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Integration and Induction of Phage P22 in a Recombination-deficient Mutant of *Salmonella typhimurium*[1]

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Phage P22 can integrate as prophage into a recombination-deficient (Rec^-) strain of *Salmonella typhimurium*. At 37 C, the integration efficiency is only 10% that in Rec^+ infection, but at 25 C the efficiencies in Rec^- and Rec^+ hosts are similar. Rec^- lysogens cannot be induced by ultraviolet irradiation or by treatments with the chemical inducing agents streptonigrin or mitomycin C. Heat induction of Rec^- cells lysogenic for a temperature-sensitive c_2 mutant (*ts* c_2) is normal, showing that the Rec^- cell has the machinery necessary for prophage excision. Ultraviolet irradiation of Rec^- (*ts* c_2) lysogens prior to heat induction does not prevent the formation of infective centers after temperature shift. Thus, the noninducibility of Rec^- lysogens is not due to destruction of the prophage as a result of ultraviolet irradiation. Deoxyribonucleic acid-ribonucleic acid (RNA) hybridization experiments demonstrate that no increase in phage-specific RNA synthesis occurs after ultraviolet irradiation of a Rec^- (c^+) lysogen. The Rec^- mutant appears to lack part of the mechanism required to destroy the phage repressor and allow the initiation of early phage functions such as messenger RNA synthesis. A similar conclusion was reached previously for an *Escherichia coli* Rec^- strain.

A previous paper (13) described the isolation and characterization of a recombination-deficient, ultraviolet (UV)-sensitive mutant of *Salmonella typhimurium* strain LT-2. In addition to being recombination-deficient after conjugation, the strain is unable to undergo general transduction by phage P22 (13), a process which involves recombination between the bacterial chromosome and a bacterial deoxyribonucleic acid (DNA) fragment. On the other hand, recombination between appropriately marked phage P22 mutants infecting the Rec^- bacteria occurs with normal frequencies (Wing, *unpublished data*). It appears, therefore, that recombinational events involving only bacterial DNA are prevented by the Rec^- lesion, whereas, in those involving only phage DNA, the phage genome contributes a recombinational system which overcomes the Rec^- lesion.

According to the model proposed by Campbell (2), prophage integration involves the insertion of the phage genome into the host chromosome by a recombinational process. Prophage induction is presumed to entail similar steps in the reverse order. Since integration and induction involve recombination between phage and bacterial DNA, it was of interest to determine whether the phage recombinational system could promote these processes in a Rec^- host. It is shown in this paper that prophage integration does take place in a Rec^- strain. Induction of prophage by UV irradiation, however, does not occur. Various explanations for this finding were investigated by using lysogens of a heat-inducible phage P22 mutant.

Results similar to some of those described here have been recently reported for an *Escherichia coli* Rec^- strain lysogenic for phage λ (1).

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains employed are all derivatives of *S. typhimurium* strain LT-2: *leu197* (from the collection of Demerec); *rec*$^-$ *leu197* (13); R17, a UV-sensitive, non-host-cell-reactivating (Wing, *unpublished data*), leucine-requiring strain isolated by H. O. Smith; and the wild-type strain.

Phage strains used were: wild-type phage P22 (c^+); c_1, a clear mutant altered in a gene required

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for the establishment of lysogeny (6); c_2, a clear mutant altered in the structural gene for phage P22 repressor (6, 8); and *ts* c_2 (8), which contains a temperature-sensitive mutation in the c_2 cistron. Plaques of *ts* c_2 phage incubated at 25 C are turbid, but those incubated at 40 C are clear. Lysogens of *ts* c_2 are stable at room temperature, but are induced to produce phage when growing cells are transferred to 40 C.

Media. L broth, soft agar for top layers, buffered saline (6), M9, and supplemented M9 (11) have all been previously described. Tryptone agar contains 10 g of tryptone (Difco), 5 g of NaCl, and 12 g of agar (Difco) in 1 liter of distilled water.

