Proc. Nat. Acad. Sci. USA
Vol. 68, No. 5, pp. 1070–1074, May 1971

Fate of Transforming DNA After Uptake by Competent *Bacillus subtilis*: Failure of Donor DNA to Replicate in a Recombination-Deficient Recipient*

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Communicated by George K. Hirst, March 5, 1971

ABSTRACT **The fate of radioactively-labeled transforming DNA was studied in a recombination-deficient strain of *Bacillus subtilis* that carried the *recB2* mutation (Rec⁻) and was sensitive to radiation. Experiments performed with extracts of this strain after transformation showed that the recovery of donor transforming activity and the appearance of recombinant transforming activity occurred to the same extent as in the Rec⁺ strain. Sucrose gradient analyses revealed that donor–recipient complex is also formed to the same extent in the Rec⁻ and Rec⁺ strains, but that 80–90% of the donor genetic material in the complex failed to replicate in the Rec⁻ mutant.**

Bacterial transformation is an advantageous system for the study of recombination, since the fate of purified donor DNA can be studied in the course of the different steps before, during, and after integration. Recombination-deficient mutants of bacteriophage and bacteria have been useful in the study of recombination processes (1–10). Recombination deficient, radiation-sensitive mutants of *Bacillus subtilis* have been isolated and described by several investigators (11–14). This paper reports the results of experiments in which the fate of transforming DNA was examined in one of these mutants (*recB2*). Our results indicate that in *recB2*, the donor–recipient complex is formed to the same extent as in an isogenic Rec⁺ strain, but that donor DNA fails to replicate in the recipient after formation of the complex.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used were derivatives of *Bacillus subtilis 168*. Strains BD170 (*thr-5 trp-2*) and BD191 (*thr-5 trp-2 recB2*) were derived from the same parent and were isogenic for all markers except the *recB2* marker, which was introduced by transformation as was described (15). The *recB2* marker is derived from the mitomycin-sensitive strain *mc-1* (12). *RecB2* has been shown to be radiation sensitive and deficient in both transformability and transducibility (12–14). Strain BD204 (*hisB2 thy*) was used to prepare radioactively-labeled transforming DNA.

BD55 (*trp-2 hisB2*), known also as SB25, was used to assay the biological activity of DNA fractions isolated from the transformed cells. The *trp-2* and *hisB2* markers are co-transformed at a frequency of about 60%.

Abbreviations: TCA, trichloroacetic acid; Rec⁺, strain BD170 of *B. subtilis* 168; Rec⁻, strain BD191(*recB2*) of *B. subtilis* 168.

* The preceding paper in this series is ref. 16.

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Media and solutions

The media for plating and for the development of competence were prepared as described (16). Transformed cells were washed in SP buffer (0.15 M NaCl–0.02 M K_2HPO_4, pH 7.5). The washed samples were resuspended in lysis medium (0.05 M NaCl–0.1 M EDTA, pH 6.9).

SPII medium contained Spizizen salts (17), 0.02% casamino acids, 0.1% Difco yeast extract, 0.5% glucose, 0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 50 μg/ml of each required amino acid.

Re-extraction experiments

Competent cells were prepared and frozen as was described (16). Frozen competent Rec⁺ and Rec⁻ cells were quickly thawed and incubated for 5 min at 37°C. To begin the reaction, [^{3}H]DNA, isolated from BD204 as described (16), was then added to a final concentration of 1–2 μg/ml. The specific activity of the DNA preparations ranged from 4 to 6×10^5 cpm/μg. The mixture was incubated at 37°C for 7 min or 9 min, at which time an excess (100 μg/ml) of salmon-sperm DNA (Calbiochem) was added to stop further DNA uptake. In some experiments the reaction mixture was then transferred to 30°C. At different times after addition of the salmon-sperm DNA, samples of the transformed cells were chilled rapidly, washed three times by centrifugation in ice-cold SP, and resuspended in lysis medium. A portion of the washed cells was assayed for radioactive DNA uptake, and the rest was used for DNA extraction. A sample of cells, taken at 30 min, was plated for *Trp*⁺ transformants on properly supplemented minimal plates. The relative transformation values are obtained by dividing the number of transformants per ml by the uptake of radioactive DNA per ml determined 1 min after the addition of the heterologous DNA.

