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Transfection of *Escherichia coli* Spheroplasts

V. Activity of *rec*BC Nuclease in *rec*$^+$ and *rec*$^-$ Spheroplasts Measured with Different Forms of Bacteriophage DNA

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The in vivo activity of the *rec*BC nuclease was assayed by transfection of isogenic *rec*$^+$ and *rec*$^-$ spheroplasts with bacteriophage DNA of various origin and structure. The results indicate that the *rec*BC nuclease can limit transfection at several stages during the production of an infective center; such limitations depend primarily on whether the DNA is in, or assumes, a nuclease-sensitive structure. The first stage of limitation can occur when a nuclease-sensitive transfecting molecule enters the spheroplast. Other potential limitation points occur during replication and maturation of the bacteriophage DNA. The initial stage can be bypassed by using *rec*BC nuclease-resistant molecules such as circular forms. Through analysis of results with other DNA structures, we found that in vivo the effects of the double-strand exonucleolytic activity of the *rec*BC nuclease predominated. The effects of the single-strand nuclease activities seem to be modified from those observed for the purified enzyme in vitro (Karu et al., 1974). Inside the cell, the single-strand exonuclease activity is very weak and the single-strand endonuclease activity is abolished almost completely.

Bacterial nucleases can greatly limit the efficiency of transfection and transformation of competent cells. In *Escherichia coli*, the most destructive seems to be the nuclease complex encoded by the *rec*B and *rec*C genes (10, 29, 30, 41, 43).

The *rec*BC nuclease has at least four activities demonstrable in vitro: an ATP-dependent double-strand exonuclease; an ATP-dependent single-strand exonuclease; an ATP stimulated single-strand endonuclease; and an ATPase (15, 27, 28, 47). This enzyme complex appears to degrade single- and double-stranded DNA in both the 3′ to 5′ and the 5′ to 3′ directions, producing mainly small oligonucleotides (15, 21, 47). The *rec*BC nuclease is influenced in some way by the product of the *rec*A gene. The extensive DNA breakdown observed after UV irradiation of *rec*A mutants is not seen in *rec*$^+$ and *rec*AB strains (45). In addition, the nuclease activities seem to be more active in extracts of *rec*A than in *rec*$^+$ cells (8, 20).

Pilarski and Egan (30) and Wackernagel and Radding (43) have noted the destructive effect of the *rec*BC nuclease on infectivity of bacteriophage lambda DNA fragments. In addition, Wackernagel (40) suggested that the infectivity of T4 DNA could be markedly increased by using *rec*B spheroplasts for transfection. We have previously observed large differences in transfection efficiency among preparations of linear double-stranded, single-stranded, and circular double-stranded DNA for *rec*$^+$ spheroplasts (24). The following report summarizes the results from our systematic examination of the role of DNA structure and the *rec*BC nuclease during transfection of *E. coli* spheroplasts. In these analyses, we transfected spheroplasts made from isogenic strains of *rec*$^+$, *rec*A, *rec*B, and *rec*AB bacteria with different forms of DNA derived from several bacteriophages, including T4, T5, T7, P22, λ, and φX174. Our results clearly show that the efficiency of transfection depends on the structure of the transfecting DNA. Moreover, we find that the *rec*BC nuclease is a major factor limiting transfection of *E. coli* spheroplasts by linear phage DNA; circular DNA molecules are relatively resistant to the nuclease complex.

MATERIALS AND METHODS

The following isogenic bacteria were a gift of A. J. Clark: *E. coli* JC2918 *rec*$^+$; JC2926 *recA*$_{13}$; JC5495 *recA*$_{13}$*B*$_{21}$ and JC5743 *recB*$_{21}$. Other markers carried by these strains include F$^-$, *thi*$_1$, *thr*$_1$, leu$_6$, *lacy*$_1$,

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mtl$_1$, *xyl*$_5$, *ara*$_{14}$, *galK*$_2$, *gal*$_{14}$, *his*$_4$, *proA*$_2$, *strR*$_{31}$, *argE*$_3$, *tsx*$_{33}$, and *supE*$_{44}$.

