

Escherichia coli *recA* gene product inactivates phage λ repressor

(proteolytic cleavage/ATP/SOS functions)

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ABSTRACT Phage λ repressor is inactivated and cleaved into two detectable fragments during incubation with purified *Escherichia coli* *recA* gene protein *in vitro*, in a reaction that requires ATP. This reaction reproduces the *recA*-dependent inactivation of repressor that occurs *in vivo* during induction of the SOS functions. The proteolytic activity may reside in the *recA* protein itself and may be a fundamental activity of it.

The protein encoded by the *recA* gene of *Escherichia coli* is essential not only for genetic recombination, but also for the expression of the "SOS" functions, a set of responses to agents that threaten the cell by disrupting the structure or synthesis of its DNA (1, 2). A typical such threat is damage to bacterial DNA caused by ultraviolet irradiation. The SOS functions include an inducible mode of repair of damaged DNA named W-reactivation, induced mutagenesis that may result from inaccurate DNA repair during W-reactivation, inhibition of cell division, and the induction of temperate bacteriophages (2). Some mutations in the *recA* gene (designated *recA*⁻) abolish both genetic recombination and the expression of the SOS functions, whereas other mutations in *recA* (e.g., *tif*, *lexB*) either enhance or depress SOS expression without markedly affecting recombination (3). Thus, the *recA* gene has an important role in regulating these processes, although the biochemical function of its product has not been known.

The immediate cause of the *recA*-dependent induction of bacteriophage λ is the inactivation of the λ immunity repressor, a protein that prevents transcription from the critical early phage promoters. We have found that this inactivation event occurs *in vitro* during incubation of λ repressor in an extract of a bacterial strain (DM1187) that constitutively expresses all SOS functions (4). This reaction requires ATP and Mg²⁺ and is accompanied by proteolytic cleavage of the repressor molecule, as also occurs during *recA*-dependent induction of λ *in vivo* (5). This finding raised several questions that we now address: What cellular components are involved in the inactivation and cleavage reaction? Is proteolytic cleavage the direct activity of the inactivating function, or is it a secondary consequence of inactivation? What is the biochemical basis of the SOS constitutive phenotype of strain DM1187?

We have purified the factor responsible for repressor inactivation and we report here that it is the product of the *recA* gene. The repressor inactivation reaction apparently requires only this protein and small molecules, so that the product of the *recA* gene presumably directly inactivates repressor by promoting its cleavage.

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MATERIALS AND METHODS

Growth of Bacteria and Preparation of Extracts. The sources of bacteria are shown in Table 1. Bacteria were grown and extracts were prepared as described (4), with the following modifications: strain 294(pKB252) was grown in a low-sulfur medium (4) to an OD₅₅₀ of 0.5-1.0; bacteria used for preparation of *recA* protein were grown in a medium containing 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter, to an OD₅₅₀ of approximately 1. Strain JA200(pLC30-20), and sometimes strain DM1187, were treated with 40 μ g of nalidixic acid per ml 30 min before harvest.

Purification of Proteins. *recA* protein was prepared by a procedure to be described elsewhere. In brief, it consists of selecting protein that binds to polymin P and elutes between 0.5 and 1.0 M NaCl (11), precipitating with 0.28 g of ammonium sulfate per ml, chromatographing on DNA-agarose (12) or phosphocellulose, and sedimentating on a sucrose density gradient. *recA* protein sediments at 6-7 S and thus is probably a tetramer of the 40,000-dalton polypeptide (13). We assume arbitrarily that a 1.0 mg/ml solution of *recA* protein has an A₂₈₀ of 1.0. λ repressor was purified as described (14).

Other Procedures. Purified rabbit IgG against *recA* protein and λ repressor were prepared as described (15). Double-antibody precipitation was performed as described (5). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (NaDodSO₄) was performed as described, except that the gradient was 10-25% acrylamide. λ repressor was assayed as described (4).

Materials. ATP was obtained from P-L Biochemicals or Boehringer. Nalidixic acid was obtained from Calbiochem. Polymin P was obtained from Miles.