DNA uptake was measured by precipitating 0.1 ml of transformed, washed cells with cold 5% trichloroacetic acid (TCA) and collecting the precipitate on nitrocellulose (HA type) Millipore filters. The filters were washed with 5% TCA, dried, and counted with Omnifluor (New England Nuclear) in a Beckman LS-200B scintillation counter.

DNA extraction

Washed, resuspended cells were treated at 37°C with 500 μg/ml of lysozyme for 15 min. Lysis was completed by the addition of sodium dodecyl sulfate to a final concentration of 1 mg/ml, followed by incubation for 2 hr at 48°C with 0.5 mg/ml of Pronase (Calbiochem). The DNA samples were dialyzed against 0.1 × SSC (i.e., 0.015 M NaCl–0.0015 M Na citrate).

Native and denatured DNA of high molecular weight that had been added to washed cells together with lysozyme were recovered by this procedure with virtually unaltered sedimentation properties (unpublished observations). This, plus the detection of processes with short time-constants (see Figs. 2 and 3, ref. 16), give some assurance that alteration of transforming DNA does not occur during isolation.

Sucrose gradient sedimentation analysis

Centrifugation in sucrose gradients was performed with 5–20% linear sucrose gradients, containing 1 M NaCl and 0.05 M potassium phosphate, pH 7.0. A 0.8-ml cushion of 60% sucrose was placed at the bottom of the gradient to facilitate recovery of high molecular weight DNA species. Centrifugation was in an SW 50.1 rotor at 49,000 rpm at 20°C for 85 min. [^{14}C]Thymidine-labeled DNA from coliphage T_7 was included as a sedimentation standard. The molecular weight of this DNA was taken as 2.53×10^7 (18). 3-drop samples were collected after puncturing the bottom of the tube and were counted in Omnifluor that contained Scintisall solubilizer (Isolab, Inc.). The recovery of radioactivity from the gradients ranged from 85 to 100%.

RESULTS

Table 1 shows the transformation frequency relative to DNA uptake (*Trp*⁺/cpm) obtained in three experiments using Rec⁻ and Rec⁺. DNA uptake varied among the experiments because of differences in the competence of the culture and in the specific activity of the DNA used. The efficiency of transformation of the Rec⁻ strain, when normalized to DNA uptake, was 10–25% of the value obtained with the *Rec*⁺ strain. The value shown for experiment 1 was atypically high, since in most experiments the normalized transformation frequency obtained with the Rec⁻ strain was about 10% of the wild-type value. Thus, the *recB2* marker partially blocks the transformation process, in accord with previous results (12–14).

Re-extraction experiments were performed with (Rec⁺ and Rec⁻ strains) as recipients. Fig. 1 shows the amount of TCA-precipitable radioactivity in the washed cells at different times after DNA uptake had been arrested. A progressive loss of counts was observed in both Rec⁺ and Rec⁻ cells. This loss of counts, which reflects a degradative process (unpublished observations), proceeds to about the same extent in the Rec⁻ and Rec⁺ strains. In four different experiments, the final amounts of radioactivity in both strains after 60 min was 40–60% of the initial value.

