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Protease-Sensitive Transfection of *Streptococcus pneumoniae* with Bacteriophage Cp-1 DNA†

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The transfecting activity of pneumococcal phage Cp-1 DNA was destroyed by treatment with proteolytic enzymes, although these enzymes did not affect transfection with bacteriophage Dp-4 DNA. This transfection was stimulated by calcium ions. Protease-treated Cp-1 DNA competes for binding and uptake with transforming pneumococcal DNA as well as with transfecting Dp-4 DNA to approximately the same extent as does untreated Cp-1 DNA. In addition, [^{3}H]thymidine-labeled Cp-1 DNA, treated with proteases or untreated, was absorbed with the same efficiency. These data suggest that uptake of Cp-1 DNA is not affected by protease treatment. [^{3}H]thymidine-labeled Cp-1 DNA showed remarkable resistance against surface nuclease activity of competent wild-type cells. The monomeric form of the Cp-1 DNA-protein complex showed a linear dose response in transfection.

A feature of the genomes of several small *Bacillus* phages is their association with protein (33). The biological activity of the DNA of these phages (transfection) is sensitive to proteolytic enzymes, suggesting a role for the DNA-associated protein in the transfection process (1, 13, 14). Several reports have demonstrated that the presence of the protein is essential for the replication of the φ29 phage DNA in vivo (21, 45) and in vitro (25, 44). It has also been shown that a protein (p3) is covalently linked to φ29 DNA through a phosphodiester bond between serine and 5′-dAMP (12). On the other hand, Chase and Benzinger (7) have reported the role of a 65,000-dalton polypeptide noncovalently associated with the DNA of bacteriophage Mu in the transfection of *Escherichia coli* spheroplasts.

Recently, several pneumococcal phages have been isolated and characterized which show a morphology completely different from that previously described for the streptococcal phages (29). The DNA of the pneumococcal phage Cp-1 is a double-stranded, linear molecule of 12×10^6 daltons (29). Results obtained in our laboratory (9a) demonstrate that this DNA has a protein with an apparent molecular weight of 28,000 covalently linked to it.

Transfection with mature Dp-4 (30, 31) and ω3 phage DNAs as well as with the replicating form of ω3 DNA has been reported in *Streptococcus pneumoniae* (11, 26). In addition, a substantial knowledge on the transfection of several species of *Streptococcus* has been achieved recently (10, 23). To our knowledge, the existence of a transfection system in procaryotic cells that is sensitive to proteolytic enzymes and uses intact competent cells as receptors has not been described so far, apart from the case of *Bacillus* species.

In the present communication, we show that the transfecting activity of the DNA obtained from Cp-1 phage was destroyed by treatment with proteolytic enzymes, although these enzymes did not affect transfection with DNA isolated from the pneumococcal phage Dp-4. Nevertheless, the uptake of Cp-1 DNA by competent pneumococci was not affected by the proteolytic treatment of the DNA-protein complex. We also discuss the dose response obtained with the different forms of aggregation present in the DNA-protein complex.

MATERIALS AND METHODS

Bacterial strains. Strain R6, a derivative of *S. pneumoniae* R36A (Rockefeller University stock), was used mainly as an indicator for the phages described in this paper. A streptomycin-resistant strain, R6st, was constructed by genetic transformation and used for the preparation of phage Cp-1.

The nuclease-deficient mutant (*end* 1–*exo* 2), a mutant lacking about 90% of the wild-type nuclease activity, and strain 470, a thymidine-requiring mutant (18), were kindly supplied by S. Lacks (Brookhaven National Laboratory).

Phages. Bacteriophages Cp-1 and Dp-4 and details of the preparation and purification of these phages have

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been described elsewhere (29, 31) except that tryptic soy broth was used instead of K-CAT medium for the preparation of Cp-1.

Media and chemicals. Tryptic soy broth supplemented with 50 mM potassium-phosphate buffer, pH 8.0 (Difco Laboratories) was used to grow R6st. Semisynthetic growth medium (C medium) has been described before (41, 42). This medium contained salts of three divalent cations (38): $MgCl_2$ (2.5 mM), $CaCl_2$ (0.02 mM), and $MnSO_4$ (0.0001 mM). These concentrations were considered in the experiments described below. To avoid the introduction of additional amounts of divalent cations contained in some batches of the casein hydrolysate used in the C medium, different batches of this product were analyzed for calcium content in an Eppendorf emission spectrophotometer and for magnesium and manganese contents in a Perkin-Elmer 403 atomic absorption spectrophotometer. The batch of casein hydrolysate used in our media contained very low amounts of the three ions, which, nevertheless, were considered in the experiments presented in Table 2. DNase I and RNase A were from Worthington Diagnostics. Proteinase K and trypsin were obtained from Merck. Soy bean inhibitor was from Sigma Chemical Co., and [*methyl*-^{3}H]thymidine (25 Ci/mmol) was purchased from the Amersham Radiochemical Centre. All chemicals were reagent-grade, commercially available products.

