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Early events and mechanisms in the induction of bacterial SOS functions: Analysis of the phage repressor inactivation process *in vivo*

(inducing agents/DNA degradation/*recB* gene/strand scissions)

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ABSTRACT **Different inducing agents and treatments produced distinctly different kinetic patterns of inactivation of prophage repressor molecules. The different patterns were related to differences in the initial altered states of DNA that were produced. The timing of appearance of DNA degradation was correlated with the time needed for repressor inactivation. These characteristics suggest that all the inducing treatments lead to the formation of a final predegradative DNA structure(s) (probably involving scissions) that is acted on by specific DNases, including the *recBC* DNase, to produce the signals for the induction of prophage.**

When bacterial DNA replication is inhibited by damage inflicted on the DNA or by other means, a sequence of events is initiated that ultimately leads to induction of prophage and also encompasses various other phenomena termed "SOS functions" (1, 2). These phenomena include filamentous cell growth, increased DNA repair, and inducible mutagenesis. The X protein associated with induction of SOS functions (3) and the *recA* gene protein product (4) were recently demonstrated to be identical (5–7). There is evidence of protease involvement in the inactivation of λ repressor molecules (8) and possibly in other SOS functions (9). However, little is known about the early events that trigger induction of the SOS functions.

Recently, we reported a new biochemical assay procedure for analyzing phage repressor inactivation (10). This procedure is based on the phage-controlled read-through transcription of bacterial tryptophan genes translocated into the phage genome. In this assay method, when a φ80 lysogen of an *Escherichia coli* (*trpE*$^-$) strain is infected with such φ80*ptrp* phages in medium containing tryptophan, expression of the tryptophan operon is observed only when the phage repressor is inactivated. Completion of inactivation of the phage repressor molecules is signaled by the appearance of anthranilate synthase [ASase; chorismate pyruvate-lysate (amino-accepting), EC 4.1.3.27], the product of the first two *trp* operon genes, *trpED*. This method allows one to readily follow the kinetics of repressor inactivation biochemically by simply assaying for the appearance of ASase activity.

In this paper, we present experimental evidence suggesting that the DNA structures initially produced by various inducing treatments are all converted to a common final predegradative DNA structure(s) that is susceptible to the action of specific DNases, including the *recBC* DNase, and this DNase activity produces the signal that triggers induction.

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MATERIALS AND METHODS

Bacterial and Phage Strains. The following isogeneic (except BT10266) *E. coli* K-12 strains were used throughout the study: M01512 (a derivative of AB1157) carries the *trpE9758* mutation, an *end*$^-$ mutation (endonuclease I negative), and genetic markers from AB1157 *thr-1 leu-6 argE3 thi-1 pro-A2 ara-14 xyl-5*); M01505, the same as M01512 but also has a *thy*$^-$ mutation (requires 50 μg of thymine per ml); M01518, the same as M01512 but also carried *recB21;* M01513, a φ80 lysogen of M01512; M01506, a φ80 lysogen of M01505; and M01519, a φ80 lysogen of M01518. The φ80 lysogen of a temperature-sensitive *dnaE*$_{ts}$ strain (BT10266) used here was supplied by Y. Hirota and has the following genetic characteristics: *thy PolA1 polB1 dnaE*$_{ts}$/F′(*arg*$^+$ *thy*$^+$ *lys*$^+$). Phage φ80*ptrp190h*(*trpO*$^+$-*E*$^+$*D*$^+$*C*$^+$*B*$^+$*A*$^+$) was obtained from F. Imamoto. The phage was purified as described (10).

Chemicals. The chemicals and antibiotics used in this study were obtained from the following sources: bleomycin, Bristol-Myers Co.; streptonigrin, National Cancer Institute; mitomycin C, Sigma Chemical Co., novobiocin, Sigma Chemical Co.; and colicin E2, a generous gift from A. Maeda of Kyoto University. Radioactive materials used here were purchased from New England Nuclear.

Assay of Prophage Induction. The assay of prophage induction by read-through transcription of the tryptophan operon integrated into a φ80 genome has been described in detail (10).

