. 7). This indicates that the early termination of stage III DNA replication occurred at various points on the template DNA. This early termination produces a phage particle that contains ss circular DNA smaller than the unit length of the template DNA. The sizes of all such deleted DNA were similar to those of group B DNA (see Fig. 6). Only the phage particles carrying DNA with sizes similar to those of group B retained infectivity because of the size requirement for infectious phage production (Fig. 2). Such early termination of stage III reaction may occur either by ligating the pre-existing nick on the template DNA or by nicking/closing activity of φX174 gene A protein. In either case, this produces the recombinant plasmids that have mutations in the origin region of φX174 DNA replication. These mutant DNA produced infectious phage with several different efficiencies (Table II). Therefore, these mutants may be useful to study the interaction between origin region of DNA and gene A protein or other proteins required for stage III reaction.

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* A. Aoyama and M. Hayashi, unpublished observation.