Preparation of DNA. Cp-1 DNA-protein complex was obtained as follows. Purified phage Cp-1 (starting solutions contained about 10^{12} PFU/ml, unless otherwise stated) was incubated for 5 min at 65°C in TBT buffer (10 mM Tris-chloride, pH 7.8, 0.5 M NaCl, 10 mM $MgCl_2$) and 2% sodium dodecyl sulfate. After cooling at room temperature, the disrupted phages were shaken gently with TBT-saturated phenol and centrifuged. The DNA-protein complex at the interphase was exhaustively dialyzed against SSC (0.15 M NaCl, 0.015 M sodium citrate) or 0.05× SSC (see below). Dialysis bags were treated as previously described (8) to reduce the sticking of the material to the bag. Protease-treated Cp-1 DNA and Dp-4 DNA were isolated as described before (29, 31).

Radioactive phage DNA was prepared as follows. Strain 470 was grown in tryptic soy broth supplemented with 2 μg of thymidine per ml and 50 mM potassium phosphate buffer (pH 8.0) and incubated at 37°C without shaking. At a cell concentration of 5×10^7 CFU/ml, the bacteria were infected with Cp-1 (multiplicity of infection = 10); after 30 min at 30°C, 5 μCi of [*methyl*-^{3}H]thymidine per ml was added and the incubation was continued at the same temperature until the culture lysed (ca. 5 h later). The phage was purified as previously described (29), and [^{3}H]thymidine-labeled DNA-protein complex was obtained as described above for unlabeled Cp-1 DNA. The procedures used to determine total and DNase I-resistant DNA binding to the cells and surface nuclease activity have been previously published (35–37). Preparation of [^{3}H]thymidine-labeled Dp-4 DNA has been described (30).

Assay for infectious DNA and transformation procedures. Competent cells were prepared by standard methods used for *S. pneumoniae* (41, 42); several of the experimental procedures have been described previously (i.e., the assay for transformants [41]). Transfection with phage Dp-4 DNA has been described previously (31). Transfection with Cp-1 DNA was tested as follows. Cultures of competent wild-type or nuclease-deficient cells were diluted 1:10 with C medium, C medium without phosphate, or C medium containing 20 mM phosphate buffer (see below), and the DNA was added. The mixture was incubated at 30°C for 90 min without shaking; at this time, 0.1 ml of a 1% solution of fetal calf serum was added and incubation was continued at 37°C for 10 min. The reaction was stopped by adding DNase (5 μg/ml). Samples were taken and plated in K-CAT medium in the presence of catalase as previously described (29).

Antiserum. Anti-Cp-1 phage serum (K = 1,700 min^{-1}) was obtained by following a procedure similar to that previously described for obtaining an antiserum against phage Dp-1 (20).

RESULTS

Effect of enzymatic treatment on the infectivity of Cp-1 DNA. Previous observations have shown that Cp-1 DNA obtained by treatment by the proteinase K method has no biological activity in transfection, whereas if the DNA is prepared in the absence of proteases this material shows

TABLE 1. Effect of enzymatic treatment on the infectivity of Cp-1 and Dp-4 DNAs[a]

Enzyme	Conc (μg/ml)	No. of infective centers per ml	
		Cp-1 DNA (× 10^2)	Dp-4 DNA (× 10^4)
Control		5	2.5
Proteinase K	5	0.2	2.5
	50	0	2.4
Pronase	5	0.4	2.0
	50	0.05	2.3
	100	0	2.4
Trypsin	5	0.3	2.5
	50	0.02	2.6
Inhibited trypsin[b]	50	4.8	2.4
DNase	5×10^{-3}	0	0
RNase	50	5.5	2.5

[a] Samples (0.1 ml) containing 5 μg of Cp-1 DNA or 10 μg of Dp-4 DNA were treated with the different enzymes for 15 min at 37°C. Then, 0.1 ml of competent nuclease-defective mutant and 0.8 ml of CpH 8 medium containing yeast extract and bovine serum albumin were added to the samples, which were incubated at 30°C for 55 min (Dp-4 DNA) or 100 min (Cp-1 DNA) and then plated for transfectants as described in the text.

[b] Inhibited trypsin was prepared by mixing equal amounts of trypsin and soybean inhibitor and incubating the mixture for 15 min at 37°C.

transfecting activity. This finding, together with other data (29), suggested the presence of a protein(s) required for transfection and associated with the DNA of Cp-1. The results shown in Table 1 demonstrate that the infectivity of Cp-1 DNA was remarkably reduced when the DNA was treated with proteolytic enzymes. In contrast, Dp-4 DNA was not affected by the treatment with proteases as previously reported (31). The fact that the inactivation could be prevented by the specific protease inhibitor indicated that we were analyzing an effect which must be attributed to specific protease activity and not, for example, to contaminating nucleases.

Several conditions must be followed to obtain a succesful transfection with Cp-1 DNA-protein complex. (i) The DNA has to be freshly prepared. After 8 to 10 days of storage at 4°C, the level of transfection dropped about 1 log unit. This was probably due to the formation of multimeres (see below) or to the extreme sensitivity of the protein bound to the DNA to contaminating proteases or both. The terminal protein of the complex was severely degraded when this complex was treated with micrococcal nuclease, probably due to the presence of proteolytic contaminants. In fact, phenylmethylsulfonyl fluoride, a protease inhibitor, protects the protein from degradation (9a). (ii) Fresh preparations of competent receptor cells are also required. Competent cells that keep the level of competence for transformation over several months show a reduction in the number of transfectants after 15 days of storage at −75°C (data not shown).

