Initiation of Infection by Deoxyribonucleic Acid From Bacteriophage λ

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ABSTRACT

Kinetic studies conducted on the early stages of infection of *Escherichia coli* K-12 by deoxyribonucleic acid (DNA) isolated from bacteriophage λ indicate a rapid adsorption of the phage DNA to receptor sites at the bacterial surface prior to deoxyribonuclease-insensitive incorporation. A direct relationship found between the number of DNA molecules adsorbed per bacterium and the multiplicity of helper phage infection indicates a requirement for helper function during the attachment process. An apparent lack of attachment specificity with regard to the source of the DNA preparation, to the size of the inhibiting fragment, to the base ratio of the inhibiting DNA molecule, and to "cohesive" ends suggests a nonspecific interaction between the infectious DNA and the sites of helper phage attachment.

Deoxyribonucleic acid (DNA) isolated from bacteriophage λ can infect *Escherichia coli* K-12 in the presence of adsorbed helper phage (11, 12). The manner in which λ DNA enters the cell and the possible role of helper in the penetration process is still not clear. Kinetic studies demonstrate an initial lag of 55 to 80 sec for the phage DNA to become deoxyribonuclease-insensitive at 33 C (1). The lag period is not dependent on the concentration of either helper-infected bacteria or λ DNA.

The present studies were undertaken to determine the possible role of helper phage during the penetration of λ DNA. Experiments concerning the initial stages of infection are presented and discussed in terms of a kinetic model.

MATERIALS AND METHODS

Media. Plating bacteria were grown in 1.0% tryptone broth (Difco) with 0.5% NaCl (TB medium). Bacteria to be used as recipients for λ DNA infection were grown in H medium (11) supplemented with 5 mg/ml of glucose. The agar derivatives of TB medium consisted of TB soft agar (0.7% agar) and TB plate agar (1.0% agar).

Bacteria. *E. coli* K-12 W3104, a galactose-negative mutant deficient in galactose-1-phosphate uridyl transferase, was used as an indicator strain for infectivity assays. *E. coli* K-12 W3104 lysogenic for either λ i⁴³⁴ or λ i^h was used as a selective indicator strain during the plating of the assay mixtures to prevent plaque forma-

tion by the particular helper phage used for the infectivity assay. *E. coli* K-12 C600 and *Bacillus subtilis* SB-19 were used as sources of bacterial DNA.

Bacteriophage. Phages λ i^h and λ i⁴³⁴ were obtained by ultraviolet induction from their lysogenic derivatives of *E. coli* K-12 W3104. The phage stocks were purified by CsCl density gradient centrifugation according to the procedure described by Hogness and Simmons (9).

Preparation of phage and bacterial DNA. Infectious phage DNA was prepared by aqueous phenol extraction according to the procedure described by Kaiser and Hogness (12).

Bacterial DNA from *B. subtilis* was isolated by the technique described by Bresler (3).

The DNA samples were dialyzed against 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 7.1), MgSO₄, and CaCl₂ (TCM) before use.

Breakage of phage DNA. Solutions of λ DNA at an optical density equal to 0.2 were stirred in a 100-ml beaker at a volume of 20 ml. The stirring apparatus consisted of a stirring shaft, aligned with the axis of the beaker, with a propeller consisting of a VirTis 4.0-cm blade having a razor-edge on one side. This shaft was driven by a Heller GT21 motor whose speed could be varied or held constant. The stirring temperature was kept near 24 C.

The breakage of the phage DNA was followed by determining the viscosity of the DNA solutions at 30.00 $\pm$ 0.01 C with a capillary viscometer (9).

Polymerase-catalyzed reaction. *E. coli* DNA polymerase, a λ phosphocellulose fraction separated from exonuclease III by acrylamide gel electrophoresis, was a generous gift of A. Kornberg. This preparation had an activity of 4,200 dAT units/ml.

For the inactivation of the biological activity of λ DNA by DNA polymerase, 5 μ g/ml of λ DNA was

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incubated at 15 C for the reported time intervals with 0.5 μ mole each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate, 100 μ moles of Tris-chloride buffer (pH 7.1), 10 μ moles of $MgCl_2$, 5 μ moles of 2-mercaptoethanol, and 22 units of DNA polymerase, in a total volume of 1 ml. After each reaction mixture was incubated for the desired time interval, it was heated to 70 C for 5 min to denature the enzyme.