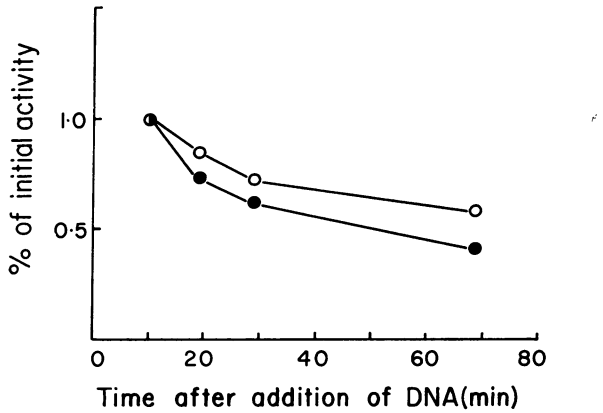

FIG. 1. TCA-precipitable radioactivity in Rec⁺ and Rec⁻ after uptake of [^{3}H]DNA. Competent cultures were incubated at 37°C for 9 min with [^{3}H]DNA from strain BD204. DNA uptake was arrested by adding salmon-sperm DNA to a final concentration of 100 μg/ml. The temperature of the reaction mixture was lowered to 30°C. At 10, 19, 29, and 69 min, aliquots were washed and assayed for TCA-precipitable radioactivity. The initial radioactivities were 4.6×10^4 cpm/ml for the Rec⁺ (○) recipient and 5.5×10^4 cpm/ml for Rec⁻ (●).

Venema *et al.* (19) have shown that shortly after uptake by competent *B. subtilis* cells, re-extracted donor DNA exists in a form with little or no biological activity. This loss of donor transforming activity has been termed "eclipse". With time, transforming activity for the donor marker (Trp⁺) reappears, closely followed by the appearance of the recombinant marker configuration (Trp⁺His⁺). Re-extraction experiments were performed to determine whether these phenomena proceed normally in the Rec⁻ strain. Competent Rec⁺ and Rec⁻ strains were exposed for 7 min to radioactive *hisB2* Trp⁺ DNA. The reaction was stopped by the addition of salmon-sperm DNA. Samples were taken 8, 15, 30, 45, and 60 min after exposure to DNA, washed, and the DNA was extracted. The donor and recombinant marker activities were tested with SB25 (*hisB2 trp-2*) cells as recipients.

Fig. 2*a* and 2*b* show the results of this re-extraction experiment. The eclipse, and the recovery of donor marker transforming activity, as well as the appearance of recombinant

TABLE 1. *Transformation and uptake of DNA by BD170 (Rec⁺) and BD191 (Rec⁻) competent cell cultures*

Expt.	Strain	Trp⁺ transformants/ml	Viable count/ml	DNA uptake (cpm/ml of cells)	Trp⁺/cpm	Ratio of Trp⁺/cpm for Rec⁺/Rec⁻
I	Rec⁺	2.7×10^5	6.9×10^8	4.61×10^4	5.86	1
	Rec⁻	8.6×10^4	1.12×10^8	5.55×10^5	1.55	0.26
II	Rec⁺	9.0×10^6	1.2×10^9	3.8×10^3	117.1	1
	Rec⁻	3.8×10^5	6.1×10^7	1.53×10^3	12.5	0.11
III	Rec⁺	2.1×10^6	1.3×10^9	1.3×10^4	168.5	1
	Rec⁻	2.5×10^5	1.1×10^8	8.0×10^3	30.6	0.18

Competent cells were transformed by shaking with [^{3}H]DNA from strain BD204 at 37°C for 9 min. At this time, the reaction was stopped by the addition of 50 μg/ml of DNase. 1 min later, an aliquot was plated for Trp⁺ transformants and viable count; another aliquot was washed and its radioactivity was determined. DNAs of different specific activity and cell preparations of different competence were used for each experiment.