TABLE 2. Effect of divalent cations on Cp-1 DNA transfection[a]

Medium and additives	Transfectants per ml	% Transfected
C-phosphate + $MgCl_2$ (2.5 mM) + $CaCl_2$ (0.5 mM)	8.2×10^3	100
C-phosphate + $MgCl_2$	6×10^0	0.08
0.5 mM	7×10^0	0.09
5 mM	9×10^0	0.11
+ $CaCl_2$		
0.05 mM	1.1×10^1	1.4
0.25 mM	3.5×10^3	42.6
0.5 mM	6.4×10^3	78.2
1.0 mM	8.2×10^2	10.0
C + phosphate (20 mM) + $MgCl_2$ (2.5 mM) + $CaCl_2$ (0.5 mM)	7.6×10^3	93.0
CpH 8	5×10^2	6.1

[a] Transfection was determined as described in Table 1, footnote *a*.

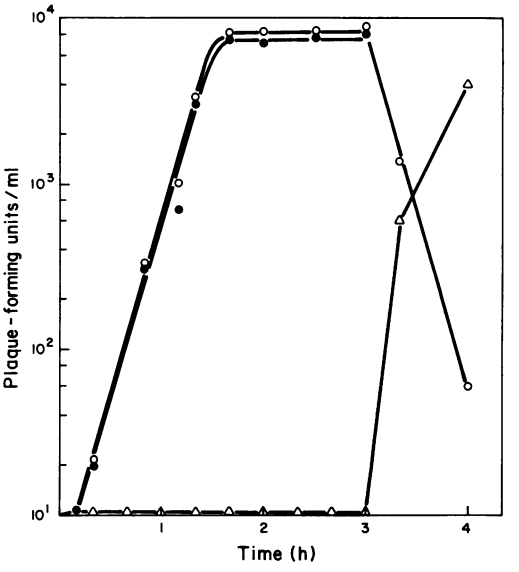

FIG. 1. Kinetics of the appearance of transfectants. Competent R6 cells (7×10^7 CFU/ml) were diluted (1:10) in C medium-phosphate supplemented with 0.5 mM $CaCl_2$. At time 0, phage Cp-1 DNA was added (final concentration, 5 μg/ml), and the culture was incubated at 30°C. At different times, 1-ml samples of the culture received DNase (10 μg), and incubation was continued for 5 min at 37°C (●). Another set of samples (○) received DNase (10 μg) and 0.1 ml of a dilution (1:100) of anti-Cp-1 serum and then were incubated at 37°C for 10 min, centrifuged (10,000 × *g*, 10 min), and resuspended in 1 ml of C medium-phosphate. Both sets of samples were assayed for transfectants. Samples of another portion of the culture were centrifuged (10,000 × *g*, 10 min) at different times, and the supernatants were tested for free phages (△).

Transfection of *S. pneumoniae* with Cp-1 DNA protein complex. Table 2 shows that transfection with Cp-1 DNA-protein complex was stimulated by calcium ions more than by magnesium ions when tested in CpH8 medium. Nevertheless, the optimal production of transfectants was achieved in the presence of both divalent ions in CpH8 medium from which the phosphate was totally or partially removed. Similar results have been found previously for transfection with Dp-4 DNA (30). The production of transfectants as a function of the time of incubation at 30°C in this medium is shown in Fig. 1. The infective centers observed in this experiment were not sensitive to treatment with anti-Cp-1 phage serum when added to the medium up to 180 min; in addition,

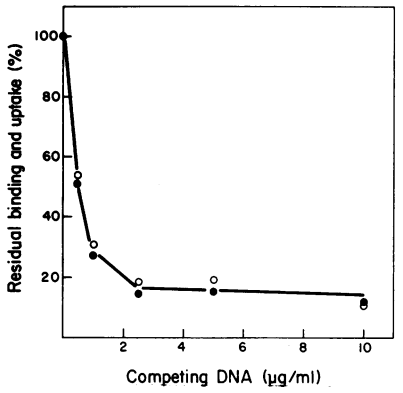

FIG. 2. Competition between phage Dp-4 DNA and phage Cp-1 DNA for binding and uptake to competent cells. Competent nuclease-defective mutant cells were incubated with 2.5 μg of [^{3}H]thymidine-labeled Dp-4 DNA per ml (specific activity, 6×10^4 cpm/μg) plus different amounts of protease-treated Cp-1 DNA for 45 min at 30°C. At this time, total (●) and DNase-resistant cell-associated Dp-4 DNA (○) was determined according to published procedures (30, 37).