EFFECT OF VARIOUS INDUCING AGENTS

To analyze the very early events in induction of prophage, we examined the kinetics of inactivation of the phage repressor molecules under various inducing conditions. As typical inducing conditions (or agents) whose modes of action on DNA replication are relatively well understood, we used bleomycin, streptonigrin, mitomycin C, colicin E2, UV irradiation, thymine deprivation of a thymine-requiring strain, and incubation of temperature-sensitive DNA replication (chain elongation) mutants (*dna*$_{ts}$) at a nonpermissive temperature. We also tried novobiocin, an inhibitor of DNA replication.

Fig. 1 presents typical kinetic patterns of ASase synthesis after a φ80*ptrp*-infected φ80 lysogen was subjected to the various inducing conditions. Quite unexpectedly, we found that the time required to inactivate phage repressor molecules varied considerably for the different inducing treatments. For example, treatment with bleomycin, streptonigrin, or colicin E2

Abbreviation: ASase, anthranilate synthase (EC 4.1.3.27).

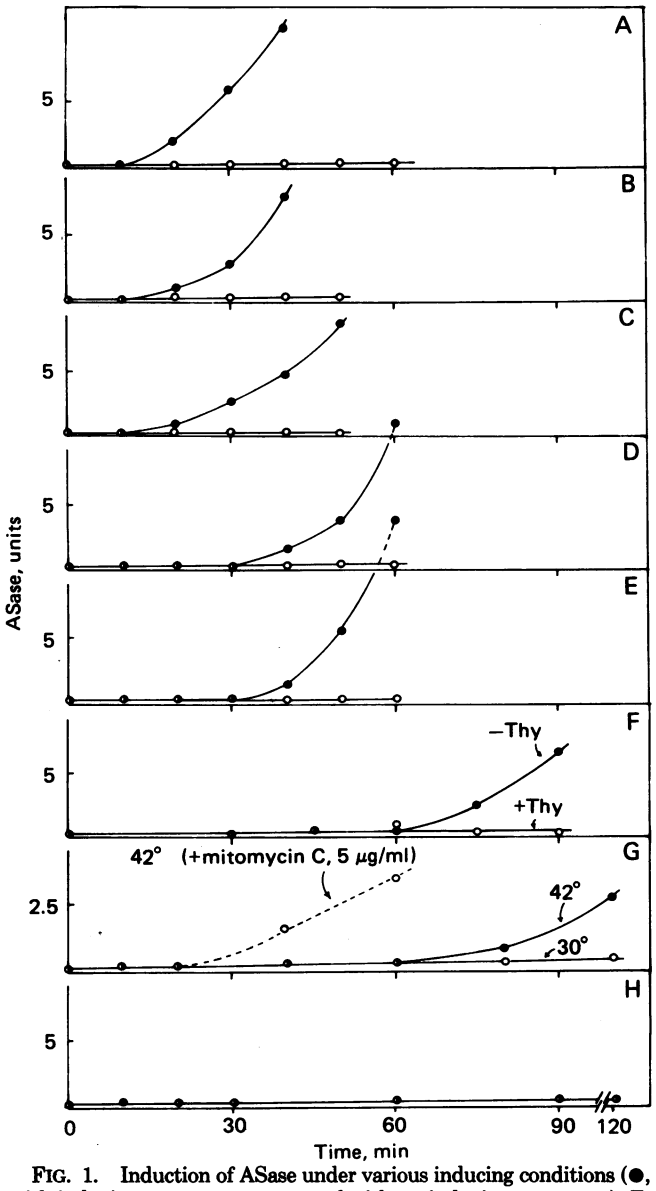

FIG. 1. Induction of ASase under various inducing conditions (●, with inducing treatment; ○, control without inducing treatment). *E. coli* strain M01513 was grown in minimal (SSA) medium (10) at 37° to a density of 2×10^8 cells per ml, infected with ϕ80*ptrp* at a multiplicity of infection, 10, and subjected to various inducing conditions. Samples (1 ml) were taken at time intervals for assay of ASase activity. The concentrations (or doses) were optimal ones that gave maximal inducing capacities (Fig. 2). The concentration of colicin E2 is given in terms of killing units (16). UV irradiation was performed after phage absorption in T1 buffer (10) in a petri dish (9 cm diameter) containing 5 ml of the samples. Four General Electric G15T8 (15 W) germicidal UV lamps were used at a distance of 52 cm, which gives an UV intensity of approximately 52 ergs/mm^2 per sec. For the thymine-deprivation experiment, a thymine-requiring strain (M01506) was grown in SSA medium containing thymine at 50 μg/ml. After infection with ϕ80*ptrp*, the cells were washed with SSA medium, divided