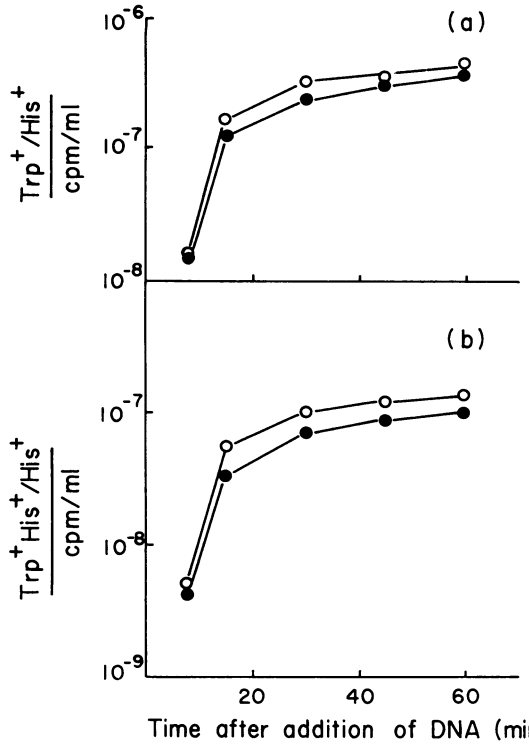

FIG. 2. Transforming activity in lysates of Rec+ and Rec− after uptake of transforming DNA. Rec+ (●) and Rec− (○) competent cell cultures were exposed for 7 min to BD204 [^{3}H]-DNA at 37°C. Uptake was stopped by the addition of 200 μg/ml of salmon-sperm DNA. Incubation was continued, samples were taken at 8, 15, 30, 45, and 60 min, and DNA was extracted. (*a*) shows the transforming activity of these preparations for the donor (Trp+) marker, and (*b*) for the recombinant (Trp+His+) marker. All values are normalized to recipient (His+) transforming activities and to the amounts of TCA-precipitable radioactivity in the two strains at 10 min.

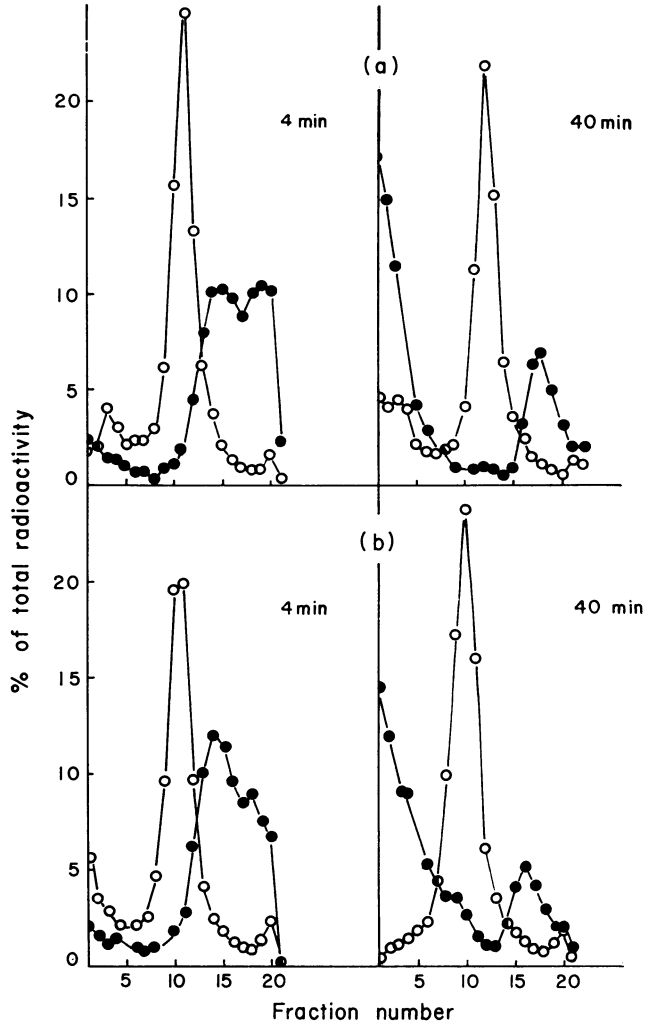

FIG. 3. Neutral sucrose gradient profiles of DNA samples extracted from Rec− (*a*) and Rec+ (*b*) cultures at 4 and 40 min after the start of [^{3}H]DNA uptake. DNA samples were extracted and were layered on 5–20% sucrose gradients. The gradients were centrifuged at 49,000 rpm for 85 min at 20°C. (●) represents the sedimentation profile of [^{3}H]DNA, and (○) that of T7 [^{14}C]DNA. The radioactivity of each fraction is expressed as the % of total radioactivity recovered. This total was 2000–3000 cpm in each case, for both ^{3}H and ^{14}C.

transforming activity, are similar in the Rec+ and Rec− strains. In fact, the final amounts of recombinant and donor transforming activity are slightly higher in the Rec− strain. In this experiment, the initial transforming activity in the Rec− strain was 20% of that in the Rec+ strain. These results have been confirmed repeatedly.