Streak test for immunity. Phage P22 c_2, at about 10^{11} phage particles per ml, was streaked across an agar plate. Colonies to be tested for lysogenic immunity were streaked across the phage streak. After overnight incubation, a break in the continuity of the line of bacterial growth indicated that the colony was sensitive to phage. The absence of a break was interpreted as evidence that the clone was exhibiting lysogenic immunity and therefore carried a prophage.

UV irradiation. Cells were grown at 23 or 37 C in L broth with aeration to a concentration of about 10^8 cells/ml, diluted into buffered saline, and irradiated in glass petri dishes. UV light was administered with a 15-w Westinghouse germicidal lamp at a distance of 50 cm, or as stated. The dose rate at 50 cm was 10 ergs per mm^2 per sec. For induction, a dose of 30 sec at 50 cm was usually used. The cells were then either assayed or diluted into L broth for further growth. All operations subsequent to exposure to ultraviolet light were carried out in dim light or in red glass vessels to avoid photo-reactivation of the irradiated cells.

DNA-ribonucleic acid (RNA) hybridization. Tritium-labeled RNA was prepared by a method similar to that described by Green (4). Bacteria were grown to a concentration of about 10^8 cells/ml in supplemented M9 medium with aeration at 37 C. At various times, 5.5-ml samples were pulse-labeled for 30 sec with tritiated uridine (4.5 μc/ml, specific activity 24.4 c/mmole; Schwarz BioResearch, Inc., Orangeburg, N.Y.). The incorporation of label was stopped by pouring the samples into a tube containing one-half volume of ice to which unlabeled uridine and NaCN had been added to a final concentration of 100 μg/ml and 0.01 M, respectively. The cells were centrifuged, washed with one volume of 0.01 M tris-(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.4) containing 0.005 M $MgSO_4$ and 0.01 M NaCN, and centrifuged again.

To the pelleted cells, 0.1 ml of a solution of 50 μg/ml of deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.) and 300 μg/ml of lysozyme (Worthington) in 0.01 M Tris (*p*H 7.4) were added and the suspension was well mixed. The cells were then frozen in an acetone-dry ice mixture, thawed, and held at 14 C for 5 min. Lysis was accomplished by addition of 10 μliters of 30% Sarkosyl (sodium *N*-lauroyl sarcosinate, Geigy Chemical Corp., Ardsley, N.Y.) and incubation at 60 C for 10 min. Water was then added to bring the volume to 1 ml.

Unlabeled phage and bacterial DNA were kindly provided by H. O. Smith. The DNA molecules were extracted as described by Smith (10). DNA was denatured by heating in boiling water for 10 min.

Hybridization was accomplished by the method of Green (4). A portion of each sample of RNA was added to three tubes. One contained 0.1 ml (30 μg) of denatured phage DNA, the second contained 0.1 ml (30 μg) of denatured bacterial DNA, and the third, 0.1 ml of water. The third tube was a control to determine nonspecific binding of label to filters. Each tube also contained saline citrate buffer to a final concentration of two times SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate). The volume in the tubes was approximately 0.3 ml. The tightly stoppered tubes were incubated 4 to 6 hr at 60 C, then diluted with water to a final volume of 1 ml. Ribonuclease (Sigma Chemical Co., St. Louis, Mo.), preboiled to destroy any traces of deoxyribonuclease activity, was added at a final concentration of 10 μg/ml to degrade single-stranded unhybridized RNA; the reaction mixtures were incubated at 37 C for 10 min. The samples were then diluted fivefold with 0.5 M KCl-0.01 M Tris (*p*H 7.4) at room temperature, filtered through nitrocellulose membrane filters (Schleicher and Schuell Co., Keene, N.H.; 24 mm, type B-6, presoaked in the KCl-Tris solution), and washed with at least 40 ml of the buffer. RNA-DNA hybrid molecules stick to the filter, whereas single-stranded molecules do not (9). The filters were dried and counted in toluene scintillation fluid. All samples were corrected for nonspecific binding.

