Transfecting Deoxyribonucleic Acid of *Bacillus* Bacteriophage ϕ 29 That Is Protease Sensitive

(trypsin/CsCl centrifugation/sucrose gradient centrifugation/SPP1, SPO1, and SP50 bacteriophages)

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ABSTRACT The transfecting activity of *Bacillus* phage ϕ 29 DNA, extracted either by sodium lauroyl sarcosine-phenol or by 2 M perchlorate, was destroyed by treatment with proteolytic enzymes, although these enzymes did not effect transfecting DNAs of SPP1, SPO1, and SP50. These facts suggest that a protein is associated with transfective ϕ 29 DNA. Stabilization of protease-resistance during transfection appeared earlier than that of DNase-resistance, indicating that the protein associated with ϕ 29 DNA is necessary for initiation of the incorporation of DNA molecules into competent cells. The physical nature of ϕ 29 DNA before and after the trypsin treatment was investigated by sucrose and CsCl density gradient centrifugations. The trypsin treatment did not alter the sedimentation rate of the unit ϕ 29 DNA; however, it did convert the sedimentation rate of the aggregated material in the untreated DNA to that of the unit ϕ 29 DNA. The density of the trypsinized DNA was 0.009 g/cm³ greater than that of the untreated DNA. The possible location of the protein on the DNA is discussed.

After Avery *et al.* (1) reported that the transforming principle is probably DNA, efforts were made to prepare DNA free from protein (2, 3). It was then established that small amounts of protein, remaining in DNA preparations, were not important in transformation. Similarly, for transfection by bacteriophage DNA, the protein remaining in the DNA preparation had no influence on its biological activity (4-6).

Transfecting ϕ 29 DNA, composed of nonpermuted linear duplex molecules of 11×10^6 daltons (7), purified by sodium lauroyl sarcosinate (SLS)-phenol, shows the characteristic DNA absorption spectrum. However, several observations, such as shapes of DNA molecules and thermosensitive infectivity of ϕ 29 DNA, obtained in our laboratory (8) suggest that some substance other than DNA is associated with ϕ 29 DNA. Recently, Ortin *et al.* (9) reported that a protein was involved in the circularization of ϕ 29 DNA. A DNA-protein complex in plasmid DNA of *Escherichia coli* (10, 11) and in DNA of P1 transducing phage (12) has also been reported. With respect to a regulation-mechanism of DNA replication, an analysis of protein that has binding affinity to DNA, such as gene 32 protein of T4 phage (13), is becoming an important field of investigation.

In this paper, I will report evidence that (a) a protein is bound to purified ϕ 29 DNA, probably at the end of the molecule, and (b) this bound protein is necessary for initiation of

the incorporation of DNA into competent cells. At the same time it is confirmed that the infectivity of other transfecting DNAs, SPP1, SP50, and SPO1, was not sensitive to proteolytic enzymes. Thus, ϕ 29 DNA appears to be unique among transfecting DNAs.

MATERIALS AND METHODS

Phages and Bacterial Strains. *Bacillus* phages ϕ 29 and SPO1 were kindly supplied by B. E. Reilly (5) and W. R. Romig (23), respectively. SP50 and SPP1 were stocks of Trautner's laboratory, Berlin. *Bacillus amyloliquefaciens* strain H, called originally *B. subtilis* H and obtained from Reilly, was used in the preparation of ϕ 29 stock cultures. SPO1 and SP50 were grown in *B. subtilis* 168, and SPP1 was grown in its substrain 168-2, isolated by Trautner (14). The strain 168-2 was also used in transfection as the source of competent cells.

Media. TY medium (15), was supplemented with 0.4% glucose and 1 mM MnSO₄. LTT plates (14) were used. The phage-diluting solution was 0.1 M Tris buffer containing 0.1 M NaCl and 0.01 M MgCl₂ (pH 7.5). Standard saline citrate (SSC) contains 0.15 M NaCl and 0.015 M Na₃ citrate.

Enzymes and Reagents. Trypsin (1 $\times$ crystallized, 160 EU/mg), α -chymotrypsin (3 $\times$ crystallized), Pronase-P, trypsin-inhibitor (soy bean), bovine serum albumin, calf-thymus DNA, and phenyl methylsulfonyl-fluoride (PMSF) were from Serva Feinbiochemica, Heidelberg, Germany. DNase (pancreatic endonuclease) was from Worthington Biochemical Corp., N.J., USA, and sodium lauroyl sarcosine (SLS) from Mann Research Laboratories, N.Y., USA. Anti- ϕ 29 phage serum (K = 150) was made according to Hagen *et al.* (15). Trypsin activity was assayed by the hydrolysis of *p*-toluenesulfonyl-L-argininemethylester (Serva Feinbiochemica) (22). [6-³H]Thymidine (2 Ci/mmol) and [2-¹⁴C]thymidine (57 Ci/mmol) were from the Radiochemical Center, Amersham, England.