We modified the procedure described by Henner et al. (19) for making spheroplasts as follows. All slants, cultures, and spheroplasts were kept in the dark. To prepare *rec*$^+$ and *rec*A spheroplasts, a loopful of inoculum from a fresh slant was transferred to a flask with 20 ml of modified Fraser-Jerrel medium containing 10 μg of required growth factors per ml and 10 μg of thymine per ml. This "starting culture" was grown anaerobically (stationary) or aerobically by shaking a flask at 2 rps and 37 C in a New Brunswick water bath shaker. While still in exponential phase (or close to it), this culture was diluted with fresh, prewarmed medium to yield 400 ml of bacteria at 0.05 absorbancy units at 550 nm (A_{550}). The 400-ml culture was shaken at 37 C to an A_{550} of 0.2 and harvested by centrifugation at room temperature. Conversion of the cells to spheroplasts was as previously reported (19). The cell pellet was first gently suspended in 1.0 ml of 1.5 M sucrose. (It is important to avoid excessive foaming and to disperse the pellet uniformly). Next, 0.3 ml of 30% Povite serum albumin (from Serum Biotest Institut, GMBH, Frankfurt/Main, Germany) was added, followed by 0.06 ml of freshly made lysozyme (2 mg/ml in 0.25 M Tris, pH 8.1). The lysozyme was activated by adding 0.12 ml of unbuffered 0.01 M EDTA (disodium salt). After 2 min (additional time may be necessary for some strains), 25 ml of minimal PA medium (100 g of sucrose, 1 g of glucose, and 0.5 g of Casamino Acids [Difco] per liter of water) was added. This mixture was kept without stirring at room temperature for 12 min. Next, 0.6 ml of 10% $MgSO_4 \cdot 7H_2O$ (20.5 g/100 ml), 0.075 ml of 1% protamine sulfate, and 0.025 ml of a fresh solution of spermine (250 mg/ml) were added. The spheroplasts were stored on ice for 3 h, and then their competence was tested by using φX174 DNA (2). It is important to keep the spheroplasts on ice at all times to maintain optimal competence. Spheroplasts of *rec*AB cells were prepared similarly, except that lower cell densities (A_{550} of 0.5 to 1.2) of the overnight cultures were necessary for maximum activity. We determined empirically that 45 μg of cyclic AMP per ml included with the spermine tetrahydrochloride and protamine sulfate stabilizes *rec*AB spheroplasts. Such treatment did not change the relative transfection efficiency of standard DNA preparations. Spheroplasts of *rec*B cells were prepared similarly using low cell densities for overnight cultures, but cyclic AMP was not needed for stability.

All *rec*$^-$ spheroplasts showed a tendency to lyse; thus, preliminary transfection assays with φX174 DNA were performed to insure that high competence levels had been attained. Most of the experimental results reported here derive from spheroplast preparations that were approximately 15 h old. Lysis of spheroplasts could sometimes be prevented by substituting 20% sucrose for 10% sucrose in the PA medium.

The *rec* phenotype of the spheroplasts was verified by measuring the UV inactivation of colony-forming ability (data not shown). Such experiments indicated that, if the inactivation rate for *rec*$^+$ was set at 1, *rec*B spheroplasts were inactivated at a relative rate of 3, and *rec*A and *rec*AB at a relative rate of 10. (These rates are difficult to measure accurately because the high and variable optical density of the spheroplast preparations resulted in variable self-absorption of UV light). The relative inactivation rates are in reasonable agreement with published results for intact bacteria of the same phenotype (45).

The preparation of native, denatured, and renatured T4, T5, T7, P22, and φX174 phage DNA has been described previously (24). Lambda DNA was labeled with tritiated thymidine and isolated as described by Enquist and Skalka (13). Hydrogen-bonded lambda circles (Hershey circles) were prepared as follows. The annealing mixture of 1 ml contained 5 μg of lambda DNA per ml, 0.6 M NaCl, 0.01 M Tris buffer (pH 7.4), and 0.01 M EDTA. The mixture was heated at 75 C for 10 min in a covered water bath. The bath was shut off and allowed to cool overnight. The next day, the mixture was dialyzed against three 1-liter changes of 0.1 M NaCl, 0.01 M Tris buffer, pH 7.4, and 0.001 M EDTA. Such preparations were used with no further purification and contained from 65 to 85% Hershey circles, as judged by sedimentation in neutral sucrose gradients. For preparations of λ "half-molecules," lambda DNA was sheared hydrodynamically and purified as described by Skalka (35). For preparations of "inverted linears," λ half-molecules were annealed under the conditions described for producing Hershey circles. The size and quality of the half-molecules and inverted linears were monitored by sedimentation through alkaline and neutral sucrose gradients (13). For preparation of "filled-in linears," λ DNA extracted from purified phage was treated with *E. coli* DNA polymerase I as follows. Lambda DNA at 5 μg/ml was heated in 0.01 M NaCl and 0.001 M sodium citrate in a sterile glass tube at 64.5 C for 3 min to disjoin cohered ends. This DNA was immediately added to a fivefold-concentrated reaction mixture at 0 C to yield a final concentration of 0.067 M Tris buffer (pH 7.4), 0.0067 M $MgCl_2$, 0.01 M mercaptoethanol, 0.05 mM of each of the four nucleotide triphosphates, and 4 μg of λ DNA per ml. Endonuclease-free DNA polymerase I (a gift of Cliff Harvey, Hoffmann-LaRoche, Inc.) was added to a final concentration of 15 U/ml. The mixture was incubated for 15 min at 15 C. NaCl and EDTA were added to a final concentration of 0.1 and 0.01 M, respectively. The resulting solution was mixed gently on ice and extracted with 1 ml of phenol saturated with 0.05 M Tris buffer, pH 7. The aqueous layer was removed and re-extracted with an equal volume of phenol. The final aqueous layer was dialyzed against three 1-liter changes of 0.01 M NaCl, 0.01 M Tris (pH 7.4), and 0.001 M EDTA. This preparation, called filled-in linears, was unable to form Hershey circles, as expected for molecules with repaired cohesive ends (data not shown). Such preparations yielded DNA which cosedimented with native λ linear DNA in neutral sucrose gradients. To prepare DNA from λ *cI*$_{857}$ *bet*$_{113}$ *gam*$_6$ *Sam*$_6$ purified phage, a *rec*B$_{21}$ lysogen of the mutant was constructed using standard phage techniques. Thermal induction of this lysogen yielded a reasonable burst of phage and minimized

the chance of picking up secondary mutations that bypass the specific growth defects of this mutant. When linear "native" λ DNA was to be used for transfection, the solutions (at about 1 μg/ml in 0.01 M Tris buffer, pH 8.0) were heated for 3 min at 65 C and then quickly chilled before use to disjoin any end-to-end aggregates.