no free phages were detected in the supernatants within 3 h after the DNA was added to the competent culture, indicating that the infected centers were due to plating of infected bacteria. The use of a long period of incubation to obtain a high level of transfectants is not new in the literature. Trautner et al. have previously found (43) that uptake of transfecting DNA is strongly temperature dependent, e.g., for SPP1 the plateau values were obtained at about 40 min at 37°C, whereas a period of 90 min was necessary at 30°C. On the other hand, the maximum uptake of DNA was achieved at 60 min, and the burst occurred about 90 min or more after the start of infection with SPO1 phage DNA (24). Furthermore, in our current studies on replication of bacteriophage Cp-1 DNA we have recently found (data not shown) that the incorporation of [^{3}H]thymidine into Cp-1-infected *S. pneumoniae* in the presence of 6-(*p*-hydroxyphenylazo)-uracil, which selectively inhibits host DNA synthesis (19), starts about 60 min after the culture is infected, and the liberation of the phage progeny appears at 180 min when experimental conditions similar to those described above for transfection are used.

Binding and entry of Cp-1 DNA. As shown above, protease-treated Cp-1 DNA completely lost the capacity to transfect. Nevertheless, this DNA was still an excellent competitor for binding and uptake of Dp-4 DNA (Fig. 2). Furthermore, the results summarized in Table 3 and 4 demonstrate the competition between double-stranded bacterial DNA and the transfecting DNA in transformation and in transfection, suggesting that the same receptors are used for the uptake of either DNA. Table 5 shows that [^{3}H]thymidine-labeled Cp-1 DNA either treated with proteinase K or untreated can be taken up by competent cells, suggesting that the irreversible incorporation of Cp-1 DNA was not affected by protease treatment. In contrast with the situation previously described for transfection with Dp-4 DNA, no difference in the transfec-

TABLE 3. Competition between bacterial and phage DNA in transformation[a]

Nucleic acid	Competing polynucleotide concn (μg/ml)	Transforming DNA concn (μg/ml)	Residual transformation (%)
None	None	0.05	100
Cp-1 DNA	0.5	0.05	16
	1.5	0.05	5
	5.0	0.05	3
Pneumococcal DNA	0.25	0.05	12
Dp-4 DNA	1.5	0.05	7
Micrococcus lysodeikticus DNA	1.5	0.05	6

[a] Competent R6 cells were incubated with mixtures of bacterial plus phage DNA at different relative concentrations. Transformation was assayed after 15 min of incubation (to limit cellular lysis caused by phage production), whereas transfection was tested after 90 min of incubation. Data in Fig. 1 clearly show that addition of DNase within 15 min of incubation of the bacteria with the phage DNA suppresses transfection. Thus, the low transformation cannot be due to the killing of potential transformants by phage, but rather it must represent a genuine competition of the bacterial and phage DNAs for a common step in cellular uptake.

TABLE 4. Competition between bacterial and phage DNA in transfection[a]

Nucleic acid	Competing polynucleotide concn (μg/ml)	Cp-1 phage DNA (μg/ml)	Residual transformation (%)
None	None	2.5	100
Pneumococcal DNA	0.5	2.5	28.2
	3.0	2.5	6.3
Micrococcus lysodeikticus DNA	0.2	2.5	78.2
	0.8	2.5	42.9
	2.0	2.5	22.3

[a] See Table 3, footnote *a*.

TABLE 5. Incorporation of ^{3}H-labeled Cp-1 DNA and Cp-1 DNA-protein complex in competent *S. pneumoniae* cells[a]

Sample	^{3}H-labeled DNA incorporated ($\mu g \times 10^3$ per 1.4×10^8 cells)[b]
Cp-1 DNA	5.8 ± 0.50
Cp-1 DNA-protein complex	6.0 ± 0.45

[a] A frozen culture of competent R6 strain (7×10^7 CFU/ml) was melted, centrifuged, and suspended in the same volume of C medium-phosphate supplemented with 2.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Portions (1 ml) of the cell suspension received ^{3}H-labeled Cp-1 DNA or Cp-1 DNA-protein complex (2 μg of DNA; specific radioactivity, 1.5×10^5 cpm/μg). After incubation at 30°C for 100 min, pancreatic DNase I (5 μg/ml) was added, and incubation was continued for 10 min at 37°C. Samples were chilled, centrifuged (10,000 rpm, 10 min), washed with cold medium three times, and precipitated with 10% trichloroacetic acid. The precipitate was collected on glass fiber filter disks (GF/A; Whatman), dried, and counted.

[b] Results show the mean ± the standard deviation for six experiments.

tion level was found when a nuclease-deficient mutant or the wild-type strain of *S. pneumoniae* was used as competent receptor of the Cp-1 DNA-protein complex (Table 6). Furthermore, when we analyzed the surface nuclease activity of a wild-type or a nuclease-defective strain on the donor ^{3}H-labeled Cp-1 DNA-protein complex, only moderate degradation was noted after a long incubation with either type of competent pneumococcal cell (Fig. 3C and D). It has been found previously that ^{3}H-labeled Dp-4 DNA is massively degraded during incubation with competent wild-type cells (30). In addition, when protease-treated Cp-1 DNA was analyzed for its reaction to the surface nuclease activity of both strains, the results (Fig. 3A and B) were similar to those obtained using the DNA-protein complex, indicating that the protein is not responsible for the protection of Cp-1 DNA against surface nucleases during this early step of the transfection process.