Infectivity assay. The biological assay for λ DNA was similar to the procedure described by Radding and Kaiser (17). A culture of recipient bacteria was grown to a density of 8×10^8 cells per milliliter in H medium supplemented with 5 mg/ml of glucose. The bacteria contained in 10-ml samples were sedimented and resuspended in 5 ml of 0.01 M Tris-chloride buffer (pH 7.1) and 0.01 M $MgSO_4$ (TM) at 0 C. Helper phage were added at the desired multiplicity, and the samples were incubated for 15 min at 37 C. This treatment was followed by sedimentation and resuspension of the bacteria in cold TCM. After keeping this suspension at 0 C for 60 to 120 min, 0.2 ml of helper-infected bacteria was added to samples containing 0.2 ml of properly diluted DNA, and the samples were incubated at 30 C without shaking for time intervals ranging from 5 to 10 min. To stop the reaction, pancreatic deoxyribonuclease was added to a final concentration of 10 μ g/ml and the samples were chilled to 0 C. The samples were then mixed with 2 ml of soft agar containing 0.1 ml of the selective indicator strain at a density of 10^9 cells per milliliter, and the mixtures were poured over TB agar plates. After incubation overnight at 37 C, the plates were scored for plaques produced by the infectious DNA of either genotype i^h or i^{434} .

RESULTS

DNA dose-response curves. To determine a possible mechanism for the reaction between λ DNA and competent bacteria, samples of recipient bacteria were infected with low multiplicities of helper phage and exposed to increasing concentrations of infectious DNA. The time of exposure to DNA was held constant and kept sufficiently brief to ensure that the number of plaque-forming units (PFU) formed during the time interval was a measure of the initial velocity.

When bacteria infected with helper phage at multiplicities of either 5×10^{-2} or 5×10^{-3} are incubated with high concentrations of λ DNA, saturation of the measured rate of infection occurs (Fig. 1). Although the rate of infection is directly proportional to the helper-infected bacterial density, the concentration of DNA required to saturate the rate appears to remain constant, regardless of the concentration of either total bacteria or adsorbed helper.

In terms of permeability, such saturation curves are familiar forms describing a unidirectional flux through a permeability barrier utilizing sites

FIG. 1. Effect of λ DNA concentration on the rate of infection. Symbols: $\square$, helper multiplicity = 5×10^{-3} , bacteria undiluted; $\circ$, helper multiplicity = 5×10^{-2} , bacteria diluted 10-fold before incubation with DNA; $\bullet$, helper multiplicity = 5×10^{-2} , bacteria diluted 10-fold after incubation with DNA.

of limited capacity. The reaction can be interpreted in terms of a rapid adsorption of the phage DNA to fixed receptor sites at the bacterial surface, followed by a slow irreversible penetration into the bacterium (1). Since the infectivity resides in a single molecule of λ DNA, the reaction sequence can be written as

The proposed reaction sequence can be treated in a manner analogous to a conventional enzyme-substrate reaction (7). The rate of formation of the complex, $d\varphi/dt$, will then equal $k_1(d)(b) - k_{-1}\varphi - k_2\varphi$, and the rate of infectivity, $d(p)/dt$, will equal $k_2\varphi$.

At high DNA concentrations, the concentration of unbound DNA, (d) , can be taken as the total DNA concentration, (D) , if the concentration of helper-infected bacteria is small in comparison.

At rate-limiting bacterial densities, $(b) = (B) - \varphi$, where (B) is the total bacterial concentration.

If the time of exposure to DNA is held constant and kept sufficiently brief, the PFU formed during the measured time interval can be used as an estimate of the initial velocity. The maximal PFU formed at saturating DNA concentrations, P_a , will then be proportional to the maximal velocity. Applying a steady-state approximation to the concentration of the DNA-bacterial complex (7), the PFU formed during the time interval can then be expressed as

$$p = P_d \frac{(D)}{K + (D)} \quad (1)$$

where $K = (k_2 + k_{-1})/k_1$.

At high helper-infected bacterial densities and rate-limiting λ DNA concentrations, $(d) = (D) - n\phi$, where n is equal to the average number of DNA molecules adsorbed per bacterium, being directly proportional to the average number of receptor sites. Applying the same steady-state treatment as in equation 1, the PFU formed at high bacterial concentrations can be expressed as

$$p = P'_b \frac{(B)}{K' + (B)} \quad (2)$$

where $P'_b = P_b/n$ and is the maximal PFU formed at saturating bacterial densities with n receptor sites. K' will be equal to K/n .