Transfection assays were performed as described previously (2). DNA solutions were first diluted to 1 μg/ml in 0.05 M Tris, pH 8, except for λ DNA, which was diluted in 0.01 M Tris, pH 8. (It is essential to dilute the DNA out of any EDTA- or citrate-containing buffers). Next, 0.2 ml of various dilutions of the DNA were placed in tubes at 30 C. After the DNA had warmed to 30 C, 0.2 ml of ice-cold spheroplasts was added. Infectious centers were scored by adding 3 ml of melted indicator agar and pouring the mixture over solidified tryptone-agar plates. Indicator agar contains 1 ml of 30% Povite albumin and 1 ml of 5×10^9 indicator bacteria per 25 ml of melted sucrose agar (10 g of tryptone broth, 5 g of NaCl, 7 g of agar [Difco], 100 g of sucrose, and 1 g of glycerol per liter of water. After autoclaving, add 20 ml of 10% $MgSO_4$). The indicator bacteria have been described previously (2). All λ DNA preparations carried the S_7 amber mutation; therefore, λ transfection assays were performed with *E. coli* QD5003 *su*III$^+$ indicator.

RESULTS

Method for Standardization. As shown by in vitro studies, the *rec*BC nuclease is remarkably destructive for linear double- and single-stranded DNA. However, these same studies also demonstrated that the enzyme did not digest circular double-stranded DNA even if it contained nicks (47) or gaps of less than about five nucleotides (21). One may ask if similar activities can be directly demonstrated in vivo. Our approach to this question has been to use a variety of DNA molecules that are either linear or circular (or potentially circular) and ask if such molecules can efficiently transfect in the presence (*rec*$^+$ and *rec*A spheroplasts) or absence (*rec*AB and *rec*B spheroplasts) of the *rec*BC nuclease. Before using these DNA preparations, however, one technical problem had to be solved. Different strains of *E. coli* give widely different efficiencies of transfection with the same coliphage DNA (2). Such differences may also be expected for *rec*$^+$ and *rec*$^-$ spheroplasts, since the *rec*$^-$ cells often segregate dead or very slowly dividing cells (7). We realized that a standard transfecting DNA would minimize these problems and allow us to compare transfection efficiencies directly for other coliphage DNA preparations on *rec*$^+$ and *rec*$^-$ spheroplasts. For this purpose we chose the replicative form of φX174 DNA largely because it had been shown to be completely resistant to the *rec*BC nuclease in vitro (47). In our assays, then, the yield of infective centers for other phage DNA samples was always corrected by multiplying it by the ratio of φX174 infectivity on *rec*$^+$ spheroplasts to φX174 infectivity on the spheroplasts being tested. By using a standard that we presumed to be insensitive to the *rec*BC nuclease, we were able to test the hypothesis.

Table 1 presents the results of transfection assays comparing the infectivity of single- and double-stranded φX174 DNA preparations for the four isogenic *rec*$^+$ and *rec*$^-$ strains. The difference in competence levels of the strains is apparent. Independent spheroplast preparations of *rec*$^+$ and *rec*A strains generally give more reproducible competence levels than do those of *rec*B or *rec*AB strains. About one in seven preparations of *rec*B or *rec*AB spheroplasts were exceptional in that their competence was more than 10-fold higher than *rec*$^+$ or *rec*A spheroplasts prepared simultaneously. This last observation supports our assumption that the marked differences among the *rec* strains for infectivity of φX174 DNA reflect only competence levels of these strains and do not indicate, for example, a requirement of the *rec*BC enzyme for φX174 development. For a given preparation, the competence level remained constant for at least 15 to 20 h.

In characterizing φX174 DNA, we noticed that circular single-stranded DNA was always less infective than circular double-stranded DNA (see also [24]). However, the ratio of double- to single-stranded DNA infectivity did not seem to change significantly in strains deficient in the *rec*BC nuclease (Table 1). If the *rec*BC single-strand endonuclease were active, we would expect to find a much higher ratio for *rec*$^+$ and *rec*A spheroplasts than for *rec*AB and *rec*B preparations. We conclude that the single-strand endonuclease activity of the *rec*BC enzyme detected in vitro does not significantly affect transfection by φX174 single-stranded circular DNA.

Transfection efficiency of various bacterio-

TABLE 1. *Plating efficiency of double- and single-stranded φX174 DNA on rec$^+$ and rec$^-$ spheroplasts*

Spheroplast genotype	No. of infective centers for 10^7 molecules		Ratio of double- to single-stranded infectivity
	Double-stranded φX174 DNA	Single-stranded φX174 DNA	
rec$^+$	1,340	273	4.9
*rec*A	726	74	9.8
*rec*AB	55	21	2.4
*rec*B	36	6	6.0

phage DNA structures in rec$^+$ and rec$^-$ spheroplasts. Table 2 presents the data obtained from transfection experiments with a variety of bacteriophage DNA samples, using ϕX174 DNA to normalize for competence differences. Hereafter in the text the terms "transfection efficiency" and "infectivity" will refer to values which have been normalized to ϕX174. When expressed in this way it can be seen that DNA preparation responded to the presence or absence of the *rec*BC nuclease in characteristic fashion.