RESULTS

Prophage integration. Integration of phage P22 DNA into the bacterial chromosome is a very late function, occurring between the first and third division after infection (12). Smith and Levine have defined *integration efficiency* as the ratio of phage carriers to total cells after many generations. To obtain this value in infections of Rec$^-$ and Rec$^+$ cells, the following experiment was carried out. Log-phase cells were infected with phage c^+ at a multiplicity of about 20. After a 5-min adsorption period, the cells were diluted to about 10^3 cells/ml in L broth containing anti-phage serum (K = 3) and maintained at this concentration by repeated dilutions. At intervals, samples were plated on tryptone-agar plates. Individual colonies were picked from the tryptone plates and tested for lysogenic immunity by cross-streaking. About 100 colonies were tested for each point and the percentage of phage carriers and sensitive segregants was determined.

The segregation pattern of Rec$^+$ (*leu 197*) cells infected with phage c^+ at 37 C is shown in Fig. 1A. After a lag in division of about one generation time, the survivors (80% of the infected cells) began to divide; thereafter, the total number of cells increased logarithmically. All the cells were phage carriers for the first few divisions, but eventually segregation of sensitive cells occurred

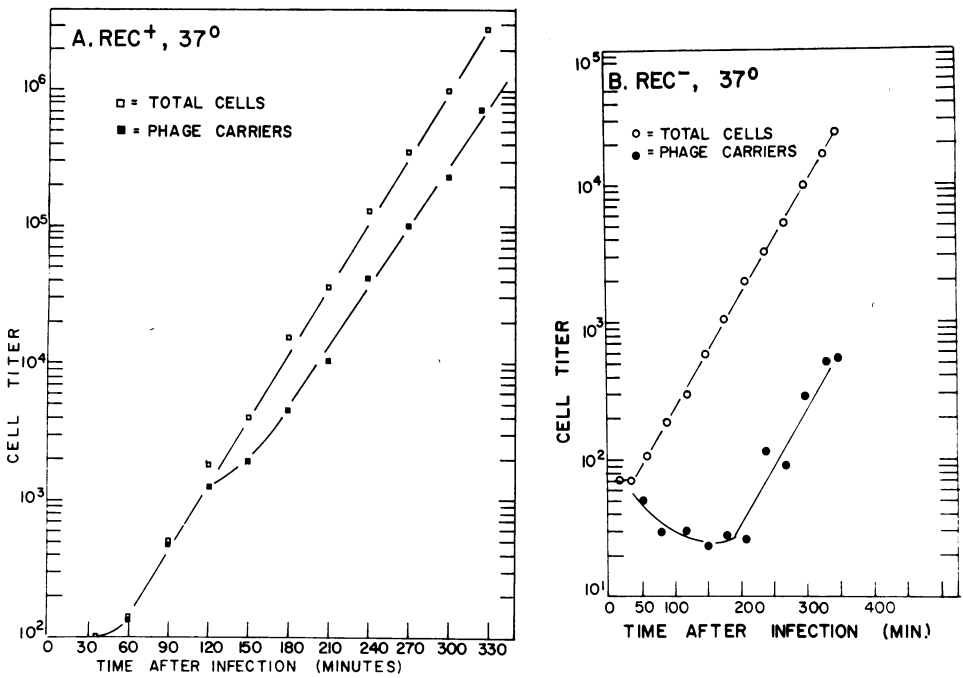

FIG. 1. *Segregation patterns for phage c⁺-infected Rec⁺ and Rec⁻ cells at 37 C.*

until a constant ratio of phage carriers to total cells was reached. This ratio, the integration efficiency, is about 25% for the Rec⁺ culture at 37 C. The initially high proportion of phage carriers is due to the several intact phage genomes present in each cell after infection. These genomes are distributed to separate cells in subsequent divisions.

The segregation pattern for the c^+-infected Rec⁻ cells (Fig. 1B) was quite different. The number of phage carriers did not increase for the first 3 hr. When the number of phage carriers began to increase exponentially, the integration efficiency was found to be only about 2%, less than 10% that for Rec⁺ infection.

The experiment was repeated at 25 C, a temperature at which integration in wild-type cells proceeds with much greater efficiency than at 37 C (12). Figures 2A and 2B show that, for the Rec⁺ strain, the integration efficiency at 25 C was about 98%, while the efficiency for infected Rec⁻ increased to 75%. Thus, Rec⁻ cells are able to integrate phage genomes, although with a reduced efficiency and greater temperature sensitivity than Rec⁺ cells.