Production and Purification of Phages. Logarithmically growing cells of *B. amyloliquefaciens* strain H in TY medium were infected with ϕ 29 at a multiplicity of infection of 5-10. Complete lysis occurred within 1.5 hr at 37°. Crude phage sediments were collected by differential centrifugation and were purified by centrifugation through a discontinuous CsCl gradient (15). The phage suspension thus obtained had a titer of 5×10^{12} infective centers/ml. SPP1, SP50, and SPO1

Abbreviations: PFU, plaque-forming units; SLS, sodium lauroyl sarcosinate; SSC, standard saline citrate (0.15 M NaCl-0.015 M Na₃ citrate); PMSF, phenyl methyl-sulfonyl-fluoride.

phages were obtained by the same method, except that a different bacterial strain was used.

Extraction of Phage DNA. Phage $\phi 29$ in phage-diluting solution was diluted to 10^{12} plaque forming units (PFU)/ml with SSC. Then SLS at a final concentration of 2.0% was added to the phage suspension, which remained at room temperature 5–10 min. Without SLS, most of the $\phi 29$ DNA could not be separated from denatured proteins in phenol extraction. An equal volume of distilled phenol saturated with SSC was added to the suspension. The phage-phenol mixture was rocked for 10 min at room temperature, and the DNA fraction was separated by centrifugation at 7000 rpm for 5 min ($6000 \times g$). After a second phenol extraction, the DNA was dialyzed extensively against SSC at 4°. The ratio of A_{260}/A_{280} nm of the purified $\phi 29$ DNA was 1.8–2.0. 10^6 – 10^8 phage equivalents of the DNA gave rise to one infective center with competent cells of 168-2. Other phage DNAs were extracted by the same method without SLS.

$\phi 29$ DNA was also prepared by the 2 M sodium perchlorate procedure of Anderson and Mosharafa (7).

Labeled Phage DNA. To obtain $\phi 29$ labeled with [3H]-thymidine, 250 μCi of [3H]thymidine was added to the culture of strain H in TY medium 15 min before the addition of phage. The cells were infected with $\phi 29$ at a multiplicity of infection of 3. The phage were purified as described above. Purified DNA having 4×10^4 cpm/ μg was obtained. SPP1 DNA labeled with [^{14}C]thymidine was prepared in a similar manner, except that strain 168-2 was used as host cells.

Proteolytic Enzyme Treatment. Trypsin, chymotrypsin, and Pronase were dissolved in SSC just before use. Pronase solution was autodigested for 2 hr at 37° to destroy nuclease

activity. 0.1 ml of DNA was mixed with 0.1 ml of enzyme solution and incubated for 15–30 min at 30°. This 0.2 ml of treated DNA was added directly to 0.2 ml of competent cells for transfection assay. When 0.1 ml of trypsin-inhibitor was added to the treated DNA, this mixture was incubated 15 min more at 30°, and then 0.2-ml samples were removed for transfection.

Transfection. Competent cells of strain 168-2 were prepared (14). 0.2 ml of competent cells was mixed in a plastic tube with 0.2 ml of $\phi 29$ DNA to be assayed and incubated at 30° for 40 min without shaking. For transfection with SPP1, SPO1, and SP50, the incubation was done at 37°. Transfection was terminated by addition of 0.1 ml DNase (200 μg /ml of phage-diluting solution) for 5 min. As plating bacteria, 0.1 ml of a late logarithmic culture of 168-2 (noncompetent) was added to the transfecting tube. 0.2 ml of the transfected culture was spread on an LTT plate in duplicate and incubated at 30°. The number of infective centers was counted after about 16 hr of incubation.

No effect of trypsin on the competent cells and on the infection of $\phi 29$ phage was observed in cultures treated with trypsin.

Sucrose Gradient Centrifugation. A gradient was made with 4–20% sucrose in SSC in Spinco SW50L tubes. 0.5 ml of 60% sucrose was put in the bottom of tubes as a cushion for fast sedimenting materials. Usually, 40 μl of a solution containing less than 2 μg /ml of DNA was layered on the top of the gradient. After centrifugation at 40,000 rpm ($130,000 \times g$) at 20° for 2.5 hr, about 38 fractions were collected on glass filters. Radioactivity on dried glass filters was directly counted in Permafluor scintillation fluid (Packard) in a Packard Liquid Scintillation Spectrometer.