(i) T4 DNA. Transfection by native T4 DNA was markedly affected by the *rec*BC nuclease. In the *rec*B or *rec*AB spheroplasts which do not contain this nuclease, transfection efficiency increases at least 100-fold, and in some exceptional spheroplast preparations, over 2,000-fold. Linear single-stranded T4 DNA obtained by denaturation of native DNA is about 10-fold more infective than native DNA in the *rec*$^+$ and *rec*A spheroplasts. Like the double-stranded native form, single-stranded infectivity increases markedly in the absence of the *rec*BC

TABLE 2. *Relative infectivity of various forms of coliphage DNAs on rec$^+$ and rec$^-$ spheroplasts*[a]

Phage DNA	Preparation and probable structure	DNA molecules/ spheroplast	Infective centers/10^7 DNA molecules[b]			
			rec$^+$	*recA*	*recAB*	*recB*
T4	Native	1.0	1.0	0.3	150	280
	Denatured	0.5	13.0	5.7	380	650
	Renatured	0.5	68.0	43.0	210	260
T7	Native	2.0	0.04	0.04	2.5	1.1
	Denatured	1.0	1.0	1.0	1.0	11.0
λ	Native	0.2	50.0	45.0	51.0	55.0
	Hershey circles	0.1	59.0	60.0	58.0	57.0
	Halves	1.2	0.01	0.05	0.9	0.7
	Inverted linears	1.2	0.02	0.09	1.8	1.2
	Filled-in linears	0.3	0.5	0.5	0.4	
	red gam native	0.2	4.0[c]	2.7[c]	13.0	56.0
	red gam Hershey circles	0.1	3.2[c]	1.6[c]	13.0	21.0
	red gam filled-in linears	0.5	0.05[c]	0.08[c]	0.7	3.6
P22	Native	10.0	0.003	0.0003	5.5	0.6
	Denatured	0.4	1.0	1.3	18.0	75.0
	Renatured	0.2	5.0	5.0	20.0	19.0
T5	Native	2.0	1.0	0.7	17.0	12.0

[a] Competence level differences have been corrected by normalizing infective center numbers of ϕX174 RF competence; for *rec*$^+$ spheroplasts (the standard) an average of 2,000 ϕX174 infective centers were obtained; for *rec*A, 1,500; for *rec*AB, 400; and for *rec*B, 100. To obtain actual transfection efficiencies, divide *rec*A values by 1.3, *rec*AB values by 5, and *rec*B values by 20. Each assay contained 8×10^8 spheroplasts. The average of three to 10 experiments is presented, except for renatured P22 DNA, for which the results of a single experiment are shown. For *rec*$^+$ and *rec*A spheroplasts, variation was ±50%. For *rec*AB and *rec*B spheroplasts, variation was higher, in many cases as much as 50-fold.

[b] Infective centers/10^7 DNA molecules means corrected infective centers/10^7 double-stranded DNA equivalents.

[c] These plaques were extremely small; for comparison with the other assays, the numbers should be corrected upwards by at least twofold (the *red gam* lambda phage plates on these *rec*$^+$ and *rec*A bacteria with an efficiency of 0.5 that of the wild-type phage).

nuclease. Renaturation of the single-stranded material produces an interesting effect: this double-stranded DNA is more than 60 times more infective than the original native DNA in *rec*$^+$ and *rec*A spheroplasts. Again, infectivity of the renatured DNA is significantly increased in *rec*B and *rec*AB spheroplasts.

(ii) T7 DNA. In comparison to T4 native DNA, T7 native DNA transfects poorly in cells containing the *rec*BC nuclease. However, about a 50-fold increase in activity can be seen when *rec*B or *rec*AB spheroplasts are used. Like denatured T4 DNA, denatured T7 DNA is 10 to 25 times more infective in *rec*$^+$ or *rec*A cells. However, the infectivity of denatured T7 DNA does not increase as much as that of denatured T4 DNA when *rec*B, and especially *rec*AB, instead of *rec*$^+$ or *rec*A spheroplasts are used.

(iii) Lambda DNA. Experiments with λ DNA provide more insight into the role of the *rec*BC nuclease in transfection. The native form of λ DNA is unique in that it has complementary ends which rapidly circularize in vivo (12) and in vitro (44). Moreover, the product of the lambda gene, *gamma* (*gam*), is a specific inhibitor of all four activities of the *rec*BC nuclease (37, 38). A 5′ to 3′ exonuclease involved in general recombination is encoded by the λ *red* gene, *exo*. We compared lambda DNA from wild-type phage with that of the lambda mutant red_{113} gam_5 that lacks both the *rec*BC nuclease inhibitor and general recombination ability. The results are that wild-type native and Hershey circle DNA are both equally infective in all hosts: the presence or absence of the *rec*BC nuclease has little effect. In fact, lambda DNA is about 50 times more infective than native T4 DNA and over 100 times more infective than T7 DNA in *rec*$^+$ and *rec*A spheroplasts. With the *red gam* mutant DNA, we find that for both native and Hershey circle forms transfection efficiency is significantly lower in *rec*$^+$ and *rec*A cells. Unlike wild-type DNA, *red gam* Hershey circles and native samples gain infectivity in *rec*AB and *rec*B spheroplasts. In fact, their infectivity approaches wild-type levels in these strains. The slightly reduced levels of *rec*AB and *rec*B transfection are not surprising because the presence of the *red* mutation lowers the burst size by two- to fivefold (13, 48).