Spontaneous induction. A log-phase culture of *S. typhimurium* LT-2 lysogenic for phage c^+ contains approximately one free phage per 1,000 cells. These phage, normal by all criteria, are released by the occasional spontaneous lysis of a lysogenic cell. In contrast, an exponentially growing Rec⁻ (c^+) culture contains 100-fold fewer free phage. Furthermore, a substantial proportion—up to 30%—of the free phage are clear mutants, and another 5 to 10% are of the V_1 (14) or Mnt (M. Gough, Bacteriol. Proc., p. 159, 1968) phenotype. These are phages mutant in a gene necessary for the maintenance of lysogeny. Complementation tests (6) showed that the majority of the clear mutants are of the c_2 type, also altered in a gene necessary for the maintenance of lysogeny (6, 8). Of the remaining normal-appearing turbid plaque formers, about 50% also appear to be defective in maintaining lysogeny (Wing, *unpublished data*). Only a minority of the plaques are wild type by available tests. Thus, a large proportion of phages spontaneously released from Rec⁻ lysogens are defective in their ability to maintain lysogeny.

UV and chemical induction. Production of phage by Rec⁻ lysogens could not be induced by UV doses between 5- and 90-sec duration. Nor were the lysogens induced by treatments with streptonigrin (10 to 25 μg/ml, 5 to 15 min, 37 C) or mitomycin C (4 to 10 μg/ml, 10 to 15 min, 37 C), which induce Rec⁺ cells (7). However, the Rec⁻ strain was extremely sensitive to killing by all of these inducing agents (13).

Three possibilities may be considered to ex-

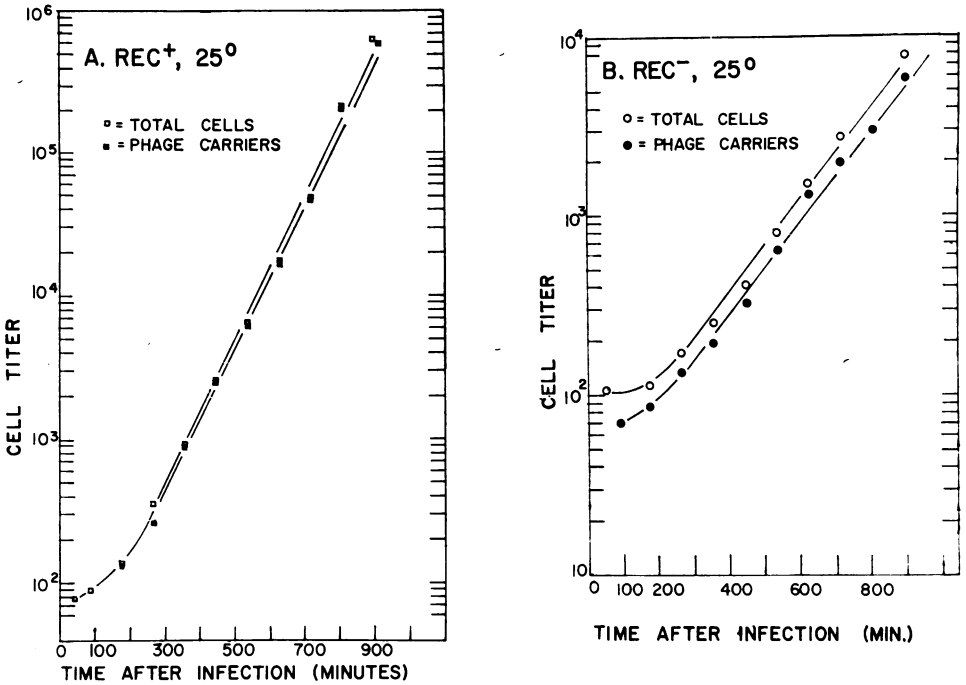

FIG. 2. *Segregation pattern for phage c⁺-infected Rec⁺ and Rec⁻ cells at 25 C.*

plain the noninducibility of the Rec⁻ lysogen. (i) Since prophage detachment probably involves a recombinational step, the Rec⁻ lysogen may be unable to detach the prophage from the bacterial chromosome. (ii) Rec⁻ cells degrade their DNA extensively during normal growth, and even more so after UV irradiation (13). The integrated prophage may share the increased UV sensitivity of the Rec⁻ bacterial DNA and may be degraded after an inducing dose of UV light. (iii) The Rec⁻ strain may lack part of the mechanism which results in inactivation of the phage repressor after UV irradiation.