into two portions, and incubated in fresh SSA medium in the absence and presence (50 μg/ml) of thymine. In the experiment with the *dnaE*$_{ts}$ mutant, strain BT10266 was grown in SSA medium at 30°. After infection with ϕ80*ptrp* the culture was divided into two portions, one incubated at 30° and the other incubated at 43°. (*A*) Bleomycin, 30 μg/ml; (*B*) streptonigrin, 10 μg/ml; (*C*) colicin E2, 10 killing units per cell; (*D*) UV, 2500 ergs/mm^2; (*E*) mitomycin C, 5 μg/ml; (*F*) thymine deprivation; (*G*) *dnaE*$_{ts}$ at 42° with and without mitomycin C and at 30°; (*H*) novobiocin, 100 μg/ml.

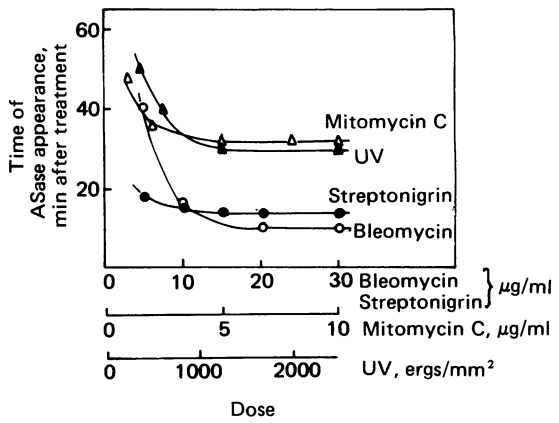

FIG. 2. Time required for completion of repressor inactivation as a function of concentration of various inducing agents. Strain M01513 was grown in SSA medium at 37° to 2×10^8 cells per ml; after infection with ϕ80*ptrp* (multiplicity of infection, 10), the cells were treated with various concentrations of the inducing agents as indicated. The time required for completion of the repressor inactivation was determined from the earliest appearance of ASase activities by extrapolating the individual curves. (For details, see Fig. 1 legend.)

led to phage repressor inactivation within a very short time (10–15 min). Inactivation of the repressor molecules by mitomycin C or UV treatment required approximately 30 min. Thymine deprivation or incubation of a *dnaE*$_{ts}$ mutant at the nonpermissive temperature required a considerably longer incubation period, of at least 60 min, to bring about repressor inactivation. In a separate series of experiments, we found that inactivation of the phage repressor molecules in the other available *dna*$_{ts}$ mutants (*dnaB*$_{ts}$, *dnaC*$_{ts}$, *nrdA*$_{ts}$, and *dnaG*$_{ts}$) all required 60–90 min of incubation at the nonpermissive temperature (unpublished data). We did not detect any induction of ASase synthesis after novobiocin treatment at concentrations up to 100 μg/ml, even after extended incubation periods. Essentially the same results with the various inducing treatments were obtained for inactivation of λ repressor in a λ*ptrp* assay system (data not shown).