When lambda DNA cannot circularize, transfection efficiency drops sharply, even if the DNA used has the capacity to synthesize the *gam* inhibitor protein. This can be seen by studying lambda wild-type half-molecules (see also [43]). It should be noted that for infective center formation, at least two halves, containing between them a genome's worth of information, must cotransfect (43). Since we used a multiplicity of halves slightly more than one half-molecule per spheroplast, we assume that we observed infective centers from cells which had received only two halves of lambda. Under these conditions, half-molecules are decidedly less infective in *rec*A and *rec*$^+$ cells than in *rec*B and *rec*AB preparations. It should be noted that, even in the absence of the *rec*BC nuclease, half molecules are more than 50-fold less infective than molecules that can circularize. Because lambda halves must recombine to generate a complete molecule, we suggest the *rec*BC enzyme degrades many of the participants with the subsequent loss of this potential infective center. In *rec*$^-$ spheroplasts, such recombination is accomplished by the general and site-specific recombination system of lambda and residual host recombination functions (8, 43). In our hands, *red gam* halves give no detectable infectivity in *rec*$^+$ and *rec*A hosts (data not shown).

By annealing the complementary ends of the half-molecules, we produced "inverted linears." Such molecules contain, on the average, a complete genome's worth of information, but the molecules are "inside out"; they cannot circularize normally. In all spheroplasts, these molecules were only slightly more infectious than separated half-molecules. These observations suggest that the *rec*BC nuclease limits the infectivity of lambda halves and inverted linears, even though these molecules have the capacity to synthesize an inhibitor of the *rec*BC complex. Apparently the *rec*BC nuclease attacks the double-stranded ends of these molecules before enough *gamma* protein is made. Alternatively, molecules that cannot circularize may not be able to transcribe efficiently. The fact that, in *rec*AB or *rec*B spheroplasts, inverted linears still were 50-fold less infective than normal lambda DNA suggested that something more than the *rec*BC enzyme might be blocking infectivity. Our data are consistent with the results of Sternberg and Weisberg (personal communication), who have studied the ability of λ *doc*R (*doc*R carries the right cohesive end and the right DNA half of the normal phage, but the DNA cannot circularize when injected into the host because it lacks the left cohesive end [25]) to express lambda endolysin in a *rec*A and a *rec*B host. Whereas λ *doc*R produces five times more endolysin in a *rec*B host than in a *rec*A host, the level made in the former host is only 5% of that produced by normal lambda.

To produce molecules that could not circularize, without having been subjected to the harsh

methods of preparation described above, we used DNA polymerase to repair the complementary ends of wild-type and *red gam* DNA. These molecules, unlike halves or inverted halves, have their genetic information intact; no genes are disrupted. In *rec*$^+$ or *recA* spheroplasts, DNA in this preparation was about an order of magnitude more infective than that of halves or inverted linears, but still more than 100-fold reduced from the infectivity of lambda that could circularize. It is significant that this preparation (like the native DNA) gave no increase in infectivity when *rec*AB spheroplasts were used. When the ability to make the *gamma* inhibitor and ability to recombine were removed by mutation, a preparation of filled-in linears was about 10-fold less infective in *rec*$^+$ and *rec*A cells than the corresponding wild-type molecules. As with the native *red gam* DNA, infectivity with the preparation of filled-in molecules increased significantly in *rec*B and *rec*AB spheroplasts, reaching levels equal to those of wild-type filled-in molecules. The simplest explanation of these findings is that our preparations of filled-in linears contained a low-level contamination with normal λ DNA molecules. Our screening and purification methods could not eliminate contamination at levels approaching 1 or 2%. Given the high infectivity of native λ DNA, such contamination could easily mask what we presume to be the low infectivity of filled-in linears.

(iv) P22 DNA. Although *Salmonella typhimurium* is the natural host for phage P22, all components required for P22 growth are present in *E. coli* (6). Both T4 and P22 phage DNAs are circularly permuted; accordingly, the transfection pattern for denatured and renatured P22 DNAs is closely similar to that observed for the corresponding T4 DNAs (Table 2). However, native P22 DNA is at least 30-fold less infective for all types of spheroplasts than expected on the basis of the native T4 DNA transfection results. A simple explanation for this discrepancy is that the multiplicity of native P22 DNA molecules per cell (10; see Table 2) was too high and that higher relative efficiencies of transfection would have been observed at lower DNA multiplicity. Complications introduced by the weak *E. coli* K-specific host-controlled restriction system for P22 DNA (1) will be considered in the Discussion.

T5 DNA. The special problems affecting transfection with T5 DNA have been described previously (3) and are discussed below. In our studies we find that native T5 DNA transfects *rec*$^+$ and *rec*A spheroplasts with about the same efficiency as native T4 DNA. Infectivity increased significantly in *rec*B and *rec*AB cells, though not to the extent observed with native T4 DNA.

