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Proteolytic Cleavage of Bacteriophage Lambda Repressor in Induction

(mitomycin C/*recA*/λc_1*ind*$^-$)

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ABSTRACT **The bacteriophage lambda repressor, a protein that maintains the lysogenic state of a bacterium containing a lambda prophage, is cleaved when the lysogen is induced by mitomycin C or ultraviolet light. This cleavage does not occur when induction is prevented by mutational alteration either of the phage repressor or of the host *recA* gene product. Proteolytic cleavage may be the primary mechanism of repressor inactivation in this induction pathway, or it may follow a different event which causes the initial inactivation.**

The lysogenic state of a bacterium carrying bacteriophage lambda is maintained by the phage-encoded immunity repressor, a protein which prevents expression of the viral genes required for lytic growth (1). A λ prophage is induced to grow by various treatments of the lysogen which destroy repression: these include irradiation with ultraviolet light, growth in the presence of the antibiotic mitomycin C, and inhibition of bacterial DNA synthesis by other means (2–4). Such treatments lead to inactivation of the repressor molecule through a complex metabolic process which requires the products of several bacterial genes, metabolic energy, and probably protein synthesis (2, 5). Although one can generalize that induction of λ is initiated by damage to bacterial (and/or) prophage DNA or interference with DNA synthesis, little is known about the induction process and the mechanism by which the phage repressor is inactivated. It has been observed that the DNA binding activity of λ repressor cannot be detected in extracts of induced lysogens, and that this activity cannot be restored by dialysis of the induced extract (ref. 7 and unpublished results of P. Chadwick). Thus induction of λ may involve a different mechanism from the classical case of induction of the lactose operon (6), in which inactivation of the repressor occurs through reversible binding of a small molecule.

We have examined the induction mechanism by exploring the fate of phage repressor molecules in induced cells. We find that λ repressor undergoes proteolytic cleavage upon induction, producing at least one fragment approximately half the size of repressor; this breakdown accounts for the lack of repressor activity in extracts of induced cells. We infer from its kinetics that cleavage either closely follows the inactivation of repressor or itself constitutes the inactivation event.

MATERIALS AND METHODS

Bacterial and Bacteriophage Strains. Escherichia coli W3102, W3102(λ*N7N53*), W3102(λ*N7N53*c_1*ind*$^-$), W3102*recA3*(λ-*N7N53*), W3102(λi^{434}), and wild-type bacteriophage λ were obtained from M. Ptashne. All wild-type λ lysogens were constructed from the same source of bacteriophage. *E. coli* JC4728, JC2926, JC2917, and JC9239 containing respectively the *recA142*, *recA13*, *recA12*, and *recF143* alleles were obtained from A. J. Clark.

Cell Growth. For radioactive labeling of proteins cells were grown in a low sulfur medium containing per liter: 7.0 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 1.0 g of NaCl, 0.1 g of $MgCl_2$, 3 μmol of $FeCl_3$, 100 μmol of $CaCl_2$, 1.0 mg of thiamine, 2.0 g of glucose, and either 40 or 60 μmol of $MgSO_4$. For growth of JC4728, JC2926, JC2917, and JC9239 the medium was supplemented with threonine, leucine, proline, histidine, and arginine to 60 μg/ml of each. Cultures to be labeled were inoculated to an OD_{550} of 0.01 from an overnight culture grown to glucose starvation in the same medium supplemented to 0.2 mM $MgSO_4$. Labeling was initiated by the addition of $H_2{}^{35}SO_4$ to an exponentially growing culture. The generation times of the various strains growing in this medium at 37° ranged from 65 to 80 min. Nonradioactive carrier cells were nonlysogenic W3102 grown in the same medium to an OD_{550} of 1, concentrated 20-fold, and stored frozen in medium lacking glucose.