Determination of the kinetic parameters. As shown by rearrangement of equation 1, a Lineweaver-Burk plot of $1/p$ versus $1/(D)$ will yield extrapolated values for both K and P_d . In the graph shown in Fig. 2, an application of this plot yields an extrapolated value for P_d equal to 6.7×10^2 PFU. The extrapolated value for K is $0.33 \mu\text{g/ml}$ or 6×10^9 molecules/ml, assuming a molecular weight for λ DNA of 3.3×10^7 (15).

Applying a reciprocal plot of equation 2, extrapolated values for P'_b and K' can be obtained. An application of this plot is shown in Fig. 3. At a multiplicity of helper phage infection of 0.1, a bacterial density of 4×10^9 per milliliter was not sufficient for saturation of the rate of infection. This is the result expected if λ DNA could adsorb to the surface of helper-infected bacteria only. At multiplicities of 5 and 10, saturation of the rate occurred. The extrapolated values for P'_b at the multiplicities of 5 and 10 are 6.7×10^2 PFU and 2.5×10^2 PFU, respectively. The values for K' at the multiplicities of 5 and 10 are 1.7×10^9 bacteria per milliliter and 8.3×10^8 bacteria per milliliter, respectively.

Estimation of the number of receptor sites. Since the kinetic parameters are for the same reaction, i.e., the infection of a bacterium by λ DNA, and are determined by the PFU formed during the same time interval, P_d should equal P_b when the rate-limiting concentrations of DNA and bacteria are equal. Therefore, the ratios K/K' and $P_d(D)/P'_b(B)$ should yield independent estimates of the number of receptor sites per bacterium. Using the values obtained for the kinetic parameters at both DNA and bacterial saturating conditions, estimates of the number of sites were obtained (Table 1). The values calculated from the ratios of both parameters at each multiplicity of helper infection

FIG. 2. Reciprocal plot of the rate of infection versus the λ DNA concentration. The helper-infected bacterial density = 8×10^6 per milliliter. The reaction mixtures were incubated for 10 min prior to the addition of deoxyribonuclease.

FIG. 3. Reciprocal plot of the rate of infection versus the bacterial concentration. The λ DNA concentration = $1.5 \times 10^{-3} \mu\text{g/ml}$. The reaction mixtures were incubated for 10 min prior to the addition of deoxyribonuclease. Symbols: $\square$, helper multiplicity = 0.1; $\circ$, helper multiplicity = 5; $\bullet$, helper multiplicity = 10.

appear to be in reasonable agreement and approximate the average number of helper phage adsorbed per bacterium. This result is expected if adsorbed helper acted in some manner as an attachment and penetration site during the infection process.

Effect of helper multiplicity on the rate of infection. If helper phage does act as an attachment site for λ DNA, the experimental values for both P_b and K should decrease upon an increase in the multiplicity of helper phage infection, the multiplicity being directly proportional to n . Since rearrangement of equation 2 yields

$$P_b/p = n + K/(B) \quad (3)$$

a plot of $1/p$ versus the multiplicity of helper phage infection should yield a straight line that crosses the abscissa where $K = -n(B)$. In Fig. 4,

TABLE 1. Estimates of the number of attachment sites

Multiplicity of infection	Ratio ^a	Estimate of sites
5	$K/K' = 6 \times 10^9 / 1.7 \times 10^9$	4
	$P_a(D)/P_b'(B) = \frac{(6.7 \times 10^3)(3 \times 10^7)}{(6.7 \times 10^2)(8 \times 10^6)}$	4
10	$K/K' = 6 \times 10^9 / 8.3 \times 10^8$	7
	$P_a(D)/P_b'(B) = \frac{(6.7 \times 10^3)(3 \times 10^7)}{(2.5 \times 10^2)(8 \times 10^6)}$	10

^a The values of K and (D) are given in DNA molecules per milliliter.

a plot of equation 3 is shown for multiplicities of infection ranging from 4 to 14. Since the growth of the recipient bacteria is inhibited at multiplicities higher than 14 (12), this multiplicity was considered a maximal level. From the plot, a value of 5×10^9 bacteria per milliliter was obtained for K , in reasonable agreement with the value obtained in Fig. 2. These results indicate that, if helper phage does function as an attachment site, it acts in an independent manner during the attachment process.