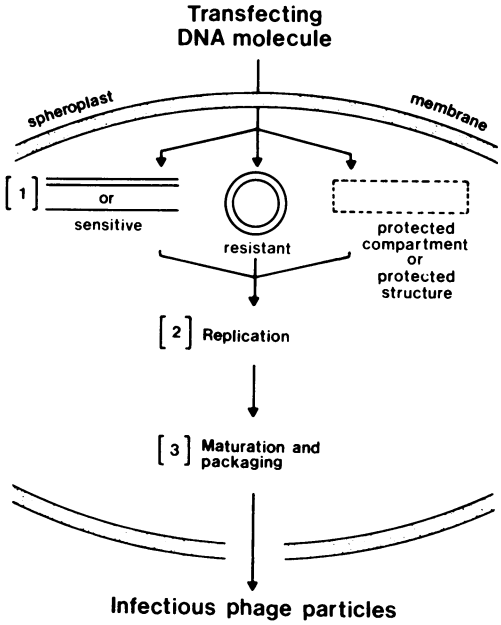

FIG. 1. *Three stages for the possible destruction of infective centers by recBC nuclease. (Stage 1) Entrance of transfecting DNA into the cell. (Stage 2) Replication of the transfecting DNA with the formation of potentially sensitive intermediates. (Stage 3) Maturation and packaging of the DNA into infectious phage particles with the formation of potentially sensitive intermediates.*

DISCUSSION

recBC nuclease in vivo. The effect of the *rec*BC nuclease on transfecting DNA molecules varies according to the structure of the DNA, the capacity of the DNA to encode inhibitors of the *rec*BC nuclease, and the particular mode of DNA replication of the virus. The order of importance of these variables is not absolutely fixed. The reasons for such ambiguity are shown schematically in Fig. 1. In our assays, we measure the ability of an infecting phage DNA molecule to produce an infective center, that is, a spheroplast capable of producing at least one infectious phage particle. We can conceive of at least three stages where infective center formation could be blocked by the *rec*BC nuclease (Fig. 1). The transfecting DNA molecule may be degraded as it enters the spheroplast (stage 1). It can survive this first stage in at least three ways: (i) by having a *rec*BC-resistant structure (e.g., a circular molecule); (ii) by immediately

entering an environment not accessible to the *rec*BC enzyme (the "protected compartment" or "protected structure"); or (iii) by rapid conversion of a sensitive form to an insensitive form (e.g., the circularization of lambda DNA). DNA molecules that escape stage 1 degradation may again be exposed to the *rec*BC nuclease when replication begins (stage 2). During replication, *rec*BC-sensitive intermediates may be formed and destruction of such forms would block the ability to produce an infective center. DNA molecules can bypass stage 2 degradation in several ways. (i) The replicative intermediates may not be *rec*BC sensitive; (ii) an inhibitor of the *rec*BC nuclease may be made (e.g., the lambda *gamma* protein); or (iii) sensitive molecules may replicate in isolated cellular compartments and escape degradation. The final stage where *rec*BC enzyme could conceivably block infectious center formation would be during morphogenesis and packaging of phage DNA into infectious particles (stage 3). DNA molecules that pass these three potential *rec*BC degradation stages appear in our assay as an infective center.

In the following discussion, we will present evidence for the existence of stage 1 and stage 2 degradation in our spheroplast system. We will show that at least stage 1 degradation can be bypassed through in vitro construction of circular molecules or by using single-stranded DNA. Finally, we will discuss evidence that stage 2 degradation can be blocked via a phage-specified inhibitor.

We believe that one of the three nuclease activities (the single-strand endonuclease) present in the *rec*BC complex functions inefficiently in our spheroplasts. This follows from our observations that infectivity of ϕX174 single-stranded circular DNA is not affected by the *rec*BC nuclease (Table 1). As will be discussed later, linear single-stranded DNA molecules seem to escape some nuclease digestion as well (see denatured DNA, Table 2). Such an effect may reflect the low in vitro activity (0.003% of the exonuclease activity [14]). Recent results of MacKay and Linn (manuscript in preparation) indicate that single-stranded DNA complexed with purified *E. coli* DNA unwinding protein (33) is not attacked by the *rec*BC single-strand endonuclease. Attack by the single-strand exonuclease is also inhibited at high concentrations of this protein. These workers also observed that double-strand exonuclease activity is relatively unaffected by unwinding protein, a fact consistent with the protein's low affinity for double-stranded DNA. They have suggested that the unwinding protein may play a role in the modulation of the activities of the *rec*BC enzyme in vivo. Our data strongly support this view. Results from our in vivo assays of this enzyme's activities suggest that the single-strand endonuclease has almost no activity and the single-strand exonuclease has little activity inside the cell. The in vivo and in vitro results are consistent, if we simply assume that most of the single-stranded DNAs that we have studied are covered with DNA unwinding protein soon after they enter the cell. Moreover, these results may reflect the fact that all single-stranded DNA inside *E. coli* cells is complexed with the DNA unwinding protein. It is also possible that some single-stranded circular DNA escapes stage 1 degradation by rapid conversion to the double-stranded form (perhaps involving unwinding protein) or that the complementary strand is formed in a *rec*BC nuclease inaccessible compartment.