Extract Preparation. Samples of 0.5 ml (or occasionally 1.0 ml) of a labeled culture were pipetted into 1.0 ml concentrated carrier cells at 0° prepared as above and supplemented to 0.01 M NaN_3. The cells were pelleted and frozen. For lysis, cell pellets were thawed in 25 μl of a buffer containing 0.01 M Tris·HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.05 M KCl, and 0.6 mg/ml of lysozyme, and were subjected to five cycles of freezing and thawing. After addition of 50 μl of a buffer containing 0.01 M Tris·HCl, pH 7.9, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.01 M KCl, 5% glycerol, 20 μg/ml of deoxyribonuclease I, and 10 μg/ml of ribonuclease A, the extracts were incubated at room temperature for 30 min. Debris was removed by centrifugation for 15–30 min at 27,000 × *g* in a Sorvall SS-34 rotor.

Antibody Precipitation. All steps were performed at 0–4°. The extract (80–100 μl) was diluted with 100 μl of a buffer containing 0.05 M Tris·HCl, pH 7.5, 2.0 M KCl, and 2% Triton X-100. Sufficient purified rabbit anti-λ repressor IgG was added to complex at least 10 to 12 times the amount of repressor present. Pilot experiments were performed to determine the appropriate amount of antibody. After an incubation of 10–15 hr to form the primary repressor–antibody complex, the rabbit IgG was precipitated by addition of an

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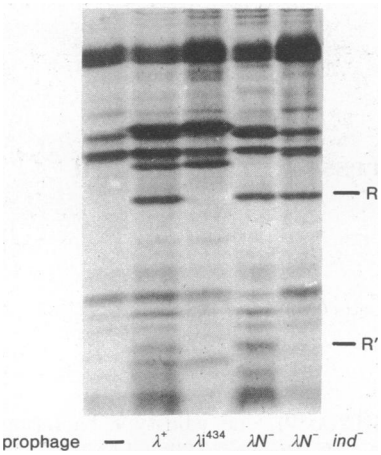

FIG. 1. Identification of λ repressor by antibody precipitation. *E. coli* W3102 cells containing various prophages were grown at 37° in low sulfur medium containing 40 μM $MgSO_4$. After growth until the OD_{550} of the culture reached 0.10, $H_2{}^{35}SO_4$ was added to 0.40 mCi/ml. Growth continued until the OD_{550} reached 0.60, when nonradioactive Na_2SO_4 was added to 7 mM. After a 5-min chase, samples of 0.50 ml were taken, processed, and subjected to electrophoresis as described in *Materials and Methods*. The direction of electrophoresis is from top to bottom in the figures. In addition to numerous bacterial polypeptides which appear in all samples, there exist several bands which occur in both λ^+ and λi^{434} lysogens and are absent in the parent cell. These are probably phage-specific products synthesized in spontaneously induced cells, because they are not produced by the λN^- prophage, which is defective in lytic growth; they are also absent in a lysogen of $\lambda N^+ c_I ind^-$, which does not induce spontaneously (data not shown). Contaminating phage proteins are enriched over contaminating bacterial proteins in the precipitate because the nonlysogenic nonradioactive carrier cell proteins dilute only labeled bacterial proteins. The pattern of background proteins is not precisely reproducible and varies somewhat in the experiments shown here. The molecular weight of fragment R' was determined by electrophoresis in parallel with phage T4-specific polypeptides of the bacterial RNA polymerase which have been carefully sized (20); it migrates between polypeptides of 15,000 and 12,000 molecular weight and is closer to the former, so that a molecular weight of 14,000 is assigned to it.

equivalent amount of goat antiserum to rabbit IgG. After at least 1 hr at 0°, the precipitates were pelleted (15 min at 27,000 × *g*); dispersed in 0.5 ml of 0.05 M Tris·HCl, pH 7.5, 1.2 M KCl, and 1.2% Triton X-100; pelleted again; suspended again; pelleted again; suspended in 0.5 ml of 0.05 M Tris·HCl, pH 7.5, and 0.1 M NaCl; pelleted; and subjected to electrophoresis.