We have observed that after uptake by Rec+ cells, donor DNA is converted to duplex fragments, with a molecular weight of about 9×10^6, and to single-stranded fragments of about 1×10^5 daltons (unpublished observations). Donor radioactivity is gradually lost from these slowly-sedimenting forms and appears in the donor–recipient complex; with our isolation procedure for DNA, the complex has a molecular weight of about 1×10^8 (16). Thus, sedimentation in sucrose gradients provides a convenient technique for monitoring the appearance of the donor–recipient complex. The sedimentation behavior of DNA samples from both Rec− and Rec+ strains, obtained at different times after exposure to DNA, was studied by centrifugation through neutral 5–20% sucrose gradients (Fig. 3). In the sedimentation pattern obtained with the Rec+ sample taken at 4 min, most of the radioactivity was found in slowly-sedimenting material. The two slow-sedimenting forms described above were poorly resolved under the conditions of this experiment. A small accumulation of rapidly-sedimenting material at the bottom of the tube was apparent. In the sedimentation pattern obtained with the material isolated from the Rec+ strain at 40 min, the amount of slow-sedimenting material had decreased in favor of the rapidly-sedimenting donor–recipient complex. A comparison of the sedimentation profiles of the Rec+ and Rec− strains showed no significant difference between the two strains. Faster-sedimenting material in the 40-min sample of the Rec− strain contains 67% of the total radioactivity, while 30% of the labeled DNA is found in the slower-sedimenting forms. In the 40-min sample obtained from Rec+ cells, 72% is in the dense, and 25% in the less-dense forms. The initial normalized transformation efficiency of Rec− in this experiment was 10% of that in the Rec+ strain. Using Rec+ cells (16), we have shown that the most rapidly sedimenting form is a complex of donor and recipient DNA that contains all of the recombinant and recipient, and almost all of the donor transforming activity found

in each sample. The results presented in Fig. 3, as well as those of Fig. 2, therefore demonstrate the formation of donor–recipient complex in the Rec⁻ strain to about the same as in the Rec⁺ strain.

Although donor–recipient complex does form in the Rec⁻ recipient, it is possible that the donor moiety fails to replicate. Competent Rec⁺ and Rec⁻ cells were incubated for 30 min with [³H]DNA from strain BD204, and further uptake was stopped by the addition of salmon-sperm DNA (100 μg/ml). The transformed cultures were washed with fresh, warm SP11 medium and were diluted 1:20. An aliquot was withdrawn to determine the total TCA-precipitable radioactivity in washed cells from each culture. When the optical density of the cultures, determined in a Klett colorimeter, had doubled, they were diluted two-fold, and one-half of each culture was removed and used for DNA extraction. An additional aliquot of the cultures was plated for Trp⁺ colony-forming units and for viable count. This procedure was repeated for a total of eight (optical density) doublings. The extracted DNA samples were tested for Trp⁺ and His⁺ transforming activities.