CsCl Density Centrifugation. $\phi 29$ [3H]DNA was dissolved in CsCl-1/10 SSC solution of density 1.700 g/cm³ in a polyallomer tube. Centrifugation was done in the Spinco SW50L rotor at 35,000 rpm and 15° for 60 hr. Then successive 0.08-ml fractions were collected.

The radioactive DNA in each fraction was collected on Millipore filters (HAWP 02500; 0.45 μm) and precipitated with 5% cold trichloroacetic acid in the presence of carrier calf-thymus DNA (100 μg /ml). The samples were washed with 5 ml of cold 5% acid three times, and then washed with 95% ethanol. They were then dried and counted in 5 ml of scintillation fluid.

The buoyant density distribution of $\phi 29$ DNA in CsCl solution was also followed in the Heraeus Christ analytical ultracentrifuge 9100 equipped with UV-optics and photoelectric scanner at 44,770 rpm at 25° for 24 hr. As a density reference, SPO1 DNA (1.736 g/cm³) was used at the same time.

RESULTS

Biological evidence for protease sensitivity of $\phi 29$ DNA

Effect of Protease. The infectivity of $\phi 29$ DNA previously treated with trypsin, chymotrypsin, and Pronase was assayed by transfection. Table 1 shows that the infectivity was remarkably reduced by these protease treatments. However, these treatments caused no reduction of the infectivity of SPP1 DNA. Since SPP1 DNA is highly sensitive to DNase, the loss of infectivity of $\phi 29$ DNA was not due to contami-

TABLE 1. Effect of proteolytic enzymes on transfecting DNAs*

Enzymes (μg /ml)	No. of infective centers per plate	
	$\phi 29$ (0.3 μg /ml)	SPP1 (0.025 μg /ml)
Trypsin (0.0)	305	175
(1.5)	97	167
(15.0)	19	211
(150.0)	0	213
Inhibited trypsin† (15.0)	315	—
PMSF-trypsin‡ (15.0)	287	—
Chymotrypsin (0.0)	308	173
(1.5)	93	179
(15.0)	60	246
(150.0)	2	243
Pronase (0.0)	302	549§
(2.0)	134	474
(20.0)	30	513
(200.0)	2	632

* DNAs were treated with protease for 30 min at 30°.

† The trypsin was inhibited with soy bean inhibitor.

‡ PMSF-trypsin was made as follows: 1 volume of trypsin (30 μg /ml) was mixed with an equal volume of 10 mM PMSF and was stirred for 60 min at room temperature, then dialyzed against SSC at 4°.

§ Concentration was 0.05 μg /ml.

nation by DNase. It was confirmed that trypsin inactivated either by soy bean inhibitor or by PMSF had no effect on the infectivity of $\phi 29$ DNA. There was also no reduction of infectivity of SP50 and SPO1 DNAs treated with trypsin. These results indicate clearly that the infectivity of $\phi 29$ DNA was specifically destroyed by the proteolytic action of enzymes. In addition, $\phi 29$ DNA extracted by 2 M NaClO₄ appeared to be equally sensitive to trypsin treatment (unpublished data).

During trypsin treatment, residual infectivity of the DNA was measured by transfection after the addition of trypsin-inhibitor. The action of trypsin on the infectivity was fast; more than 90% of the infectivity was destroyed within 5 min of incubation in the presence of 15 μ g/ml of trypsin.

Effect of Other Proteins and Anti- $\phi 29$ Serum. As one control for protease action, the effect of several nonproteolytic proteins on $\phi 29$ transfection was investigated. Cytochrome *c* and ribosomal proteins did not affect the transfection. The presence of albumin or of anti- $\phi 29$ serum significantly enhanced the number of infective centers, although the mechanism is not understood.