These possibilities were investigated by the use of a heat-inducible phage c_2 lysogen.

Heat induction of Rec⁻ lysogens. Rec⁻ and Rec⁺ lysogens carrying mutant phage P22 *ts* c_2 as prophage were prepared by infection at 25 C. To study the kinetics of heat induction of these lysogenic strains, the cells were first grown to log phase at 23 C. At time zero, they were diluted into medium prewarmed to the inducing temperature (40 to 42 C). Samples were removed at intervals, plated on tryptone-agar plates with and without indicator bacteria, and incubated at 25 C for 24 to 48 hr.

Heat induction of Rec⁺ (*ts* c_2) and Rec⁻ (*ts* c_2) lysogens gave very similar results (Fig. 3). Incubation of both lysogens at 41 C for 20 min resulted in the conversion of essentially all the cells to infective centers. Since heat induction affects the repressor directly, this finding shows that once the repressor is inactivated, the Rec⁻ lysogen has all the machinery for detaching and replicating the prophage. The first hypothesis, that the Rec⁻ strain cannot detach the prophage, is therefore not valid.

Effect of prior UV irradiation on heat induction. Heat-inducible lysogens can be used to separate the action of UV light as an inducing agent from its degradative effect on DNA. Induction can be triggered by heat, and the effect of UV light on subsequent phage production can then be observed. A comparison of the effects of UV irradiation on Rec⁻ and Rec⁺ *ts* c_2 lysogens was facilitated by the fact that *ts* c_2 lysogens are not UV-inducible.

To learn whether UV light causes destruction of the prophage in a Rec⁻ lysogen (hypothesis 2), log-phase cells grown at 23 C were diluted into buffered saline and samples were exposed to increasing doses of UV light. Infective centers were then assayed on tryptone-agar plates incubated at the inducing temperature (42 C). The log-phase cells were treated with antiphage serum to eliminate free phage; all plaques scored thus represented infective centers. As controls, the *ts* c_2 lysogens of Rec⁺ (wild type) and a non-host-cell-

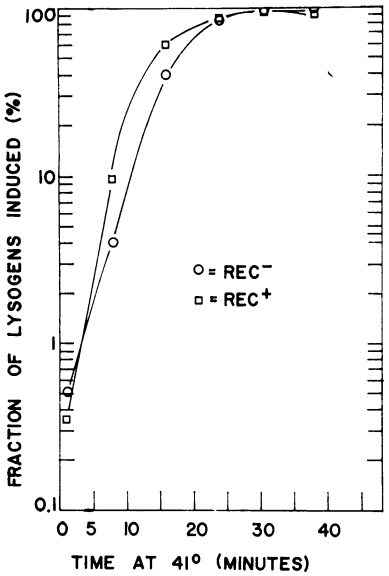

FIG. 3. *Heat induction of Rec⁺ and Rec⁻ cells lysogenic for phage ts c_2. At various times after transfer of the cultures to 41 C, a sample was removed and the number of infective centers was assayed at 25 C.*