RESULTS

Purification of Repressor-Inactivating Function. λ repressor is inactivated and cleaved when it is incubated in a crude extract of the SOS-constitutive strain DM1187 (4). In order to determine the components of the extract that are involved in this reaction, we fractionated a crude extract of DM1187 and assayed for activity that destroys the DNA-binding activity of λ repressor. Figs. 1 and 2 present a NaDodSO₄ gel analysis of protein present in a crude extract of DM1187 and in active fractions after three stages of purification. The purification selects a single predominant polypeptide, of molecular weight approximately 40,000. We argue below that this polypeptide is the product of the *recA* gene. [Since DM1187 carries the mutation *tif*⁻ (6) which maps in the *recA* gene (9, 16, 17), the protein is correspondingly modified.]

The final fractionation step for the inactivating factor is a sucrose gradient sedimentation of protein from fraction II. A NaDodSO₄ gel analysis of gradient fractions shows the distribution of protein and indicates its high purity (Fig. 2). In order

Abbreviation: NaDodSO₄, sodium dodecyl sulfate.

Table 1. Bacterial and bacteriophage strains

Strain	Relevant genotype	Source or ref.
<i>E. coli</i> DM1187	<i>tif1 sfiA11 lexA3 spr51</i>	D. Mount (6)
<i>E. coli</i> DM1180	<i>tif1 sfiA11 lexA3 spr⁺</i>	D. Mount (6)
<i>E. coli</i> DM1420	<i>tif⁺ sfiA11 lexA3 spr51</i>	D. Mount (6)
<i>E. coli</i> GC579	<i>tif⁺ sfiA11 lexA⁺ spr⁺</i>	R. Devoret (7)
<i>E. coli</i> GC467	<i>tif1 sfiA11 lexA⁺ spr⁺</i>	R. Devoret (7)
<i>E. coli</i> 294(pKB252)		K. Backman (8)
<i>E. coli</i> JC2917	<i>recA12</i>	A. J. Clark
<i>E. coli</i> DM1411	<i>recA121</i> derivative of DM1187	D. Mount
<i>E. coli</i> DM1415	<i>recA1 sfiA11 lexA3 spr51</i>	D. Mount (9)
<i>E. coli</i> JA200(pLC30-20)		J. Carbon (10)

to reveal the activity, aliquots of each fraction were incubated in assay conditions with a small amount of radioactive crude extract of the repressor overproducing strain 294(pKB252), and the cleavage of repressor was detected by specific antibody precipitation of repressor and its cleavage fragments from the incubation mixture. The peak of cleavage activity coincides with the peak of *recA* protein. Two fragments of repressor were detected, as we also had found previously (4). Since we have no convenient quantitative assay of the activity, it is difficult to perform a conventional determination of specific activity across this peak or to measure accurately specific activity during the preparation. However, we can estimate by gel electrophoresis the concentration of *recA* protein in crude extracts of strain DM1187, and from such estimates we infer that the protein shows comparable activity whether it is present in crude extract or is purified. As we discuss below, the concentration of *recA* protein both in extracts and in our reactions is substantial.

Purified λ Repressor Is Cleaved by Purified *recA* Protein. The experiment described above does not establish that *recA* protein is the only cellular component required for the inactivation and cleavage reaction, because the source of repressor was a crude extract that might have contributed other components. However, the experiment presented in Fig. 3 shows that highly purified repressor also is cleaved by purified *recA* protein to the two fragments R₁ and R₂ detected after cleavage of repressor in a crude extract (4); as in the crude system, the

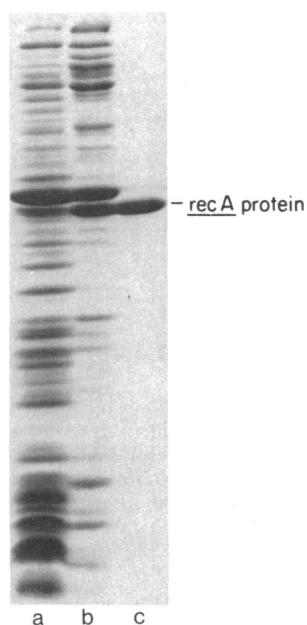


FIG. 1. Purification of *recA* protein. Samples of protein from strain DM1187 were analyzed by polyacrylamide gel electrophoresis and staining. (Gel a) 4 μ l of crude extract; (gel b) 10 μ g of protein after polymin P extraction and ammonium sulfate precipitation (fraction I); (gel c) 1.7 μ g of protein after phosphocellulose chromatography (fraction II).