Stabilization of trypsin sensitivity during transfection

The simplest explanation of the effect of trypsin on $\phi 29$ DNA would be that the trypsinized DNA could not be incorporated into competent cells. To test this possibility, trypsin was added to the transfecting culture at various times after the addition of $\phi 29$ DNA. The effect of DNase or excess amounts of calf-thymus DNA was compared with that of trypsin. The number of infective centers obtained at different times reflects the number of cells in which transfection has become insensitive to trypsin, DNase, or competing calf-thymus DNA. The stabilization of trypsin sensitivity is considerably faster than that of the DNase sensitivity (Fig. 1). Within 5 min after the incubation of competent cells with $\phi 29$ DNA, considerable stabilization had already occurred, while no stabilization of DNase resistance was observed. The DNase stabilization began about 10 min after the onset of incubation and it reached the same level as trypsin-stabilization after 40 min at 30°. The stabilization of DNA to trypsin occurred when transfection was still sensitive to DNase. Thus, the step of trypsin-stabilization precedes DNA incorporation into competent cells. If trypsin-stabilization is due to attachment of $\phi 29$ DNA to the competent cells, it must be similar to the reaction in which DNA attachment is prevented by the addition of large amounts of calf-thymus DNA. Indeed, both the kinetics of the trypsin-stabilization and of the prevention of attachment are similar in the early stage of the transfection (Fig. 1*a* and *c*).

Physical evidence for $\phi 29$ DNA-protein complexes

CsCl Density Gradient Centrifugation. Before and after trypsin treatment, the buoyant density of $\phi 29$ [³H]DNA was analyzed on CsCl density gradient centrifugation. The DNA distribution is shown in Fig. 2. The peak of trypsinized DNA shifted to a heavier density, whereas the peak of untreated DNA stayed in a lighter position, and this profile gave a skewed distribution in lighter fractions. The DNA treated with PMSF-trypsin gave the same distribution as untreated DNA. Essentially similar results were obtained by analytical

centrifugation. With SPO1 DNA as the density reference (1.736 g/cm³), the buoyant densities of untreated DNA and of trypsinized DNA were calculated as 1.686 and 1.695 g/cm³, respectively. The latter density corresponds to that reported by Mosharrafa (16) and to the base composition (35% GC) of the DNA. These results indicate that purified $\phi 29$ DNA still bound protein(s) and that the binding was stable in high concentration of CsCl. From the density difference, 0.009 g/cm³, the total amount of binding protein was about 2×10^6 daltons, which is about 2% (w/w) of the DNA.

Sucrose Gradient Centrifugation. The distribution of molecular size of $\phi 29$ DNA is presented in Fig. 3. With SPP1 DNA as reference, the position of the peak of $\phi 29$ DNA appeared to be the same and the recovery of input of $\phi 29$ DNA with regard to radioactivity was also equal before and after the trypsin treatment. Almost all of the fast-sedimenting material, aggregated forms of untreated DNA (Fig. 3*a*) disappeared after treatment with trypsin (Fig. 3*b*). Conversely, the amount of material in the peak of trypsinized DNA increased. These results indicate that trypsin did not break up $\phi 29$ DNA, but converted a fast-sedimenting material to the unit $\phi 29$ molecule.

DISCUSSION

Although the existence of a somewhat stable DNA-protein complex has been reported in plasmid DNA (10, 11), in P1 transducing DNA (12), and in $\phi 29$ DNA (9), its biological

FIG. 1. Effect of trypsin during transfection with $\phi 29$ DNA. 0.2 ml of $\phi 29$ DNA (0.2 μ g/ml) was incubated with 0.2 ml of competent cells, strain 168-2, at 30°. At various times, (a) 0.1 ml of trypsin (300 μ g/ml), (b) 0.1 ml of DNase (200 μ g/ml), or (c) 0.1 ml of calf-thymus DNA (1.8 mg/ml) was added to the transfection mixture. At 60 min, 0.1 ml of DNase was added to all tubes, which were then incubated for 5 more minutes. 0.2-ml aliquots were plated on LTT, after the addition of indicator cells (168-2, noncompetent). Incubation of plates: 16 hr at 30°.

Fig. 2. Buoyant density of $\phi 29$ DNA before and after trypsin treatment. 0.3 ml of $\phi 29$ [^3H]DNA (4×10^4 cpm/ml) was incubated with 0.3 ml of trypsin ($30 \mu\text{g}/\text{ml}$) at 30° for 30 min and was added to 2.6 ml of 1/10 SSC and 3.83 g CsCl. Centrifugation was performed in a Spinco ultracentrifuge, (SW50L rotor) at 35,000 rpm for 60 hr at 15° . Radioactivity in each fraction was counted by liquid scintillator as cold acid-insoluble material with carrier calf-thymus DNA.

importance is still obscure. This is the first evidence that a protein associated with DNA is essential for the transfecting ability of a DNA. Since the infectivity of three other *Bacillus* phage DNAs was not sensitive to proteolytic enzymes, the proteolytic sensitivity of transfective $\phi 29$ DNA appears to be unique among them. Not only the proteolytic sensitivity, but also the results of physical analysis of the DNA density, indicate clearly that transfective $\phi 29$ DNA exists as a DNA-protein complex.