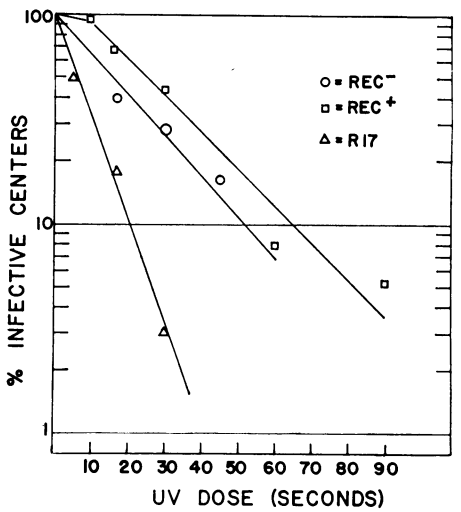

FIG. 4. *Effect of prior UV irradiation on heat induction of ts c_2 lysogens. Lysogenic cells grown at 23 C were exposed to increasing doses of UV light before transfer to 41 C. The number of infective centers subsequently produced is given as a percentage of the number produced in the absence of UV irradiation.*

reactivating (HCR⁻), UV-sensitive mutant, R17 (Wing, *unpublished data*), were used.

Figure 4 presents the results for the three strains given as percentages of the infective centers observed in the absence of UV irradiation. All three cultures evidenced an increasing loss of infective centers with increasing UV doses. The loss was greatest in the R17 strain, which cannot repair UV damage to the phage. A typical inducing dose—30 sec—reduced the number of infective centers produced by the R17 lysogen to 3% of the number produced by heat induction in the absence of UV irradiation. The curve for the Rec⁻ lysogen, on the other hand, was very similar to that for the Rec⁺. Especially significant was the finding that exposure to a 30-sec inducing dose still allowed production of 30% of the infective centers found in the absence of irradiation. This is not very different from the value of 50% observed in the Rec⁺ control. Clearly, destruction of prophage cannot account for the failure of UV light to induce a Rec⁻ lysogen.

Because the *ts* c_2 mutant used for the above experiment was not inducible by UV light, the possibility remained that the killing effect of UV light on *ts* c_2 might differ from its effect on wild-type, UV-inducible phage. To exclude this possibility, a UV-inducible, temperature-sensitive c_2 mutant was isolated. Rec⁻ and Rec⁺ lysogens were prepared and subjected to the above experiment. The results were very similar to those shown in Fig. 4, indicating that the survival of *ts* c_2 after UV irradiation was not significantly affected by its noninducibility.

The remaining possibility for the noninducibility of the Rec⁻ lysogen was that it lacks part of the mechanism required to inactivate the prophage repressor. Inactivation of the repressor allows the initiation of synthesis of large amounts of phage-specific messenger RNA, the presence of which can be detected by hybridization with phage DNA (4). It is thus possible to determine whether phage-specific messenger RNA synthesis is initiated in a Rec⁻ lysogen after UV irradiation.

An experiment was carried out in which log-phase Rec⁻ (c^+) and Rec⁺ (c^+) cells were pulse-labeled for 30 sec with ^{3}H-uridine at various times before and after exposure to an inducing dose of UV. RNA was then extracted and hybridized with bacterial and phage DNA. The results are shown in Fig. 5. The rate of phage-specific RNA synthesis in the Rec⁺ lysogen remained at the repressed level of 0.07% for about 30 min, at which time it began to rise rapidly. This rise began just before the onset of phage DNA synthesis, which usually occurs at about 35 min after UV irradiation (10). The rate of synthesis of bacterial RNA was unaffected by UV irradiation.

The Rec⁻ lysogen showed no increase in the rate of phage-specific RNA synthesis after UV irradiation. The rate of synthesis of this RNA

never rose above the initial level of 0.07%. As in the case of the Rec+ lysogen, the transcription of bacterial DNA seemed unaffected, at least for the duration of the experiment (80 min).

A similar experiment was performed on Rec− and Rec+ cells lysogenic for phage *ts* c_2, with heat induction replacing UV irradiation. In marked contrast to the irradiation results, the Rec− and Rec+ strains behaved in a similar fashion. Figure 6 shows that in both strains the rate of phage-specific RNA synthesis increases immediately after transfer to 41 C, and by 10 to 15 min ex-