Competition for attachment sites. When two DNA molecules compete for the same site, inhibition of the rate of attachment of either component should occur. If the biological activity resulting from the penetration of only λ DNA is measured, the value of K should increase by a factor $(1 + (I)/K_I)$, where (I) is the concentration of the inhibiting DNA and K_I is the inhibition constant.

To test the specificity of the sites to heterologous DNA preparations, calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J.) was used as an inhibitor. Inhibition of the production of PFU by $5 \mu\text{g/ml}$ of calf thymus DNA is shown in Fig. 5. The inhibition is competitive and from the graph, a value of $0.3 \mu\text{g/ml}$ was obtained for its inhibition constant.

To test the effect that the base ratio may have on the binding specificity of the attachment sites, *B. subtilis* DNA, having a guanine-cytosine content of 0.42 (14), was used as an inhibitor of infection by λ DNA. Inhibition of PFU production by $2.5 \mu\text{g/ml}$ of *B. subtilis* DNA is shown in Fig. 5. The inhibition is competitive and, from the graph, a value of $0.4 \mu\text{g/ml}$ was obtained for K_I .

In other studies, inhibition did occur in the presence of DNA isolated from *E. coli* K-12 C600; however, heat-denatured λ DNA at a concentration of $6 \mu\text{g/ml}$ and yeast ribonucleic acid (Worthington Biochemical Corp.) at a concentration of $100 \mu\text{g/ml}$ failed to show any measurable activity as inhibitors.

Effect of shear on attachment specificity. Mole-

FIG. 4. Effect of helper multiplicity on the reciprocal rate of infection. The λ DNA concentration = $10^{-3} \mu\text{g/ml}$ and the helper-infected bacterial density = 4×10^9 per milliliter. The reaction mixtures were incubated for 10 min prior to the addition of deoxyribonuclease.

FIG. 5. Reciprocal plot of the inhibition of the rate of infection with DNA from *Bacillus subtilis* and calf thymus. The reaction mixtures were incubated for 7 min prior to the addition of deoxyribonuclease. Symbols: $\circ$, λ DNA only; $\bullet$, λ DNA with $2.5 \mu\text{g/ml}$ of *B. subtilis* DNA; $\square$, λ DNA with $5 \mu\text{g/ml}$ of calf thymus DNA.

cules of DNA isolated from bacteriophage λ can be broken when exposed to hydrodynamic shear (11, 17). Viscosity measurements indicate quantized breaks at the appropriate stirring speeds, producing fragment populations with a length

about the population center corresponding to half and quarter molecules (9). Stirring preparations of λ DNA, of genotype i^{434} , resulted in a loss in their reduced viscosity, η_{sp}/C (Fig. 6). After stirring at a rate of 1,200 rev/min for 50 min, the viscosity of the DNA preparation decreased to a plateau value of about 55 dl/g. Upon increasing the rate of stirring to 2,800 rev/min, a new plateau value of η_{sp}/C is reached, equal to about 30 dl/g. The first and second plateau values of η_{sp}/C correspond to values expected for preparations of half and quarter molecules, respectively.

The infectivity of the phage DNA decreased rapidly with shear. The biological activity remaining in the half-molecule preparation, when tested for PFU, efficiency, was 6.7×10^{-2} of the infectivity of unbroken DNA, whereas the fraction of the original infectivity remaining in the quarter-molecule preparation was only 1.5×10^{-3} .

To test the specificity of the receptor sites to molecular size, the inhibition of the rate of i^λ infection with whole-, half- and quarter-molecule preparations was determined. In Fig. 7, a plot of the ratio of uninhibited to inhibited production of PFU, p/p_1 , versus the concentration of inhibiting DNA is shown. At a concentration of infectious DNA equal to 0.25 $\mu\text{g}/\text{ml}$, whole-, half- and quarter-molecule preparations inhibited the rate of infection with approximately equal efficiency. This result suggests a lack of any strict dependence of attachment specificity on the size of the DNA molecule, regardless of its capacity to produce plaques.