Fig. 4*a* shows the number of Trp⁺ colony-forming units and the viable count, measured after each doubling, for both strains. (The data has been corrected for dilution.) In this experiment, the optical density increased, with a doubling time of 40 min, immediately after dilution. The growth rate accelerated gradually, reaching a steady-state doubling time of 25–30 min after three generations. The viable count of both cultures increased exponentially after a lag time of one doubling. The number of Trp⁺ colony-formers increased exponentially after a lag of about two doublings, as expected from the results of Nester and Stocker (20). The Trp⁺/His⁺ transforming activity ratios, determined with DNA extracted from the two cultures and normalized to the TCA-precipitable radioactivity in each culture after 10 min of incubation with donor DNA, are presented in Fig. 4*b*. The purpose of this normalization was to adjust the initial Trp⁺/His⁺ ratios of both cultures to the same point, in order to facilitate comparison of later events in the two cultures. A clear difference in the behavior of the two strains is apparent. The normalized ratio for Rec⁺ reaches a constant value after an initial decrease. The Rec⁻ ratios, however, show a progressive decrease; they approach a value about 15% that of Rec⁺.

To interpret these results, it is important to recall that the Rec⁻ (*recB2*) lesion is partial, since 10–20% of the potential transformants survive as viable colonies (Table 1). In the Rec⁺ recipient, after replication of DNA begins, we would expect the Trp⁺/His⁺ transforming activity ratio to reach a constant value as the donor and recipient markers replicate concomitantly. Such a situation has been shown to occur in pneumococcus (21), and is apparent from the data in Fig. 4*b*. In Rec⁻, if the donor marker fails to replicate, the experimentally determined Trp⁺/His⁺ ratio should decrease reaching a plateau value 10–20% of that in Rec⁺. The kinetics of this process are described by the expression:

$$(\mathrm{Trp/His})_x = (\mathrm{Trp/His})_o\,[a + (1 - a)\exp(-g\ln 2)].$$

Where:

$(\mathrm{Trp^+/His^+})_x$ = ratio of Trp⁺ to His⁺ transforming activity at generation x, normalized to DNA uptake.

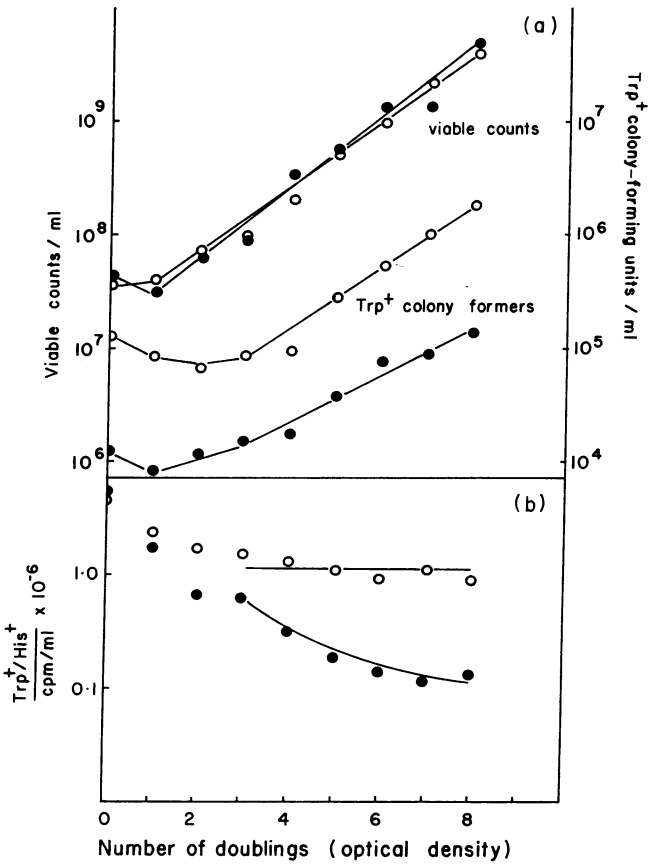

FIG. 4. Replication of the donor transforming marker (Trp⁺) in Rec⁺ and Rec⁻. *a*. Viable counts and Trp⁺ colony-forming units. Competent Rec⁺ and Rec⁻ strains were exposed for 30 min to BD204 [³H]DNA. Uptake was stopped by the addition of 100 μg/ml of salmon-sperm DNA and the culture was washed and diluted in fresh SPII medium. After each optical density doubling, the culture was diluted in half snd samples were taken for the determination of viable count and of the number of Trp⁺ colony-forming units per ml. The values plotted have been corrected for dilution.