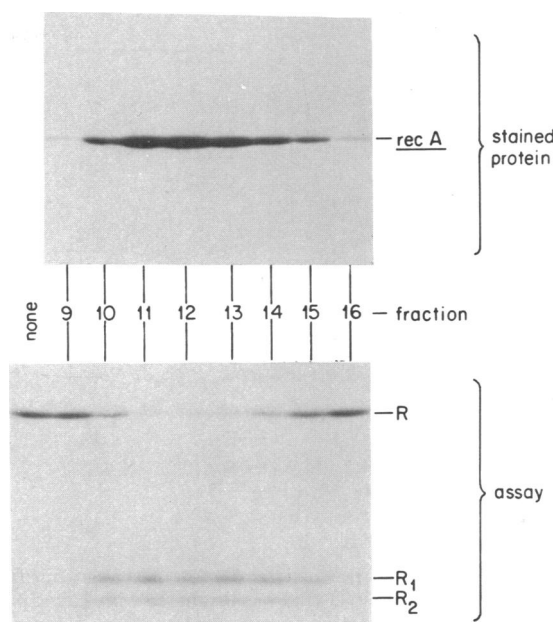


FIG. 2. Activity of sucrose gradient fractions of purified *recA* protein. Fraction II protein (1.2 mg) from strain DM1187 was sedimented on a sucrose gradient and 19 fractions were collected (fraction III). (Upper) Stained gel in which 4- μ l samples across the peak of *recA* protein were run; the sample from tube 12 contains 1.8 μ g of protein. (Lower) Autoradiogram of radioactive repressor (R) after incubation with fractions from the gradient. Each incubation mixture contained in 20 μ l: 10 mM Tris-HCl (pH 7.5), 0.20 M NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.25 mM EDTA, 5 mM ATP, 5 mM spermidine-HCl, \approx 6% sucrose (wt/vol), 200 μ g of bovine serum albumin per ml, 10 μ l of gradient fraction, and 1 μ l of ³⁵S-labeled crude extract of strain 294(pKB252) as a source of repressor. The concentration of repressor was about 1 μ g/ml; the concentration of *recA* protein in the incubation from gradient fraction 12 (the peak tube) was 220 μ g/ml. After an incubation of 30 min at 37 $^\circ$, 4 μ l of each reaction was analyzed by antibody precipitation, electrophoresis, and autoradiography. R₁ and R₂, repressor fragments.

reaction requires ATP. Thus we suspect that only repressor and *recA* protein (and small molecules) are involved in the cleavage reaction, and, furthermore, that cleavage is the primary inactivation event. A rigorous conclusion is that if any other macromolecule is essential to the reaction, it does not become limiting after a greater than 100-fold purification of both repressor and *recA* protein from crude extracts.

Only a fraction, perhaps 20%, of the purified repressor was cleaved in the experiment of Fig. 3, in contrast to the nearly complete cleavage of repressor in crude extract that we observed in the experiment of Fig. 2. This is due to the much higher concentration of repressor (80 μ g/ml) used in order that the fragments could be detected by staining. In fact, the absolute rate of cleavage is greater at the higher concentration.

Identification of Repressor-Inactivating Factor as *recA* Protein. McEntee *et al.* (13) have used a transducing phage carrying the *recA* gene to show that its product is a multimeric protein containing a single polypeptide of molecular weight approximately 40,000. This identification made possible the demonstration that the *recA* protein is identical to a previously known protein designated "protein X" (9, 16-18). Protein X had been recognized as a prominent cellular component, of unknown function, that is induced by treatments that induce the SOS functions (19). Our identification of the protein that we have purified as *recA* protein came first from the realization that it is protein X.

Protein X can be induced by growth of *E. coli* in the presence of nalidixic acid, or by growth of the mutant *tif⁻* at 40 $^\circ$ (19).

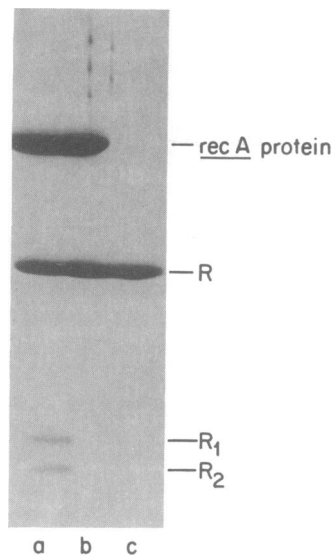


FIG. 3. Activity of purified *recA* protein against purified repressor. *tif⁻ recA* protein (13 μ g) was incubated with 4 μ g of purified λ repressor in a 50- μ l reaction mixture for 1 hr at 37° as described in the legend of Fig. 1, except that bovine serum albumin was omitted. The entire reaction mixture was analyzed by gel electrophoresis and staining to detect protein. (Gel a) Complete reaction; (gel b) ATP omitted; (gel c) *recA* protein omitted.