The double antibody precipitation (8) was chosen to render the efficiency of precipitation independent of the concentration of λ repressor (antibody was always in excess) and to facilitate handling the precipitates. It also made possible the competition analysis which provided a definitive identification of the repressor and repressor fragment.

We found that inclusion of Triton X-100 and KCl in the precipitation mixture (9) was essential to obtain a sufficiently low background of nonspecific proteins in the precipitate to allow detection of repressor and its degradation product; the detergent and salt provide at least a 10-fold decrease in the background.

Electrophoresis and Autoradiography. For electrophoresis, washed antibody pellets were dispersed in 30 μl of 0.08 M Tris·HCl, pH 6.8, containing 25% glycerol; mixed with 10 μl of 8% sodium dodecyl sulfate, 1.7 M 2-mercaptoethanol, and 0.1% bromphenol blue; and heated 2 min at 100°. Twenty microliters of the sample was layered and electrophoresed on a 10–40% polyacrylamide gradient slab gel prepared as described by Studier (10). The gels were dried (10) and subjected to contact autoradiography for 4–20 days. For staining of proteins gels were fixed 1 hr in 50% trichloroacetic acid, stained in Coomassie Brilliant Blue (11), and destained by diffusion before drying and autoradiography.

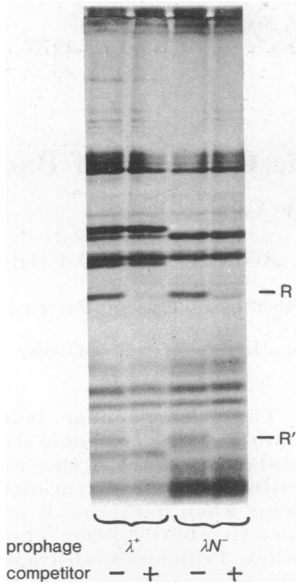

FIG. 2. Effect of purified unlabeled repressor on antibody precipitation of polypeptide R. Cultures of W3102(λ^+) and W3102($\lambda N7N53$) were grown, labeled, and sampled as described in the legend to Fig. 1. For precipitations with unlabeled competitor, 4 μg of purified λ repressor was mixed with the extract before addition of antibody.

Material. Purified λ repressor and purified rabbit anti-λ repressor IgG were gifts of K. Backman and P. Chadwick. Carrier-free $H_2{}^{35}SO_4$ was obtained from New England Nuclear; deoxyribonuclease I and lysozyme from Worthington; mitomycin C and ribonuclease A from Sigma; goat antiserum to rabbit IgG from Pentex; acrylamide and bisacrylamide from Bio-Rad; sodium dodecyl sulfate from Pierce; and Triton X-100 from Sigma.

RESULTS

To detect the λ repressor molecule in lysogenic cells, we labeled total bacterial proteins with $^{35}SO_4$, purified the repressor from radioactive extracts by precipitation with antibody to λ repressor, and analyzed the antibody precipitate by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Fig. 1 shows such a gel analysis of labeled precipitated proteins from four lysogens and their nonlysogenic parent. Numerous radioactive polypeptides appear in the antibody precipitates from all of the cells, as might be expected from this single purification step for a protein present as only 0.01% of the total. However, one polypeptide of molecular weight 27,000 (labeled R in Fig. 1) is present in the three lysogens and is absent in both the parent and the lysogen of λi^{434}, a phage nearly identical to λ but carrying a different repressor. Since this polypeptide comigrates in electrophoresis