The time needed for completion of repressor inactivation by each inducing agent was affected by the concentration up to a certain level, above which increasing amounts had no effect. As shown in Fig. 2, increasing doses of bleomycin, streptonigrin, mitomycin C, and UV irradiation led to decreasing repressor inactivation times, reaching a specific constant minimum time characteristic of each inducing agent (bleomycin, 10 min; streptonigrin, 13 min; mitomycin C, 33 min; and UV, 30 min). The differences in time required for phage repressor inactivation were not related to differences in time required to halt DNA replication. DNA replication ceased or was drastically reduced (e.g., in the case of the *dnaE*$_{ts}$ mutant) within 5–10 min after cells were exposed to the inducing conditions, as measured by incorporation of labeled DNA precursor into DNA (data not shown). Thus, despite the fact that they almost all inhibit DNA synthesis within a relatively similar time span, the different inducing treatments exhibit quite divergent kinetics in the inactivation of phage repressor molecules. These results suggest that the rate of repressor inactivation or the number of steps that lead to inactivation of repressor molecules varies according to the nature of the primary DNA structures produced by the various inducing conditions.

All the agents that cause early inactivation of the repressor molecules, such as bleomycin, streptonigrin, and colicin E2, are known to cause strand scissions in DNA molecules. Bleomycin is a complex glycopeptide whose primary action on DNA molecules *in vivo* and *in vitro* appears to be a strand scission that subsequently results in a double-strand break (11–14).

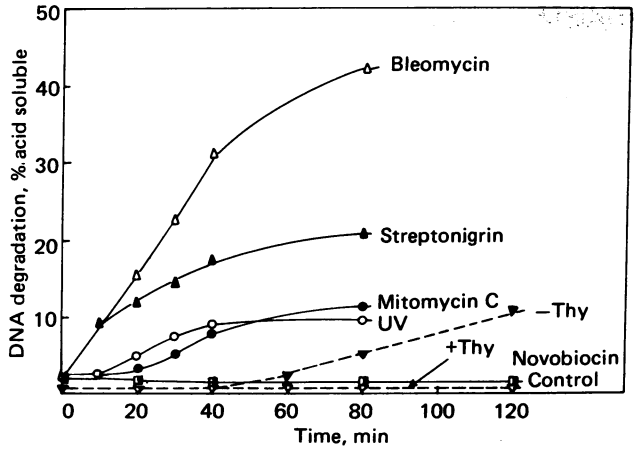

FIG. 3. DNA degradation after treatments with various inducing agents. Strain M01513 was grown for several generations in SSA medium at 37° in the presence of [5-*methyl*-^{3}H]thymidine (6 μCi/ml with 1 μg of carrier thymidine per ml). At a density of 2×10^8 cells per ml, the cells were washed, resuspended in fresh SSA medium, and distributed into culture flasks containing various inducing agents. The samples (1 ml) were taken intermittently, and trichloroacetic acid (8%, wt/vol)-soluble radioactivities were measured by a Beckman scintillation counter after 200 μl of each sample was absorbed onto Whatman glass filters (GF/D). The inducing agents and their concentrations were: bleomycin, 30 μg/ml (△); streptonigrin, 10 μg/ml (▲); UV (○); mitomycin C, 5 μg/ml (●); novobiocin, 50 μg/ml, and control (□). For UV irradiation, 10 ml of the culture in a petri dish was irradiated under UV lamps with a total intensity of 3120 ergs/mm^2. For thymine deprivation M01506 (a *thy*$^-$ derivative of M01513) was first grown in the presence of [2-^{14}C]thymidine (0.2 μCi/ml with 50 μg of thymine per ml); at 2×10^8 cells per ml, the cells were washed once with SSA medium, divided into two portions, and incubated at SSA medium in the absence (▼) and presence (▽) of unlabeled thymine (50 μl/ml).