Effect of polymerase-catalyzed inactivation on attachment specificity. DNA polymerase can catalyze the repair of single-stranded, 5' hydroxyl (or 5' phosphate)-terminated ends of molecules of DNA isolated from phage λ , resulting in a loss of infectivity (18). Incubation of λ DNA of genotype i^λ at 15 C with DNA polymerase and the

FIG. 6. Effect of shear on the reduced viscosity of λ DNA, genotype i^{434} . Symbols: $\circ$, stirring speed = 1,200 rev/min; $\bullet$, stirring speed = 2,800 rev/min.

FIG. 7. Fractional inhibition of the rate of i^λ infection with sheared DNA of genotype i^{434} . The infectious DNA concentration = 0.25 $\mu\text{g}/\text{ml}$. The reaction mixtures were incubated for 5 min prior to the addition of deoxyribonuclease. Symbols: $\circ$, unbroken inhibiting DNA; $\bullet$, inhibiting DNA sheared for 50 min at 1,200 rev/min; $\square$, inhibiting DNA sheared for 50 min at 2,800 rev/min.

FIG. 8. Polymerase-catalyzed inactivation of the infectivity of λ DNA.

four deoxyribonucleotide triphosphates leads to a rapid loss in the initial rate of infection (Fig. 8). As shown by the graph, only 2.6×10^{-4} of the initial infectivity remains after 25 min of incubation.

To determine whether the loss in infectivity is due to an inability to interact with receptor sites, inhibition studies were conducted, with the use of the DNA samples previously inactivated by DNA polymerase for the inhibition of the rate of infection by DNA of genotype i^{434} . In Fig. 9, a plot of the ratio of uninhibited to inhibited production of PFU, p/p_1 , in the presence of 1.5 $\mu\text{g}/\text{ml}$ of DNA previously incubated with DNA polymerase for the indicated time intervals is shown. If the binding specificity of the molecule is dependent on intact single-stranded ends, the inhibition should decrease rapidly, approaching a value for p/p_1 equal to one. As shown by the graph, the

FIG. 9. Fractional inhibition of the rate of i^{434} infection versus the time of incubation of the inhibiting DNA of genotype i^h with DNA polymerase. The infectious DNA concentration = 0.25 $\mu\text{g}/\text{ml}$. The inhibiting DNA concentration = 1.5 $\mu\text{g}/\text{ml}$. The reaction mixtures were incubated for 5 min prior to the addition of deoxyribonuclease. The broken line represents the fractional inhibition observed with concentrations of biologically active, inhibiting DNA equivalent in plaque-forming capacity to 1.5 $\mu\text{g}/\text{ml}$ of DNA incubated with polymerase for 25 min.

binding specificity of the inactivated DNA was not significantly altered, as expected if the specific ends are not important for attachment to receptor sites.

DISCUSSION

The kinetic studies presented in this report indicate that some form of interaction between λ DNA and helper phage initiates the infection of competent bacteria. The stoichiometric relationship found between the number of bound DNA molecules and the multiplicity of helper phage infection suggests that the interaction occurs at the cell wall level.

The mechanism for the helping function during the initial infection process is difficult to assess. The simplest hypothesis one may entertain about this function would be a localized, enzymatic degradation of the cell wall of *E. coli*, to provide a hole through which the DNA can pass. The infection process would then be a unidirectional flux of DNA through the cell membrane at the sites of phage attachment. Support for this hypothesis stems largely from reports of spheroplast infection by isolated preparations of λ DNA without the requirement of helper phage (16). Although little evidence has previously been presented that would refute this hypothesis, Barnhart (1) reported that uncouplers of oxidative phosphorylation could inhibit the incorporation of λ DNA. This report suggests that energy-requiring processes are necessary and that the penetration of λ DNA in the "free" form is more than just a nonspecific flux through a permeability barrier.

Since the mechanism for penetration could not be determined by the techniques employed in this study, the results of the kinetic studies suggest two possibilities for helper function during the initial infection process: (i) active participation during both attachment and penetration of λ DNA and (ii) participation during attachment only, facilitating the uptake of DNA, possibly by partial degradation of the cell wall to uncover fixed receptor sites on the membrane of the bacterium. The apparent lack of attachment specificity with regard to the source of the DNA preparation, to the size of the inhibiting fragment, to the base ratio of the inhibiting molecule, and to "cohesive" ends suggests a nonspecific interaction of the infectious DNA with the sites of helper phage attachment, possibly coulombic in nature. At least for the experimental conditions employed in this study, the DNA molecule would have to exist in a double-stranded configuration for interaction to take place.

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