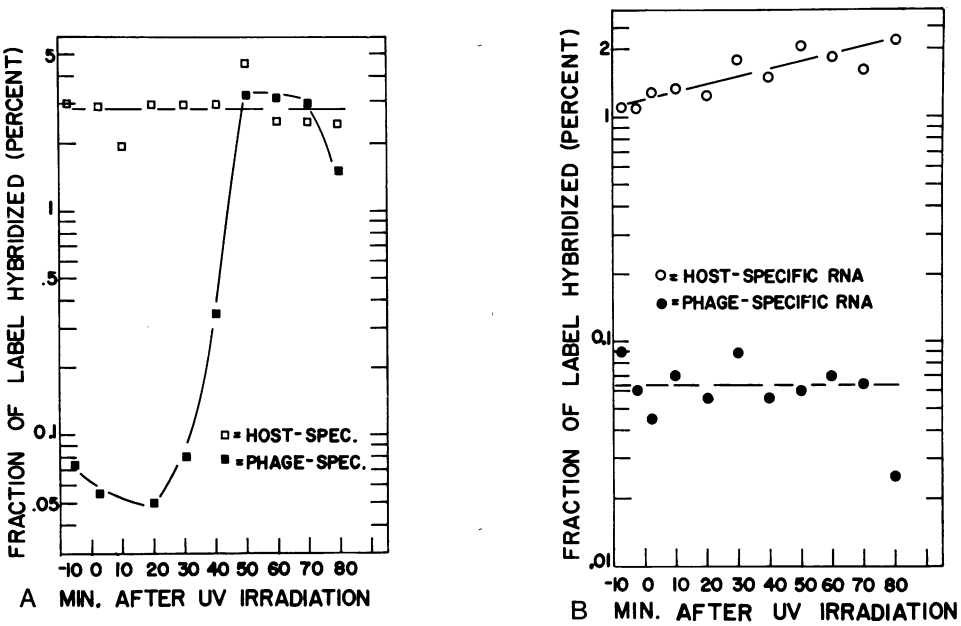

FIG. 5. *Production of phage-specific RNA by (A) Rec+ (c^+) and (B) Rec− (c^+) strains after UV irradiation. At time zero, the cells were exposed to UV light for 30 sec. Samples were pulse-labeled at 10-min intervals with ^{3}H-uridine. RNA was extracted and hybridized with unlabeled phage and bacterial DNA.*

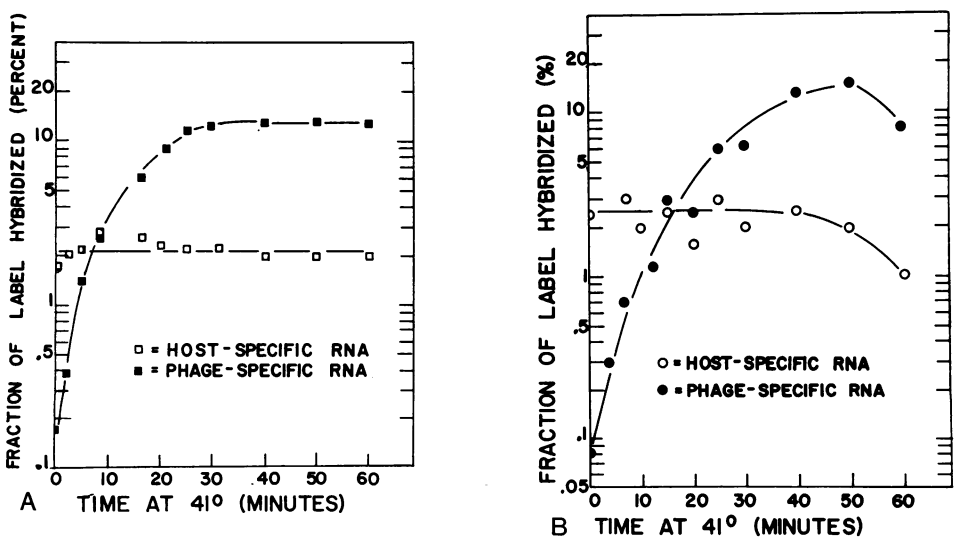

FIG. 6. *Production of phage-specific RNA by (A) Rec+ (*ts* c_2) and (B) Rec− (*ts* c_2) strains during heat induction. The cultures were grown at 23 C and at time zero were transferred to 41 C. Pulse-labeled RNA was extracted and hybridized with unlabeled phage and bacterial DNA.*

ceeds the rate of cell-specific RNA synthesis. This result was expected since heat induction occurs normally in the Rec$^-$ cells, whereas UV induction does not.