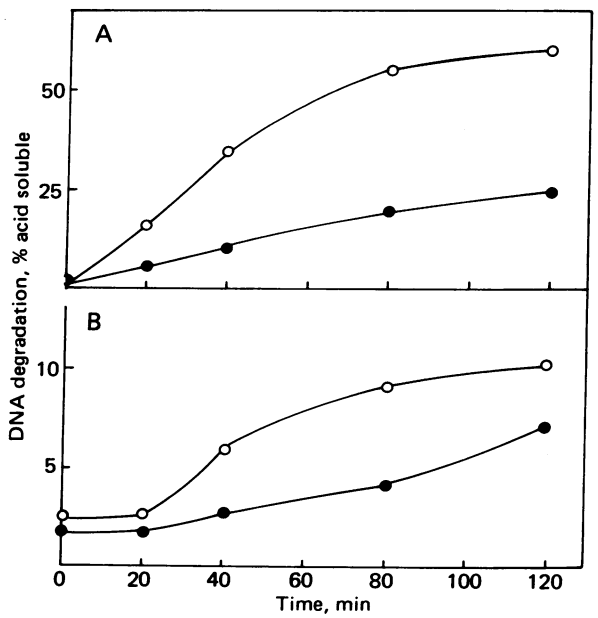

FIG. 4. DNA degradation in *recB*$^-$ and *recB*$^+$ strains after bleomycin (*A*) and mitomycin C (*B*) treatment. Strains M01512 (*rec*$^+$) (○) and M01518 (*recB21*) (●) were grown at 37° in SSA medium in the presence of [5-*methyl*-^{3}H]thymidine (6 μCi/ml with 1 μg of carrier thymidine per ml). At a density of 2×10^8 cells per ml, the cells were washed, resuspended in fresh SSA medium, and distributed into flasks containing bleomycin (30 μg/ml) or mitomycin C (5 μg/ml). Samples (1 ml) were taken intermittently as indicated. The acid-soluble radioactivities were then measured as described in the legend of Fig. 3.

Similarly, streptonigrin, an aminoquinine antibiotic, is known to cause single-strand scissions in DNA molecules (15). Recently, Schaller and Nomura (16) demonstrated that colicin E2 is a DNA endonuclease. On the other hand, none of the other inducing treatments used here is believed to cause DNA strand scission as a primary action. The principal effect of UV or mitomycin C treatment is modification of DNA [formation of pyrimidine dimers (17) or alkylations which often create crosslinks between two DNA strands (18)]. Thymine deprivation and temperature shift-up of dna_{ts} mutants cause inhibition of DNA replication by either depleting a precursor (thymine deprivation) or by inactivating the replication machinery (dna_{ts} mutants) and thereby immobilizing the entire chromosome structure.

From the experiments presented here, the following points become clear. (*i*) Various known inducing agents of conditions exhibit distinctly different patterns in the kinetics of the inactivation of phage repressor molecules. Based on time determinations with the agents we have tested so far, there appear to be three types of inducing agents and induction processes; bleomycin, streptonigrin, and colicin E2, 10–15 min; UV irradiation and mitomycin C, about 30 min; thymine starvation and temperature shift-up of dna_{ts} elongation mutants, 60–90 min. (*ii*) Damage to DNA molecules by strand scission seems to cause repressor inactivation most expeditiously, whereas prophage induction by the other types of DNA damage such as pyrimidine dimer formation or alkylation is less rapid. Induction due to inhibition of DNA replication by precursor deprivation or by inactivation of the replication machinery is a distinctly slower process. (*iii*) The inactivation of repressor molecules goes to completion in the absence of DNA replication.

DNA DEGRADATION BY *recBC* DNase AND REPRESSOR INACTIVATION

In order to obtain further information, we examined the pattern of DNA degradation after treatment of the cells with various DNA replication inhibitors (Fig. 3). Bleomycin and streptonigrin caused immediate degradation of prelabeled DNA, whereas DNA degradation induced by mitomycin C treatment or UV irradiation was a relatively slow process with a lag period of approximately 20 min. Degradation of prelabeled DNA after thymine deprivation or inactivation of the DNA replication apparatus (incubation of $dnaE_{ts}$ strain at nonpermissive temperature) (data not shown) started to appear at 60 min after initiation of treatments. Novobiocin, which inhibits DNA replication at 100 μg/ml but fails to inactivate phage repressor did not cause any degradation of DNA, even after 120 min of incubation. Essentially the same DNA degradation patterns were obtained with an isogeneic nonlysogen (M01512) (data not shown), indicating that the degradation was not due to prophage induction. These results demonstrate a distinct correlation between the timing of DNA degradation after induction treatment and the timing of repressor inactivation.