Transfection. (i) T4 DNA. By in vitro criteria, the native form of T4 DNA should be readily attacked by the *rec*BC nuclease. This prediction seems verified because T4 infectivity increases markedly in *rec*AB or *rec*B spheroplasts (Table 2). By using T4 DNA structures other than the native form, we can infer that most, but not all, of T4 native DNA infectivity is lost through degradation at stage 1. Using denatured T4 DNA, we see a 10-fold increase in infectivity over native DNA in *rec*$^+$ or *rec*A spheroplasts. This increase can only come from bypassing the stage 1 limitation, because ultimately the single-stranded DNA must enter the normal replication route common to all T4 DNA molecules, and at that point the distinction between native and denatured DNA should be lost. Denatured DNA bypasses stage 1 degradation more efficiently than the native form, but does not bypass it completely, because renatured T4 DNA is significantly more infective than the denatured form. Since T4 DNA is circularly permuted and terminally redundant, renaturation of single-stranded DNA should generate many circular molecules (36). Such molecules would be predicted to escape stage 1 degradation in *rec*$^+$ and *rec*A cells. Indeed, infectivity increases more than 50-fold over the native form in such experiments (Table 2; see also ref. 9). An even greater increase might be seen if purified T4 circles were used. Again, because these circular molecules must enter the same replication complex as native T4 molecules, we conclude that a significant portion of native T4 infectivity is lost because it is limited at stage 1. T4 does have circular replication

intermediates (4) that may be able to circumvent degradation at stage 2 because of structure. It is clear that all three DNA forms show very high and similar transfection efficiencies in *rec*BC-deficient spheroplasts. This confirms the destructive nature of the *rec*BC enzyme for transfection with T4 DNA.

(ii) T7 DNA. Analysis of T7 DNA is interesting, because the native form of the DNA should be actively degraded at stage 1 (T7 DNA is routinely used for in vitro assay of the *rec*BC exonuclease). In addition, the major replicative intermediate for T7 is a linear molecule (46). Such a molecule should be sensitive to stage 2 degradation, unless inhibitors are synthesized (13, 30). Indeed, Wackernagel and Herrmans (42) have just described the appearance of a T7 phage function which inactivates the *rec*BC nuclease shortly after infection. Native T7 DNA is much less infective than native T4 DNA in *rec*$^+$ or *rec*A spheroplasts. This increased sensitivity compared to native T4 DNA may reflect the difference in size of the terminal redundancies (more than 10^6 daltons for T4 and less than 2×10^5 daltons for T7). It may be the native T4 molecules have more DNA to give up to exonuclease attack before losing infectivity. As observed for denatured T4 DNA, denatured T7 DNA can partially bypass stage 1 degradation. These two observations together suggest that, not only is the *rec*BC single-strand endonuclease activity low or inactive, but that the single-strand exonuclease activity is also inefficient. We conclude that single-stranded DNA molecules have a high probability of bypassing stage 1 degradation. Because little increase in infectivity of the more resistant denatured T7 DNA is seen in *rec*B or *rec*AB spheroplasts, T7 transfection may be limited by *E. coli* exonucleases other than the *rec*BC nuclease.

(iii) Lambda DNA. The role of the *rec*BC nuclease in lambda growth has been analyzed in detail (13, 30). Our experiments here corroborate these studies. The similar infectivities of native and circular lambda DNA in both *rec*BC nuclease-containing or -deficient spheroplasts indicate that the native double-stranded linear lambda molecule is not sensitive to attack at stage 1. As suggested above, these molecules might be converted to an insensitive (circular) form so rapidly after they enter the cell that the *rec*BC nuclease has no time to act. Alternatively, some feature of the structure of the linear molecule might serve to protect it from the nuclease. The presence of 5'-phosphate-terminated single-stranded ends might partly inhibit the double-strand exonuclease activity of the enzyme. If the in vivo activity of the single-strand exonuclease or endonuclease is significantly lower than that of the double-strand exonuclease (as our data suggest), such inhibition could "buy" more time for the linear form to be converted to the resistant circular structure. Although there is no relevant in vitro data for the *E. coli* enzyme (Linn and Karu, personal communication), Van Dorp et. al. (39) have shown that an enzyme similar to the *rec*BC nuclease obtained from *Micrococcus luteus* degrades native linear λ DNA at a greatly reduced rate as compared to linear, native T7 DNA. It would be interesting to see if the same effect is found for the *E. coli rec*BC enzyme.

The insensitivity of linear λ DNA to stage 1 degradation is probably not dependent on the production of the *rec*BC nuclease inhibitor *gamma*, because λ *red gam* native DNA is as infectious as λ *red gam* circles in cells which contain the nuclease. Interpretation of the *red gam* results is slightly complicated by the fact that λ *red gam* molecules are sensitive to stage 2 inhibition (13), so that even though stage 1 is eliminated *gamma* must be synthesized to efficiently bypass stage 2 degradation. In *rec*B or *rec*AB spheroplasts, the stage 2 block is removed and now λ *red gam* linears and circles regain infectivity, approaching wild-type levels. By using molecules that were unable to circularize, we could show loss of infectivity in *rec*A or *rec*$^+$ spheroplasts, even though such molecules had the capacity to synthesize the *gamma* inhibitor protein. This effect is consistent with stage 1 degradation before *gamma* can accumulate. When the *rec*BC nuclease is absent, λ halves, inverted linears, and possibly filled-in linears all attain the same levels of infectivity. This level is clearly more than that found in *rec*$^+$ or *rec*A transfection, but it is much less than for molecules that can circularize. Unless these molecules are uniquely sensitive to other unknown factors, we conclude that circularity per se is a requirement for efficient λ development.