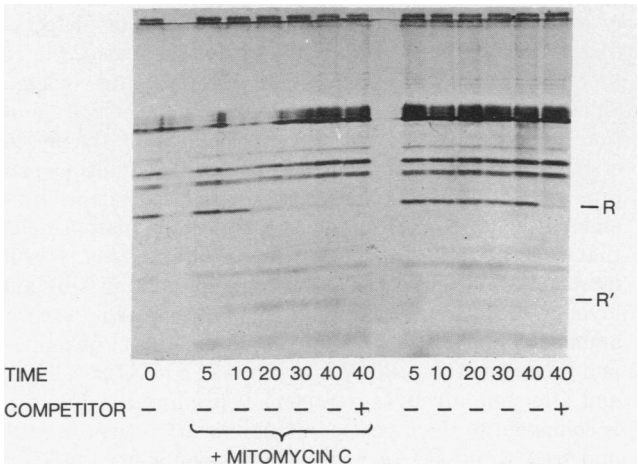

FIG. 3. Repressor breakdown upon induction. A culture of W3102(λ*N7N53*) was grown in low sulfur medium containing 40 μM $MgSO_4$ to an OD_{550} of 0.40; $H_2{}^{35}SO_4$ was added to 0.40 mCi/ml and growth was continued to an OD_{550} of 0.50. Na_2SO_4 was added to 7 mM, followed by 5 min of growth. The zero time sample was taken and the culture was split, one half receiving 5 μg/ml of mitomycin C; each portion was sampled at the indicated times (in minutes). For competition 4 μg of nonradioactive λ repressor was mixed with the extract before addition of antibody to λ repressor.

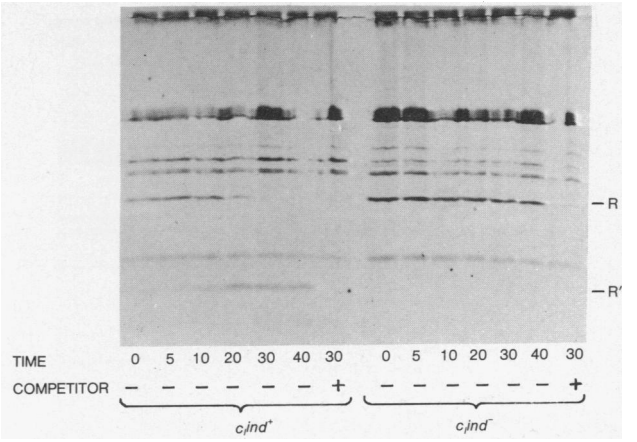

FIG. 4. The *ind*⁻ repressor is not cleaved. Cultures of W3102-(λ*N7N53*) and W3102(λ*N7N53c*$_1$*ind*⁻) were grown as described in the legend to Fig. 1, labeled with 0.40 mCi/ml of $H_2{}^{35}SO_4$ from OD_{550} = 0.10 to OD_{550} = 0.50, chased 5 min with 7 mM Na_2SO_4, sampled, induced with 5 μg/ml of mitomycin C, and sampled at the indicated intervals (in minutes).

with purified nonradioactive λ repressor (data not shown), we conclude that it is the λ repressor monomer (12).

We confirmed this assignment by demonstrating that purified nonradioactive repressor competes with radioactive band R for specific antibody sites. Fig. 2 presents a gel analysis of antibody precipitates of radioactive proteins from lysogens of wild-type λ and λN^- (a phage defective in most lytic functions), each compared to identical precipitates formed in the presence of a large excess of purified repressor. Band R is completely removed by the competition in each case, and with one exception all other proteins are unaffected by the presence of purified repressor. The exception is a polypeptide (named R′) of molecular weight approximately 14,000, the origin of which is discussed below.

In order to follow the repressor polypeptide after induction, samples of a labeled culture were examined as above at intervals after addition of mitomycin C. A culture of W3102-(λ*N7N53*) was labeled for 0.3 generations with $^{35}SO_4$, chased with nonradioactive SO_4 for 5 min, and divided into two portions; one portion was induced with 5 μg/ml of mitomycin C. Fig. 3 reveals that intact repressor disappears from the induced culture during the half hour after induction, whereas, no detectable change occurs to repressor in the uninduced culture. In fact, the repressor in uninduced cells appeared stable for at least two generations in a similar experiment (data not shown). Repressor breakdown occurs with similar kinetics after treatment of lysogenic cells by ultraviolet light, or upon induction of an N^+ prophage by mitomycin C (not shown).