This is illustrated in Fig. 4, which represents a NaDodSO₄ gel analysis of crude extracts of cells induced by these treatments, along with extracts of uninduced controls. The purified inactivating factor analyzed in parallel is a protein of the same molecular weight as protein X. This protein also is present in the crude extract of the SOS constitutive cell DM1187, our source for the inactivating factor, in an amount comparable to that in the induced cells (Fig. 1). We conclude that the inactivating factor is protein X and, therefore, is the *recA* protein.

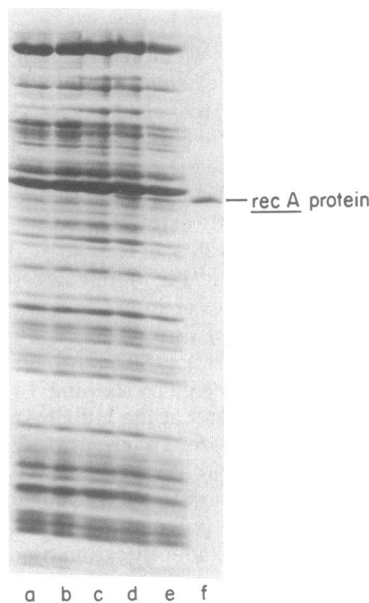


FIG. 4. Coelectrophoresis of purified inactivating factor with protein X (*recA* protein). Bacteria were grown in a minimal medium (5) supplemented with 1 mM MgSO₄, 100 μ g of adenine per ml, and required amino acids. Crude extracts were analyzed by gel electrophoresis in parallel with the purified inactivating factor, and protein was detected by staining. (Gel a) GC579 (*tif⁺*), grown at 30°; (gel b) GC579 (*tif⁺*), 42°; (gel c) GC467 (*tif⁻*), 30°; (gel d) GC467 (*tif⁻*), 42°; (gel e) GC579 (*tif⁺*), 30°, treated for one generation with 40 μ g of nalidixic acid per ml; (gel f) 1 μ g of inactivating factor.

We have confirmed this identification of the inactivating factor as *recA* protein by using the observation of McEntee (16) that the mutant *recA12* encodes a protein that has a slightly greater mobility than the wild type. We have precipitated protein from extracts of *recA⁺* and *recA12* cells with antibody to purified inactivating factor, and have analyzed the precipitates by NaDodSO₄ gel electrophoresis. An autoradiogram of such a gel is shown in Fig. 5. A polypeptide that comigrates with the inactivating factor is precipitated from extracts of both the wild-type cell (track c) and the *recA⁺ (tif⁻)* SOS constitutive cell DM1187 (track e); as expected, it is much more abundant in the latter (only 1/5 as much extract was used for track e as for tracks a and c). This polypeptide is absent in the extract of the *recA12* cell (track a) and is replaced by one of slightly greater mobility, as we expect if antibody to the inactivating factor precipitates *recA* protein. To confirm the antigenic identity of each polypeptide to the inactivating factor we performed the antibody precipitation with nonradioactive inactivating factor present in large excess over the antibody-combining sites (Fig. 5, tracks b, d, and f); it is apparent that precipitation of the polypeptide designated "*recA*" is prevented by the competitor, whereas the background of polypeptides precipitated or trapped nonspecifically is unaltered.

Role of Mutations *tif⁻* and *spr⁻* in SOS Constitutive Phenotype of Strain DM1187. The SOS constitutive phenotype of strain DM1187 is endowed by the two mutations *tif1* and *spr51* (6). [DM1187 carries other mutations that probably are not essential to the phenotype (6).] What is the role of each mutation? The mutation *tif1* maps in gene *recA* and modifies *recA* protein (9, 16, 17); we discuss below the effect of the *tif1* mutation on its activity. The mutation *spr51* maps in a gene *lexA* that regulates the expression of the *recA* gene and which, it has been suggested, might encode a repressor of gene *recA* (6, 9). The effect of *spr51* on expression of *recA* is revealed by NaDodSO₄ gel analysis of crude extracts of several strains that carry it (see also ref 9). Fig. 6, tracks a and b, shows extracts of DM1187 (*tif1 spr51*) and its parent DM1180 (*tif1 spr⁺*), which does not express SOS functions (6). The *spr⁺* strain obviously has much less *recA* protein than does DM1187. Extracts of two