Dose-response analysis of the Cp-1 DNA-protein complex. Figure 4 shows transfection as a function of phage DNA concentration; saturation of the yield of transfectants appears at DNA concentrations of about 2 to 4 μg/ml. The response of pneumococcal transfection to increased concentration of Cp-1 DNA shows a curve with a slope less than 1 (Fig. 4), indicating a still unexplained negative cooperative effect similar to that recently described for transfection of protoplasts of lactic streptococci (10). A possible mechanism to explain the slope less than 1 can be found by analysis of the results shown in Fig. 5. When we subjected Cp-1 DNA to sedimentation in neutral sucrose gradients, ^{3}H-labeled Cp-1 DNA treated with proteinase K banded as a clear peak at the middle of the tube, which corresponded to an apparent $s_{20,w}$ of 22S, whereas the ^{3}H-labeled Cp-1 DNA-protein complex showed a remarkable degree of aggregation (Fig. 5A). The biological activity of Cp-1 DNA was detected throughout the gradient, although the bulk of the activity was recovered at the 22S, 30S, and 38S positions. Therefore, it is conceivable that this negative effect may be due to the large proportion of highly aggregated DNA, which shows a relatively poor transfection capacity (Fig. 5B). In turn, the negative effect depicted in Fig. 4 could be explained if we assume that the proportion of aggregates increases with increasing DNA concentration at the expense of the monomers. In an attempt to determine the dose response of individual Cp-1 DNA-protein complexes, we used two different procedures to eliminate or diminish the presence of aggregates. Cp-1 DNA-protein complex was treated with sodium dodecyl sulfate at 65°C for 5 min, the material was immediately passed through an agarose A-5m column, and the fractions were collected in tubes treated as previously described to avoid adherence of the collected material to the surface of the tubes (8). In these conditions, the recovery of the DNA-protein complex was greater than 90% of the input (data not shown). Determination of the concentration dependence in transfection with this DNA showed a dose response of about 1 (Fig. 6A). Analysis of the collected material in sucrose gradients revealed that this material was recovered mainly as monomeric forms of the DNA-protein complex (Fig. 6B).

We have observed that when a high concentration of salt (1 M NaCl, SSC, etc.) was used during storage of the DNA-protein complex, the formation of highly aggregated DNA was more dramatic than in the experiment shown in Fig. 5A (data not shown). The analysis in neutral sucrose gradients of a DNA-protein complex obtained by phenolizing a phage stock at a low

TABLE 6. Transformation and transfection of wild type and the nuclease-defective mutant[a]

Competent cell	Transformants per ml	Transfectants per ml	
		Cp-1 DNA	Dp-4 DNA
R6	2.0×10^6	7.1×10^3	2.8×10^2
end 1, *exo* 2	1.8×10^6	6.4×10^3	5.8×10^4

[a] Transformation and transfection with Dp-4 DNA was carried out as previously described (31). Transfection with Cp-1 DNA was done as described in Table 1, footnote *a*.

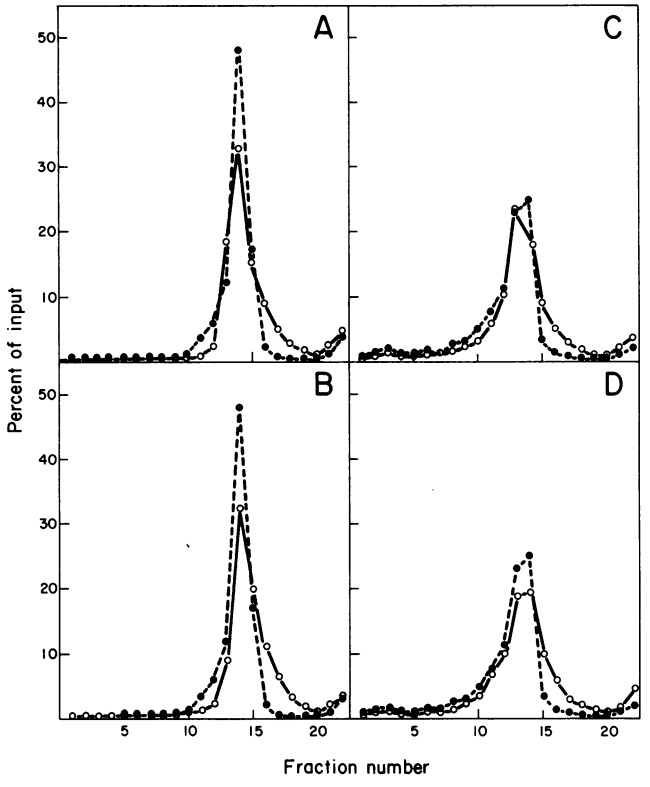

FIG. 3. Surface nuclease activity of the wild type (B and D) and the nuclease-defective mutant (A and C). Competent cultures were incubated with 2 μg of ^{3}H-labeled Cp-1 DNA-protein complex per ml (1.5×10^5 cpm/μg) (○, in C and D) or with protein-free, ^{3}H-labeled Cp-1 DNA (1.2×10^5 cpm/μg) (○, in A and B) under the standard conditions described in the text for 90 min at 30°C. The samples were centrifuged (10,000 × *g*, 10 min), and the supernatant solutions were analyzed by neutral sucrose gradient centrifugation (5 to 20%, 40,000 rpm, 120 min, at 20°C in an SW50.1 rotor). Fractions of 15 drops were collected from the bottom of the tubes. The sedimentation profile of control DNA, i.e., purified Cp-1 DNA-protein complex (●, in panels C and D) or protease-treated Cp-1 DNA (●, in panels A and B), is also shown.