A trivial explanation for the failure of radioactive repressor to appear in the antibody precipitate after induction would be the appearance of an inhibitor of the antibody–antigen reaction, for example a large excess of nonradioactive repressor synthesized during the chase. This possibility was excluded by performing cell lysis and antibody precipitation of mixed samples taken before and 30 min after addition of mitomycin C; analysis of the mixture revealed that the expected amount of repressor contributed by the uninduced sample was precipitated.

The rate of disappearance of intact repressor in induced cells is similar to the rate at which immunity is lost in cells after inducing treatments (2) and the rate at which DNA-binding activity of repressor, measured in extracts of induced cells, disappears (7); in all cases the process is complete between 20 and 30 min after the inducing treatment in usual growth conditions. Although we have not attempted to obtain a more precise correlation of the inactivation of repressor and its physical cleavage, it appears possible that these events are simultaneous and perhaps identical.

What is the fate of the repressor polypeptide which disappears from the 27,000 molecular weight position of the gel? At least part of the molecule is found as a fragment (labeled R′ in the figures) of molecular weight 14,000. The appearance of this fragment parallels the disappearance of repressor during induction. That R′ derives from the repressor polypeptide is indicated by its failure to precipitate in the presence of excess unlabeled repressor, whereas all other labeled polypeptides again are unaffected by the competitor (Fig. 3). We do not know what portion of the 27,000 molecular weight monomer is contained in R′ and we have not attempted to determine accurately what fraction of the radioactivity originally in repressor appears in R′; the band conceivably comprises both halves of the original molecule, since it appears to have exactly half the molecular weight of the monomer.

In addition to appearing as a product of the rapid degradation of repressor in induced lysogens, fragment R′ is detected (in variable amounts) in uninduced lysogens of wild-type λ and λN^- when proteins are labeled for two generations (see Fig. 2). A breakdown of repressor occurring without an explicit inducing treatment could reflect the low level of spontaneous induction which occurs in a wild-type λ lysogen. Evidence supporting this conjecture is presented below.

Induction of prophage λ by mitomycin C or by ultraviolet light can be prevented either by a mutation in the repressor gene or by any one of various mutations in the host bacterium. A lysogen of a phage carrying the repressor mutation *ind*⁻ shows little or no induction following induction treatments (2, 13). Fig. 4 reveals the fate of repressor after mitomycin C

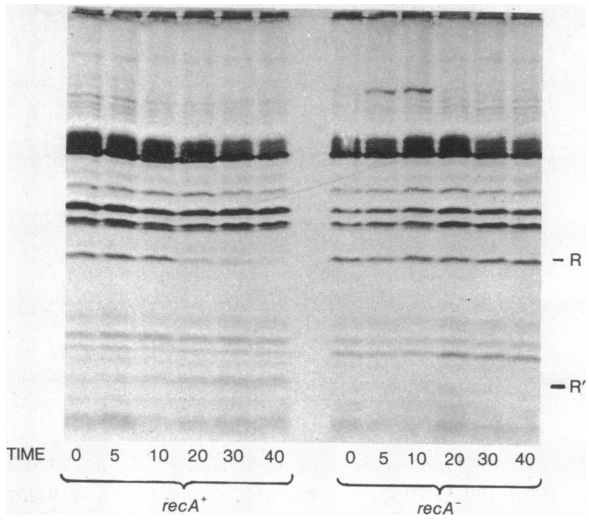

FIG. 5. The *recA*$^-$ mutation prevents cleavage of repressor. Cultures of W3102(λ*N7N53*) and W3102*recA3*(λ*N7N53*) were grown, labeled, induced, and sampled as described in the legend to Fig. 4.

addition to cultures of W3102(λ*N7N53*) and W3102(λ-*N7N53c*$_I$*ind*$^-$). Wild-type repressor disappears and fragment R′ appears as before; no detectable change occurs to the *ind*$^-$ repressor, and no trace of fragment R′ appears from it.