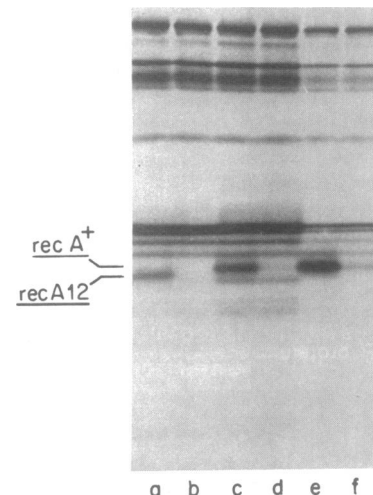


FIG. 5. Precipitation of *recA* protein by antibody to purified inactivating factor. ³⁵S-Labeled protein from crude extracts was precipitated by antibody to purified inactivating factor. For competition analysis, 1 μ g of pure inactivating factor was mixed with the extract before antibody was added. Double antibody precipitation and gel analysis of the precipitates were performed. Only a portion of the autoradiogram is shown. (Gel a) JC2917 (*recA12 tif⁺ spr⁺*); (gel b) as gel a, but with competitor; (gel c) GC 579 (*recA⁺ tif⁺ spr⁺*); (gel d) as gel c, but with competitor; (gel e) DM1187 (*recA⁺ tif⁻ spr⁻*); (gel f) as gel e, but with competitor.

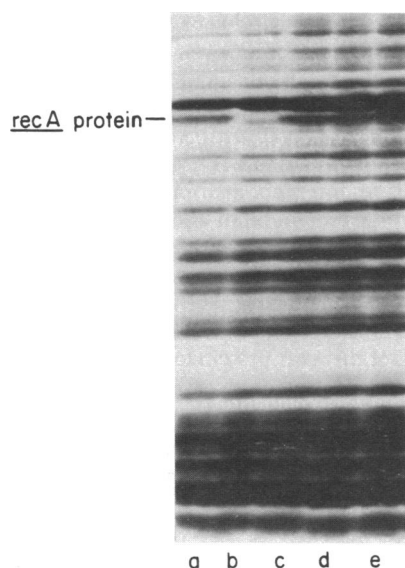


FIG. 6. Expression of *recA* protein in *spr*⁺ and *spr*⁻ strains. Equal amounts of protein from crude extracts were analyzed by electrophoresis and protein was detected by staining. (Gel a) DM1187 (*recA*⁺ *tif*⁻ *spr*⁻); (gel b) DM1180 (*recA*⁺ *tif*⁻ *spr*⁺); (gel c) DM1420 (*recA*⁺ *tif*⁺ *spr*⁻); (gel d) DM1411 (*recA*⁻ *tif*⁻ *spr*⁻); (gel e) DM1415 (*recA1* *tif*⁺ *spr*⁻).

other (uninduced) *spr*⁺ strains shown in Fig. 4 (GC467 and GC579) also contain little *recA* protein in comparison to DM1187. Since *recA* protein directly inactivates λ repressor, its low concentration in extracts of the *spr*⁺ strains accounts for their inability to inactivate repressor (4).

The mutation *spr51* also provides a high level of *recA* protein when it is combined with either of two other alleles of *recA* (Fig. 6, tracks c and e): *tif*⁺, which is the wild-type allele of *recA*, and *recA1*, a mutant that is deficient in genetic recombination and the expression of SOS functions. Unlike the *tif*⁻*spr*⁻ strain DM1187, neither of these strains displays constitutive expression of SOS functions *in vivo*, despite having a large amount of *recA* protein (6, 9). Correspondingly, crude extracts from these strains do not show the high capacity to inactivate λ repressor that we found in a crude extract of DM1187 (ref. 4 and unpublished experiments). It seems reasonable to ascribe these differences to distinct activities of *recA* protein encoded by the *tif*⁺, *tif*⁻, and *recA1* alleles.