concentration of phage (about 10^{11} PFU/ml) in the presence of 0.05× SSC during the purification procedure showed that most of the material banded at or near the position that corresponded to the monomeric forms of Cp-1 DNA (Fig. 7A). To exclude the influence of small amounts of dimers or trimers, which were the fractions that showed the highest specific activity in the transfections obtained in Fig. 5, we carried out an experiment similar to that shown in Fig. 5 in which the fractions of the gradient which corresponded to the monomeric forms of the DNA (fractions 13 and 14) were pooled and immediately used to transfect competent cells. The dose response obtained in these conditions (Fig. 7B) corresponded to a slope of about 1, similar to that depicted in Fig. 6A.

DISCUSSION

The biological importance of certain proteins complexed with DNA is a subject of current interest. In procaryotic cells, only the DNAs of the small *Bacillus* phages have been determined to contain a protein covalently linked to the DNA that plays a basic role in transfection (1, 13, 14). Protein-stimulated transfection has been observed in other viral systems (7). Protein covalently linked to the 5′ ends (6) of the DNA of adenovirus renders this DNA more infective than protease-treated adenovirus DNA in the transfection of HeLa cells (39). The presence of a protein in the DNA of Cp-1 seems to be necessary for the biological activity of this DNA (Table 1). Results obtained in this laboratory (9a) demonstrate that this protein is covalently linked to the DNA and has an apparent molecular weight of 28,000.

The importance of calcium ions for transfection of intact competent cells of *Streptococcus* species has been documented (23, 30). Calcium ions are also required for uptake of prebound transforming DNA in *S. pneumoniae* (38). The transfection system described here is another

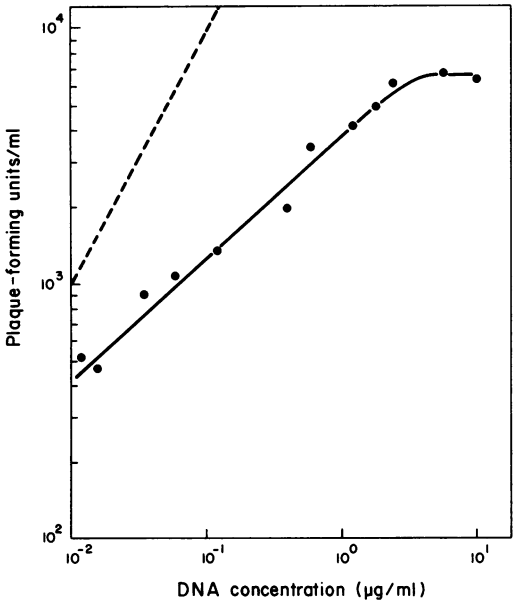

FIG. 4. Dose response of Cp-1 DNA-protein complex. Phage Cp-1 DNA (0.1 ml) at various dilutions was incubated with 0.9 ml of transfection medium containing competent R6 cells at 30°C under the standard conditions described in the text. After addition of DNase, appropriate dilutions were plated to test for transfection. The dashed line corresponds to a slope of 1.

example of the stimulatory effect of calcium ions (Table 2) probably favoring the entry of a negatively charged macromolecule (DNA) into the pneumococcus (2).

The infectivity of Cp-1 DNA previously treated with proteinase K was drastically reduced (Table 1). Hirokawa (13) originally suggested a role for the protein bound to ϕ29 DNA in the uptake of the DNA by competent cells. On the other hand, Arwert and Venema (1) have found that uptake of GA-1 DNA was not affected by protease treatment. More recently, several reports have demonstrated that the protein bound to ϕ29 DNA is essential for the replication of the DNA (16, 21, 25, 44). Cp-1 DNA treated with proteinase K was still able to compete for binding and uptake with [^{3}H]thymidine-labeled DNA obtained from the pneumococcal phage Dp-4 (Fig. 2). This DNA also competes with the homologous pneumococcal DNA for transformation and for transfection (Tables 3 and 4). In addition, the use of radioactive Cp-1 DNA treated with proteinase K demonstrated that this DNA was incorporated by competent pneumococci as efficiently as the Cp-1 DNA-protein complex (Table 5). These results strongly suggest an essential role for the protein bound to Cp-1 DNA in the intracellular development of bacteriophage Cp-1, i.e., in the replication of the DNA or protecting it from being degraded by host nucleolytic enzymes as in the case of bacteriophage T4 infection, in which the product of gene 2 is injected in noncovalent association with the phage genome and protects it from degradation by exonuclease V (40).