Induction of a λ prophage carrying the wild-type repressor is prevented by mutations in the bacterial *recA* gene, lesions which also eliminate genetic recombination in the host. An experiment identical to the preceding was performed to compare repressor breakdown in *recA*$^+$ and *recA*$^-$ cells after addition of mitomycin C. Fig. 5 reveals that, as expected, no breakdown occurs in the *recA*$^-$ lysogen and no radioactivity appears in the position of fragment R′.

The mutations examined above which prevent induction by mitomycin C also prevent the appearance of any detectable fragment R′ in *uninduced* cells. This is apparent in the zero time samples of Figs. 4 and 5 and the last sample of Fig. 1. Since both the phage *ind*$^-$ mutation and this host *recA*$^-$ mutation (*recA3*) prevent spontaneous induction of λ, the presence of R′ in uninduced cells seems to parallel spontaneous phage production. We have pursued this correlation further by examining several other lysogens with recombination defects that prevent induction of λ. The mutation *recA142* is like most *recA* lesions in that it prevents induction of λ by mitomycin C or ultraviolet light, but it differs in allowing a normal amount of spontaneous induction (5). The level of repressor and fragment R′ in wild-type lysogens of a *recA142* cell before and after mitomycin C treatment is displayed in Fig. 6 and is compared to the corresponding levels in lysogens of *recA12* and *recA13*, neither of which allows spontaneous induction. Only the *recA142* lysogen contains R′ before (or after) treatment, whereas, all three *recA*$^-$ lysogens fail to display repressor breakdown upon mitomycin C treatment. Another mutation that prevents ultraviolet light or mitomycin C induction of λ is *recF143*, a defect in a second pathway of recombination (14). The *recF* lesion does not prevent spontaneous induction, however, and the *recF143* lysogen also contains fragment R′ before induction (Fig. 6). As expected, the repressor band does not disappear in the *recF* lysogen upon mitomycin C treatment.

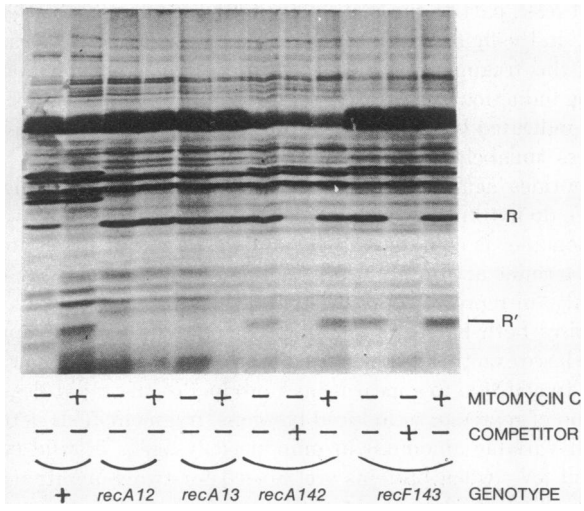

FIG. 6. The presence of R′ correlates with spontaneous induction. λ$^+$ lysogens of W3102 (wild type), JC2917 (*recA12*), JC2926 (*recA13*), JC4728 (*recA142*), and JC9239 (*recF143*) were grown in low sulfur medium containing 60 μM $MgSO_4$ to an OD_{550} of 0.10, and labeled with 0.40 mCi/ml of $H_2{}^{35}SO_4$ until the OD_{550} reached 0.60. The cultures were chased 5 min with 7 mM Na_2SO_4, sampled (1.0 ml), induced with 5 μg/ml of mitomycin C, grown 40 min, and sampled again. The competitor was 1.5 μg of nonradioactive repressor.