During induction of λ in a wild-type strain by agents such as ultraviolet light, the wild-type (*tif*⁺) *recA* protein presumably does inactivate repressor. Thus it seemed possible that the *tif*⁺ protein also might be active *in vitro*, but not sufficiently active to detect in a crude extract by measuring loss of DNA binding activity of repressor. We therefore used a more sensitive assay: we performed the inactivation reaction with partially purified and concentrated *tif*⁺ protein and with radioactive repressor, and we measured the production of the repressor fragments R₁ and R₂ by antibody precipitation. This assay showed that the *tif*⁺ protein in fact has activity (data not shown). In a similar experiment we detected no cleavage at all with the *recA1* variant (data not shown).

To provide a measure of the relative activity of the *tif*⁻ and *tif*⁺ proteins, we purified both proteins to homogeneity (fraction II or III) and determined for each the relation between the fraction of λ repressor inactivated in a standard assay and the concentration of *recA* protein present in the incubation (Fig. 7). It is apparent that 4–5 times as much *tif*⁺ as *tif*⁻ protein is required to effect the same inactivation (although we do not know the kinetic basis of this difference). Since cleavage fragments R₁ and R₂ are detected in incubations with both *recA*

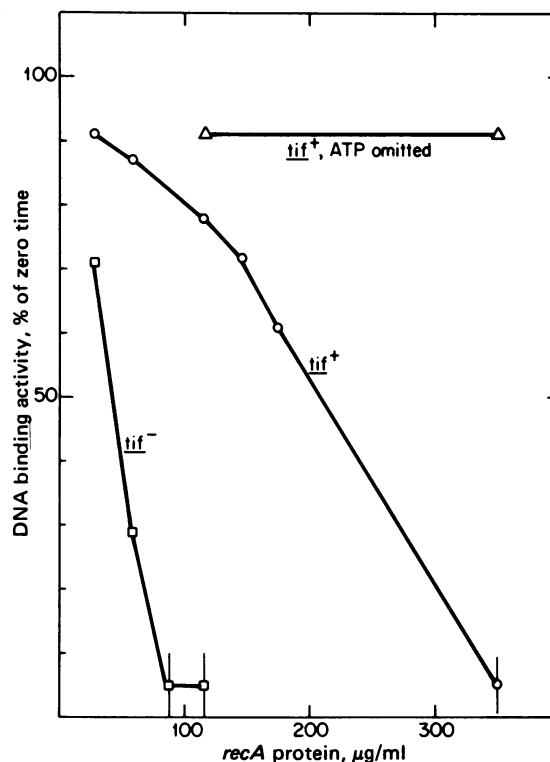


FIG. 7. Activity of *tif*⁻ and *tif*⁺ *recA* proteins. Each reaction mixture of 50 μ l contained 0.85 μ l (0.048 *A*₂₈₀ unit) of crude extract of strain 294(pKB252) as a repressor source, 100 μ g of bovine serum albumin per ml, *recA* protein as indicated, and small molecules as described in the legend to Fig. 1. The concentration of repressor was about 1 μ g/ml. Reaction mixtures were incubated for 45 min at 37° and duplicate samples were assayed for DNA binding activity as described (4). The ordinate is the percent repressor activity remaining after incubation. The *tif*⁻ protein was prepared from strain DM1187 and *tif*⁺ protein, from strain JA200(pLC30-20). An identical result was obtained with *tif*⁺ protein prepared from strain DM1420.

protein variants, it seems likely that these proteins possess the same inherent activity in forms of different potency. Considering the relatively low activity of the *tif*⁺ protein, it is reasonable that we could not detect its activity in a crude extract of the *tif*⁺ *spr*⁻ strain DM1420 (4).

In summary, both mutations *spr51* and *tif1* contribute to the high capacity of a crude extract of strain DM1187 to inactivate repressor: *spr51* provides a high level of *recA* protein, and *tif1* endows this protein with a greater activity than the wild type. We presume that these properties also are the basis of the constitutive expression of SOS functions by DM1187 *in vivo*.

DISCUSSION

We have shown that the protein encoded by the *E. coli recA* gene mediates the inactivation and cleavage of the phage λ immunity repressor. This conclusion is based upon the ability of purified *recA* protein to direct repressor inactivation and cleavage *in vitro* in an ATP-dependent reaction; it is strengthened by the observation that mutations in *recA* affect this activity in a way that is consistent with the mutant phenotypes. The direct involvement of the *recA* protein in repressor inactivation accounts for genetic evidence that the *recA* gene regulates lysogenic induction (20).