Location of the Protein on $\phi 29$ DNA. The position of the peak of trypsinized DNA was essentially the same as that of untreated DNA in sucrose gradient centrifugation (Fig. 3). This finding indicates that the protein is not linearly inserted somewhere between phosphodiester links of double-stranded DNA. It is also unlikely that the protein exists linearly in single-stranded DNA, because the presence of smaller DNA fragments could not be observed in alkaline sucrose gradient centrifugation after trypsin treatment (unpublished data).

Purified $\phi 29$ DNA has been converted from linear to circular, and to dimer, oligomer, and some novel forms. After trypsin treatment, these novel molecules disappeared (to be published). Therefore, the protein was obviously involved in the conformational change of the molecules. Recently, Ortin *et al.* (9) have made similar observations. From the DNA conformations and the results of sucrose gradient centrifugation, it is most likely that the protein is bound to the ends of the DNA.

Importance of the Protein for Transfection. During transfection of $\phi 29$ DNA, the stabilization of trypsin resistance appeared considerably earlier than that of DNase resistance. The stabilization is parallel to that of the competitive inhibi-

tion by excess amounts of calf-thymus DNA (Fig 1). These results can be explained as follows: $\phi 29$ DNA may react with competent cells at the ends of molecules where the protein is bound; then the protease sensitivity may disappear as soon as a reaction occurs between competent cells and the protein. At this time the rest of the DNA, which is still outside the cells, is sensitive to DNase. Thus, the stabilization kinetics (Fig. 1a) lead us to a hypothesis that the binding protein is primarily necessary for DNA attachment to competent cells.

We can consider two alternatives concerning the function of the protein: first, the protein maintains the infective structure of $\phi 29$ DNA, or second, it has some enzymatic function that reacts with the surface of competent cells. The second possibility, however, is unlikely. Since the DNA-protein complex was extracted by the SLS-phenol method, it is unlikely that such an enzymatic activity would remain in the protein, even if the protein bound to the DNA is rather stable to denaturation. Furthermore, the addition of the protein fraction isolated from $\phi 29$ DNA-protein complex to $\phi 29$ DNA did not change the infectivity (unpublished results).

The first possibility is more likely. In preliminary experiments, we found that $\phi 29$ DNA serves as a priming-template to DNA-polymerase at 8° (to be published), similar to λ DNA (18). Moreover, $\phi 29$ DNA was sensitive not only to exonuclease III, but also to S1-exonuclease that digests single-stranded DNA (19). These results support the idea that $\phi 29$

Fig. 3. Sucrose gradient centrifugation of $\phi 29$ DNA. 40 μl of a DNA mixture containing $\phi 29$ [^3H]DNA (10^5 cpm; $2 \mu\text{g}/\text{ml}$) and SPP1 [^{14}C]DNA (6×10^3 cpm; $0.5 \mu\text{g}/\text{ml}$) was made. (a) 40 μl of the DNA mixture was added to 40 μl of SSC and incubated at 30° for 30 min. (b) 40 μl of the mixture was added to 40 μl of trypsin ($30 \mu\text{g}/\text{ml}$) and incubated at 30° for 30 min. 40 μl of these treated DNAs were layered on the top of gradients. Centrifugation was performed by a Spinco ultracentrifuge (SW50L rotor) at 40,000 rpm, for 2.5 hr at 20° . 37-38 fractions of 8 drops each were collected on glass filters and counted directly. $\phi 29$ [^3H]DNA, heavy line; SPP1 [^{14}C]DNA, light line.

DNA resembles the structure of λ DNA on at least one end of its molecule (20, 21). Since single-stranded DNA is not incorporated into competent cells under conventional conditions of transformation, trypsinized ϕ 29 DNA having a free single strand at the ends perhaps can not become incorporated into competent cells. The infectivity of the DNA might be maintained by a binding protein that alters the structure from the random state of the single-stranded DNA to a somewhat ordered and infective one. The priming-template activity of trypsinized ϕ 29 DNA is considerably lower than that of untreated DNA (unpublished data). This finding also supports the conclusion that the binding protein maintains some ordered structure of ϕ 29 DNA.

Anderson and Mosharrafa (7) could not find any circular forms in infective ϕ 29 DNA extracted by perchlorate. However, I have found that about 10% of the DNA extracted by the same method was in a circular form, and this DNA showed the same trypsin sensitivity as phenol-extracted DNA. Therefore, my findings indicate that a protein is associated with ϕ 29 DNA, regardless of the extraction method.

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