Phage ϕ29 transfection has been characterized as exhibiting a linear dose response (15, 27). This response is predominantly caused by multimolecular, protease-sensitive aggregates of DNA that interact with a competent cell to produce a transfectant (9a). In addition, a small class of biologically active DNA molecules which sediment at the position of monomeric ϕ29 DNA shows a quadratic dose response. Geis (10) has found that the transfection curve of protoplasts of *Streptococcus lactis* shows a slope less than 1. Such an unexplained negative cooperative effect also occurred in our experimental system when the donor Cp-1 DNA-protein complex was obtained at relatively high concentration in the presence of SSC (Fig. 4). The results shown in Fig. 5A and B demonstrate a poor biological activity of highly multimeric forms of Cp-1 DNA in remarkable contrast with

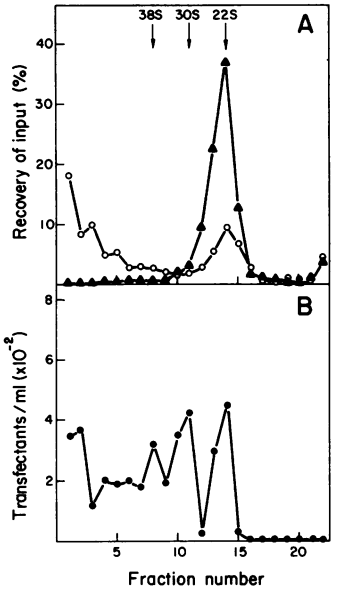

FIG. 5. Sedimentation of Cp-1 DNA-protein complex. [^{3}H]thymidine-labeled Cp-1 DNA-protein complex (0.15 ml, 260 μg/ml) was layered on the top of a neutral sucrose gradient (cushion of 0.6 ml of 60% sucrose; 4.3 ml of 20 to 5% sucrose) and centrifuged in an SW50.1 rotor at 40,000 rpm for 120 min at 20°C. Fractions (0.2 ml) were collected from the bottom of the tubes and (A) used to determine radioactivity (○) and (B) assayed for transfection (●). Protease-treated Cp-1 DNA was included in a parallel tube as a control (▲).

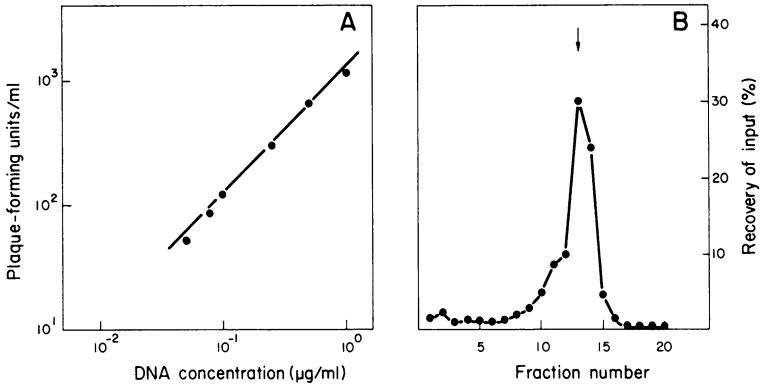

FIG. 6. Dose response and sedimentation analysis of DNA-protein complex treated with sodium dodecyl sulfate. [^{3}H]thymidine-labeled Cp-1 DNA-protein complex (0.35 ml, 260 μg/ml) was treated with 2% sodium dodecyl sulfate for 5 min at 65°C and passed through a column of agarose A-5m (0.9 by 25 cm) and eluted with 0.05× SSC. One-milliliter fractions were collected. A portion of the tube where the maximum (about 20% of the input) was detected was used to test the dose-response transfection (A). Another portion was analyzed in a neutral sucrose gradient to determine radioactivity (B) under the conditions described in the legend to Fig. 5. The arrow indicates the position where protease-treated Cp-1 DNA sedimented.

the situation previously described for ϕ29 DNA where the bulk of the biological activity of this DNA was found at the bottom of the sucrose gradient (15). The maxima of infectivity for transfection were recovered at the 22S, 30S, and 38S positions (Fig. 5). The specific activity of DNA was higher at 38S and 30S than at 22S.

We developed conditions that practically eliminated the multimeric forms of the Cp-1 DNA-protein complex from our preparation. The complex obtained corresponded (mainly) to monomeric forms of Cp-1 DNA when analyzed in neutral sucrose gradients (Fig. 6B). In our hands, transfections showing a linear dose response were always obtained when this complex was used as donor DNA (Fig. 6A). This experimental approach did not exclude completely the possibility that dimers could be present. Nevertheless, when we used the fractions obtained in the gradient shown in Fig. 7, which correspond to the position where the monomeric forms band, the dose response showed a slope of about 1.