Induction of the SOS functions of *E. coli* by treatments that damage DNA is accompanied by an increased rate of synthesis of *recA* protein, leading to its accumulation to a high concentration in the cell (19). Our results imply that this amplified synthesis is essential to phage induction because *recA* protein

is the agent of repressor inactivation. Possibly *recA* protein has a similar direct involvement in W-reactivation, mutagenesis, and other SOS functions. Induction of SOS functions by agents such as ultraviolet light requires protein synthesis (2), and it is clear that synthesis of *recA* protein could account for this requirement.

If the presence of a high concentration of *recA* protein is necessary for repressor inactivation and for the expression of other SOS functions, is it also sufficient? Our evidence that *recA* protein alone inactivates repressor suggests that it could be. For example, the constitutive expression of SOS functions by the *tif⁻ spr⁻* strain DM1187 may be simply a consequence of its high content of *recA* protein, coupled with the greater activity of the mutant *tif⁻ recA* protein. The *tif⁺ spr⁻* cell DM1420 contains a comparably high concentration of the *tif⁺* protein; the simple model that *recA* protein is sufficient for SOS expression would imply that this is not enough of the less active *tif⁺* protein to provide constitutive expression. Others (9, 16) have suggested an alternative model, that wild-type *recA* protein requires for its activity a factor produced in the cell after an inducing treatment, whereas *tif⁻ recA* protein does not require this factor. We see no way to distinguish these models, although our results would indicate that the *tif⁺* protein is partially active in the absence of this hypothetical factor.

Because purified repressor is cleaved by purified *recA* protein, it is likely that proteolytic cleavage is the primary mechanism of repressor inactivation. Nevertheless, we cannot eliminate the possibility that the *recA* protein modifies repressor so that it is recognized by a separate protease; a second enzyme could contaminate either protein, no matter how homogeneous it appears by chromatography. Thus, a rigorous demonstration that *recA* protein is a protease will require more direct evidence that the polypeptide contains the catalytic center for proteolysis. We presume that the *recA* protein at least binds to repressor in the inactivation reaction.

Several questions about the activity of the *recA* protein remain:

(i) What is the role of ATP in the reaction? ATP is hydrolyzed to ADP and P_i during incubation with purified *recA* protein under conditions of the inactivation reaction, and this ATPase activity appears to copurify with the *recA* polypeptide (unpublished experiments). We have found that neither the β-γ-methylene nor the β-γ-imido analogue of ATP substitutes for ATP in the repressor inactivation reaction, suggesting that cleavage of the terminal phosphate bond of ATP is essential.

(ii) Why is such a high concentration of *recA* protein required to effect rapid repressor inactivation *in vitro*? It is possible that the conditions that we use for the reaction are unfavorable. However, the reaction does not appear to be significantly more efficient *in vivo*. The concentration of *recA* protein that we estimate to be present in the cell during induction is similar to that used in our reactions. In fact, when we use a concentration of repressor similar to that in a λ lysogen (about 1 μg/ml), as in the experiment described in Fig. 1, the rate of cleavage approximates that which occurs *in vivo* (5). Thus, the reaction may be naturally slow. Another possibility is that only a small portion (perhaps a modified fraction) of the *recA*-protein is active both *in vivo* and *in vitro*; we have no evidence to support or contradict this alternative.

(iii) Does DNA take part in the inactivation reaction? It seems inherently likely that *recA* protein interacts with DNA

in genetic recombination and repair, and it is known to bind single-stranded DNA (19). However, DNA could not be present in significant amounts in our incubations with purified components, so that DNA cannot be essential to the reaction that we detect. It is possible that *recA* protein normally inactivates repressor while it is bound to DNA, and that somehow we have eliminated this requirement artificially.

(iv) If the *recA* protein binds and inactivates other proteins by cleavage, is this activity related to its function in genetic recombination and the induction of SOS functions? Possibly it is not; *recA* protein could have several quite separate activities. However, it is striking that the ability of the *tif⁻*, *tif⁺*, and *recA⁻* variants of the *recA* protein to cleave repressor correlates with their activity in promoting expression of SOS functions *in vivo*, and also, at least for the latter two, in promoting genetic recombination. Thus, it is reasonable to speculate that the role of *recA* protein in recombination and in induction of SOS functions may be to modify other proteins by promoting a proteolytic attack. If this is true, it should be possible to find cellular proteins that are substrates for the proteolytic activity.

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