DISCUSSION

We have shown that induction of a λ prophage by mitomycin C or ultraviolet light results in cleavage of the λ repressor molecules in the lysogen. This breakdown correlates with the induction process measured by expression of phage genes: the bacterial *recA*$^-$ mutations and the phage *ind*$^-$ repressor mutation individually block both phage induction and cleavage of the repressor molecule.

Is derepression achieved solely by the synthesis or activation of a protease in the cell, or does proteolytic cleavage follow a different change in the repressor which is the primary signal for derepression? A traditional suggestion has been that derepression results from binding of another molecule to the repressor, by analogy with the induction of numerous bacterial operons (16). Such an inducer might be a byproduct of the DNA repair processes, or a precursor of DNA synthesis that accumulates, or in fact another protein. If such an inducer exists, it might interact with the repressor (either reversibly or by covalent attachment) to simultaneously lower its affinity for the operator and expose a site for proteolytic attack. Alternatively, another molecule could interact with the repressor to render it susceptible to proteolysis but not itself cause derepression. In either case the *ind*$^-$ mutation might function by preventing the interaction between the repressor and its cofactor.

On the other hand, it is possible that proteolytic cleavage of repressor is the only event in derepression. One cellular response to damaged DNA could be the synthesis or activation of a specific protease, possibly serving to inactivate a class of proteins (repressors?) in the cell. There are precedents for specific proteolytic cleavages which modify and control the functions of proteins, including the activation of digestive proteases and the modification of bacteriophage structural proteins (15). In this model the *ind*$^-$ mutation would render the repressor molecule insensitive to attack by the enzyme.

We see no way to choose among these possibilities at this time; whatever the detailed mechanism of derepression is, however, proteolytic cleavage presumably renders the event irreversible.

It is unlikely that any phage function is required for the activity that cleaves the repressor. Phage genes are almost certainly not required for derepression (2), because essentially none (except the repressor gene) are expressed before induction; thus if cleavage *is* the primary inactivation event, it must result from a bacterial activity. Even if cleavage occurs after a different type of inactivation, it appears to be equivalent in lysogens of wild-type λ and of λ with a defect in the *N* gene; the latter phage expresses almost all lytic functions only at a very low level and might be expected to be deficient in breakdown if a phage gene were required. One phage product that is expressed in the N^- phage is that of the *tof* (or *cro*) gene (17), so that a role for it in the cleavage process cannot be excluded.

Witkin (18, 19) has observed that induction of λ is only one of a set of events which occur in a competent cell after the usual inducing treatments. A second is the expression of genes that provide repair of damage from ultraviolet light and mutagenesis, the induction of which might closely parallel the induction of prophages. In addition, inducing treatments result in filamentation of the bacterium, caused by an inhibition of cell division which may reflect the operation of a specific control mechanism. All of these events require some common metabolic step, because all require an active *recA* function. It seems a reasonable possibility that a single derepression mechanism (such as proteolytic destruction of a set of repressors) underlies all of these changes, and that the *recA* function has a central role in the process. Lesions in the *recA* gene can either abolish both spontaneous and explicit induction of λ, or allow spontaneous induction while blocking the response to mitomycin C and ultraviolet light. This apparent separation of the two processes by mutation suggests that the *recA* protein possesses a basal level of activity which provides the background of spontaneous induction, and which is greatly increased after an inducing treatment. Thus the *recA142* mutation might prevent an interaction of the *recA* protein with a previous product of the induction pathway while not impairing its inherent activity. Possibly the *recA* protein controls a protease which attacks target proteins, or conceivably it is itself a protease. Whatever its exact role may be, the fact that a specific proteolytic cleavage is a consequence of the *recA* activity suggests a mechanism which may be sought in other aspects of its function.

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