DISCUSSION

The segregation patterns of Rec$^-$ cells infected with phage P22 c^+ at 25 and 37 C have shown that prophage integration does occur in Rec$^-$ cells, although with a reduced efficiency and greater temperature sensitivity than in Rec$^+$ cells. At 25 C, the integration efficiency is 75%, as compared to 98% for Rec$^+$, suggesting that the Rec$^-$ lesion does not have a significant effect on integration. The reason for the increased temperature sensitivity is unclear. One possibility is that the extensive DNA degradation exhibited by the Rec$^-$ strain hinders integration, and there may be less degradation at 25 C.

The inability of Rec$^-$ lysogens to be induced by UV light shows that at least one step in the induction process is under the control of a bacterial function lacking in the Rec$^-$ strain. This step is not prophage detachment. The normal heat induction of Rec$^-$ (*ts* c_2) demonstrates that prophage detachment can occur in the Rec$^-$ strain. Nor can the failure of induction be due to destruction of prophage. A determination of the effect of prior UV irradiation on heat induction of Rec$^-$ (*ts* c_2) leads to the conclusion that UV light does not cause significant destruction of the prophage in a Rec$^-$ lysogen.

The bacterial function necessary for induction which is missing in the Rec$^-$ strain is concerned with the inactivation of the phage repressor. This conclusion arises from the DNA-RNA hybridization experiments, which show that Rec$^-$ (c^+) lysogens do not evidence any increase in the rate of phage-specific RNA synthesis after UV irradiation. From this, it may be inferred that the lysogenic repressor is still active in the cell, preventing the initiation of early phage functions such as the synthesis of phage messenger RNA.

Brooks and Clark (1) have demonstrated more directly that lysogenic repressor is present in an irradiated *E. coli* Rec$^-$ strain carrying prophage λ. UV irradiation normally destroys the immunity of λ lysogens to superinfecting phage and allows the development of the superinfecting phage. They found that irradiated Rec$^-$ lysogens were still immune to superinfection and therefore must contain a functional repressor.

Of the reduced number of free phage observed in a culture of Rec$^-$ lysogens, a high proportion are mutants defective in their ability to maintain lysogeny. These probably arise from spontaneous mutations which occur in the prophage. They are probably present in the cultures of all growing lysogens, but are obscured by the large number of wild-type phage particles released by the normal mechanism of spontaneous induction. This normal mechanism is missing in Rec$^-$ lysogens. Spontaneous induction of normal phage may thus occur by a mechanism the same as or similar to UV induction.

It thus appears that Rec$^-$ lysogens can be induced only if the repressor is directly destroyed (as by heat induction) or if a mutation affecting the maintenance of lysogeny occurs in the prophage. A growing Rec$^-$ culture should be an excellent source of new mutants defective in maintenance of lysogeny. A number of isolates are currently being investigated.

It has been suggested that the interruption of bacterial DNA synthesis results in prophage induction (3, 7). The Rec$^-$ lysogen is highly sensitive to killing by UV irradiation. Furthermore, a growing Rec$^-$ culture contains a large fraction of nonviable cells in which DNA replication has presumably been interrupted (13). Yet the Rec$^-$ lysogen is even more stable to spontaneous and UV induction than is the Rec$^+$ lysogen. Interruption of DNA synthesis, although perhaps necessary, is therefore not sufficient for prophage induction. Induction must be due to an indirect effect of UV irradiation, involving the formation of a specific product which inactivates the prophage repressor. A role for a specific inducing substance was first proposed by Goldthwait and Jacob (3). The formation of the specific product may be the function of the *rec*$^+$ gene. The finding that a *rec*$^+$ gene introduced by transduction into a lysogenic *E. coli* Rec$^-$ strain results in UV inducibility of the λ prophage (5) favors the idea that the *rec*$^+$ gene product functions in the formation of the inducing substance.

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