(iv) P22 DNA. Our results with P22 DNAs are generally compatible with those obtained for the corresponding T4 DNAs (Table 2); however, native P22 DNA infectivity is much lower than expected. A simple explanation for this discrepancy is that too high a multiplicity of DNA molecules was used and that an inhibition of transfection was obtained similar to previous results (1, 2).

The possibility remains that a weak host-controlled restriction of P22 DNAs by *E. coli* K-12 $r^+_K m^+_K$ spheroplasts (1) differentially affected

the infectivity of native and denatured or renatured P22 DNAs. The synergistic action of restriction enzyme and *rec*BC nuclease (34), as well as the resistance of single-stranded DNA to the restriction enzyme (26), might have to be considered. In the absence of K-12-specific restriction, the three forms of P22 DNA do transfect like the corresponding T4 DNAs (24). Final resolution of the question should be possible using low multiplicities of native P22 DNA to transfect otherwise isogenic $r_K^+m_K^+$ and $r_K^-m_K^-$, *rec*$^+$ and *rec*$^-$ spheroplasts.

Activity of recA protein. Our analyses indicate that the *rec*A protein has little, if any, effect on the *rec*BC nuclease activity that acts on transfecting DNA. The one possible exception was with P22 native DNA, which did show a reduction in infectivity due to the absence of the *rec*A product. However, both of these values were very low and are complicated by possible effects of the K-restriction system.

Transfection versus transformation. Our results corroborate those of several workers on the limitations of *E. coli* transformation exerted by the *rec*BC exonuclease (10, 11, 29, 41). Our data are not complicated by the necessity of transforming DNA to recombine with the host chromosome, using the *rec*ABC or some other recombination system. Although, as we have shown, for transfection experiments various stages of the viral growth cycle must be considered, the results suggest that *rec*$^+$ *E. coli* could be transformed quite efficiently if some way could be found to circularize the transforming DNA. Such an approach is quite feasible, using restriction endonucleases to generate cohesive ends in transforming DNA (18). Goodgal and Gromkova (16) have already used such enzymes for purifying transforming DNA.

Transfection versus infections. If the *rec*BC exonuclease in spheroplasts attacks double-stranded linear transfecting DNA so vigorously, why is normal infection by phages containing such DNA not hampered by this enzyme? One possibility is that the injection process itself provides some mechanism for protection of the DNA at stage 1. For example, in some cases the DNA may be injected into a "protected compartment" until some other mechanism for protection at stages 2 or 3 can be achieved; or the ends of the injected DNA (but not of transfecting DNA, which has been deproteinized with phenol) might be protected by DNA-binding proteins. Several lines of evidence suggest that transfecting DNA is more susceptible to nuclease attack than is DNA injected from phage particles. Our own data as well as that of others with T5 (3), SP82 (5), or HPI (17) DNA show that there is a normal one-hit dilution curve of plaque versus phage concentration for DNA injected from phage. However, in transfection assays two or more DNA molecules are needed to make a plaque. Benzinger et al. (3) showed that for T5 this difference is due to the *rec*BC nuclease. King and Green (personal communication) have evidence that the difference with SP82 is the result of an endonuclease. The data of Boling et al. (5) were more indirect; they showed that transfection by HPI DNA requires recombination, presumably to rescue the damaged pieces.

In addition to "compartmentalization" and the possible protective effects of DNA binding protein, there are other intracellular components which could modulate the activities of the BC nuclease. The intracellular level of ATP which might be different in spheroplasts as compared to intact cells, and even different in intact cells at various stages of growth, could be such a modulating component (Clark and Linn, personal communication). At 4 mM, the concentration of ATP in logarithmically growing *E. coli* (31), the single-strand exonuclease should be fairly active and the double-strand exonuclease relatively inactive (15). This might account for the prevalence, in nature, of linear double-strand DNA containing phages and the apparent absence of those containing linear single-strand DNA. It could also explain the low infectivity of phage, produced by laboratory manipulations, which contain linear single-strand DNA (32).

We, as others (40), have attempted to test the above hypothesis by raising or lowering the level of intracellular ATP in spheroplasts. Since the double-strand exonuclease activity seemed to predominate, we supposed that our starved, competent spheroplasts might contain a lower than normal concentration of ATP, optimal for the double-strand exonuclease and minimal for single-strand exonuclease. We then measured relative efficiency of transfection by double- and single-stranded DNA molecules in the presence of increasing external levels of ATP or dinitrophenol (an inhibitor of ATP synthesis). Although, in some cases, we observed the expected trends, the results were inconclusive and suggested that further attempts at such an approach would not be very fruitful.

In summary, we have found that each phage DNA has its characteristic response to the *rec*BC enzyme. Some are affected more than others, but the results are, for the most part, interpretable within a framework of the known

in vitro activities of this enzyme. Our evidence strongly suggests that linear, double-stranded DNA molecules are degraded more efficiently than single-stranded linears. Circular single- and double-stranded molecules seem to escape degradation efficiently. This may mean that in vivo, as in vitro, the (8, 21) double-strand exonuclease is the most prominent of the three *rec*BC nucleolytic activities. Recent results of Kushner, with temperature-sensitive *rec*BC nuclease mutants (22, 23), provide independent evidence that the double-strand specific exonuclease is the most biologically significant activity of the *rec*BC enzyme. These findings should be important for any consideration of the molecular mechanisms by which the *rec*BC enzyme helps to catalyze genetic recombination.

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