Barany and Tomasz (3) have demonstrated that monomeric plasmids exhibit transforming activity in *S. pneumoniae*, whereas in *B. subtilis* these conformers are inactive (5). Monomeric plasmid transformation has been confirmed in *S. pneumoniae* and in *Streptococcus sanguis* (4, 34). Plasmid transformation by monomers may represent the entry of molecules that escaped nicking or degradation by surface-bound or ex-

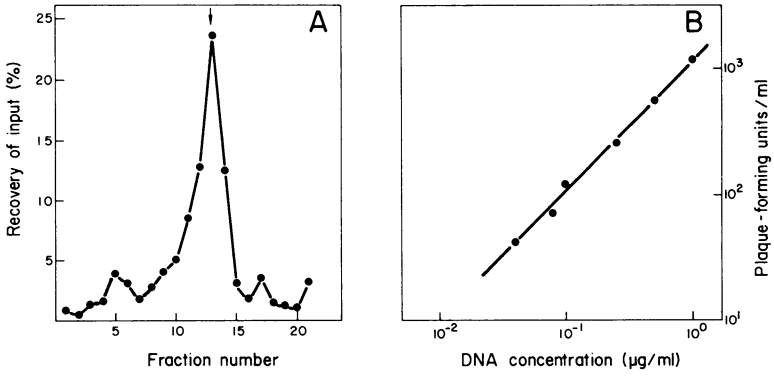

FIG. 7. Sedimentation analysis of DNA-protein complex obtained at low concentration and dose response of the monomeric forms. [^{3}H]thymidine-labeled Cp-1 in 0.05× SSC was phenolized under the conditions described in the text. The DNA-protein complex (24 μg) was centrifuged through a neutral sucrose gradient (A), and fractions 13 and 14 were pooled and immediately tested for dose-response transfection (B). The arrow indicates the position where the monomeric forms banded.

creted nucleases. Barany and Tomasz (3) have pointed out that this protective effect in the pneumococcal system, in contrast with the *B. subtilis* transformation, could be related to the use of nuclease-defective recipients and certain transformation conditions. In fact, a remarkable protection of the transfecting Dp-4 DNA against the nucleases when competent nuclease-defective pneumococci were used as receptor cells has been demonstrated previously (30). These results have been recently confirmed and extended by Goscin and Guild (11), who found that an endonuclease-defective mutant of *S. pneumoniae* with normal levels of transformability showed a higher transfection than the wild type at low DNA concentrations. In addition, these authors also suggest that, in this system, protamine sulfate enhances transfection by inhibition of the surface nuclease action that is part of the normal entry process. This is in agreement with a previous finding that a higher protection of ^{3}H-labeled Dp-4 DNA against the surface nucleases parallels a remarkably higher level of transfection (30).

Cp-1 DNA-protein complex transfected with equal efficiency competent wild-type and nuclease-defective pneumococci (Table 6), and in this case bound and adsorbed DNAs were remarkably protected in strain R6 as well as in the nuclease-defective mutant (Fig. 3). In addition, Cp-1 DNA showed a wide pattern of resistance to many restriction enzymes, including those present in pneumococcal strains (29), i.e., *Dpn*I and *Dpn*II. Analysis of the nucleotide sequence of the termini of Cp-1 DNA shows a terminal inverted repetition of 236 nucleotides as well as a homology of 97.7% within the first 352 nucleotides (C. Escarmis, A. Gomez, E. García, C. Ronda, R. López, and M. Salas, manuscript in preparation). Few known restriction enzyme recognition sequences have been found in these regions of the DNA, suggesting that this DNA may have lost the restriction sites for many endonucleases as previously reported for T7 (32). On the other hand, transformation of *S. pneumoniae* by single-stranded plasmid-phage hybrid DNA without marker rescue has been recently demonstrated (2). Under these conditions, 12 kilobases of single-stranded DNA can enter the pneumococcus intact and can also replicate to form double-stranded DNA.

All these data support the idea that a linear response in the transfection of pneumococci by the Cp-1 DNA-protein complex could represent entry of a single-stranded molecule (18 kilobases) which escaped nicking or degradation by nucleases. The opposite strand would then be formed by annealing with smaller fragments or by DNA synthesis or both as previously suggested for plasmid transformation, using either covalently closed circular or linear monomers (2, 3). It has been recently suggested (22) that in *B. subtilis* monomeric forms of plasmids containing repeats of 260 to 2,000 base pairs can transform competent cells. The presence of long repetitions at the ends (at least) of Cp-1 DNA may also account for the dose response described above when the monomeric forms of Cp-1 DNA were used in transfection. Another possibility would be the uptake, in our transfection conditions, of some molecules of double-stranded DNA as proposed for chromosomal transformation in *S. pneumoniae* (28) and more recently in *B. subtilis* (9). In fact, the analysis of DNA taken up by competent pneumococci has shown the presence of 20% double-stranded DNA (17), although the possibility that this DNA was the result of annealing of separate strands cannot be ruled out at present. Preliminary results obtained in this laboratory show that other pneumococcal phages (Cp-9 and Cp-10) with morphologies similar to Cp-1 (29) but differing in the structural proteins present in the mature viral particles as well as in the enzymatic restriction patterns also show the presence of a protein tightly bound to its DNAs. The use of these complexes in transfection would probably be of great help in our current studies on the uptake of these DNAs.

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