b. Ratios of Trp⁺/His⁺ transforming activity in DNA extracts from samples taken after each division of the transformed cultures. The Trp⁺/His⁺ values were normalized to the amount of TCA-precipitable radioactivity in each culture after 10 min of incubation with donor DNA. The *solid lines* represent the theoretical Trp⁺/His⁺ ratios, calculated as described in the text. (●), Rec⁻; (○), Rec⁺.

$(\mathrm{Trp^+/His^+})_o$ = ratio of Trp⁺ to His⁺ transforming activity at the "zeroth" generation, normalized to DNA uptake.

a = relative transforming efficiency of Rec⁻, normalized to DNA uptake, and to Rec⁺ transformation (as in Table 1, last column),

g = number of generations, determined from the optical density of the growing culture.

In Fig. 4*b*, the theoretical curve for Rec⁻, determined from this equation, is displayed. To produce this curve, an experimentally determined value of 0.17 for "a" was used. The third doubling point (from optical density) was adopted as the "zeroth" generation, since this was the time at which the normalized Trp⁺/His⁺ ratio for the Rec⁺ samples achieved constancy. The experimental Trp⁺/His⁺ ratios

for Rec$^-$ presented in Fig. 4*b* agree within experimental error with the theoretical curve.

These results indicate that most of the donor DNA that associates with recipient DNA to form donor–recipient complex does not replicate in the Rec$^-$ recipient, but is diluted as the recipient cells divide. The data also indicate that no major destruction of donor genetic information occurs. The extent of replication of the donor marker is predictable from the extent of transformation obtained with the Rec$^-$ strain. In contrast, the constancy of the Trp$^+$/His$^+$ ratio in the Rec$^+$ samples indicates that all of the donor material that enters donor–recipient complex in the Rec$^+$ strain replicates concomitantly with recipient DNA.

DISCUSSION

The data presented above show that in the Rec$^-$ strain, after eclipse, the increase of donor and recombinant transforming activity is paralleled by the formation of a donor–recipient complex that can be monitored as rapidly-sedimenting material in sucrose gradients.

Our results show no substantial difference in the course of these events between the Rec$^+$ and Rec$^-$ recipients. The observed inability of the donor markers in Rec$^-$ to proceed with DNA replication may be due to some abnormality in the structure of the donor–recipient complex, or in its association with other cell components. It is also possible that the uptake and integration of transforming DNA in Rec$^-$ cells induces a generalized failure of these cells to replicate DNA, unrelated to the structure of the donor–recipient complex.

Noncovalently-bonded donor–recipient complexes have been observed in the course of recombination in coliphage T_4 (22). We have obtained preliminary evidence for such an intermediate form in Rec$^+$ (16). An inability of the Rec$^-$ strain to complete single-strand interruptions might result in a failure of chromosomal replication in transformed cells. Such a deficiency might also explain the radiation sensitivity of *recB2* strains. Although we are pursuing this possibility, we consider it unlikely, since a noncovalently-bonded intermediate would have a low donor and recombinant transforming activity (23–25), whereas these activities are normal or slightly higher in our Rec$^-$ than in our Rec$^+$ strains.

An ATP-dependent DNase has been shown to play a role in recombination and in repair of radiation damage (7–10). Preliminary experiments performed by Dr. B. Scher in our laboratory have indicated that the *recB2* strain possesses normal amounts of this enzyme.

We would like to acknowledge valuable discussions with Drs Issar Smith and Leonard Mindich, and the expert secretarial assistance of Annabel Howard. This work was supported by National Science Foundation grant no. GB-18146 awarded to D. D. R. D. A. was supported by predoctoral training grant no. 2-TO-1-HE 05307 from the National Institutes of Health, administered by the Department of Microbiology, N.Y.U. School of Medicine.

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