It is known that the *recBC* gene product *recBC*-DNase (19) is mainly responsible for the DNA degradation that occurs after UV or x-ray irradiation (20). In Fig. 4, we show the effect of the presence of a *recB*$^-$ mutation on DNA degradation after exposure to bleomycin and mitomycin C. Although these two agents inflict entirely different types of primary damage on DNA, it is clear that the presence of a *recB*$^-$ mutation results in delayed and reduced rates of DNA degradation in both cases. These DNA degradation experiments shown in Figs. 3 and 4

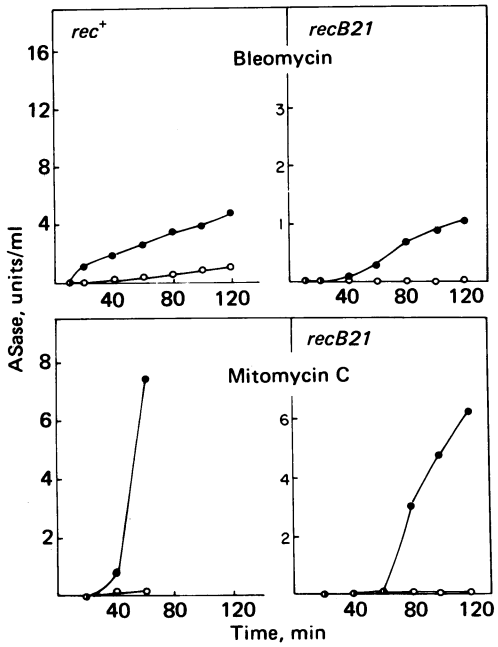

FIG. 5. Induction of ASase in a *recB*$^-$ strain after bleomycin (*Upper*) and mitomycin C (*Lower*) treatment. Strains M01513 (*rec*$^+$) (*Left*) and M01519 (*recB21*) (*Right*) were grown in SSA medium at 37°, infected with ϕ80*ptrp* at a multiplicity of infection of 10, and subjected to bleomycin (10 μg/ml) or mitomycin C (5 μg/ml) treatment. Experimental conditions were as described in the legend of Fig. 1. ●,With inducing treatment; ○, control without inducing treatment.

suggested an active role of *recBC* DNase in the DNA degradation and therefore possibly also in prophage induction. This would contradict previous *in vivo* experiments using a plaque assay procedure, which indicated that the *recBC* DNase was not involved in prophage induction (21).

Fig. 5 shows the effect of a *recB*$^-$ mutation on ASase synthesis induced by optimal doses of bleomycin and mitomycin C. The appearance of the ASase activity after each treatment was considerably delayed compared to that found with the *recB*$^+$ strain. In the case of bleomycin, for example, the time required for inactivation of the phage repressor molecules was 30–40 min with *recB*$^-$ cells as opposed to 10 min with *recB*$^+$ cells. Essentially the same delays in induction were observed with *recB*$^-$ cells after UV irradiation (data not shown). Comparison of Fig. 4 with Fig. 5 reveals a strong correlation between the changes in patterns of DNA degradation and ASase synthesis. The induction process seems to be triggered at the same time that the intracellular concentration of acid-soluble DNA degradation products reach a certain level.

The delaying effect of the *recB*$^-$ mutation on repressor inactivation appears to be a direct result of the absence of *recBC* DNase activity and not simply due to the mitomycin C or UV sensitivity associated with this type of mutation. Other UV- and mitomycin C-sensitive strains (*uvrB*$^-$ *and polA*$^-$) exhibited normal timing of repressor inactivation (data not shown). Furthermore, despite radiation sensitivity and recombination inefficiency, the viable *recB*$^-$ cells express normal cellular functions with respect to such characteristics as phage production or induced enzyme synthesis, whereas the nonviable cells produced during growth of *recB*$^-$ cells are completely inert in many aspects of cellular function including respiration (22).

Our results strongly suggest that at least bleomycin- and mitomycin C- (UV-) triggered induction involves DNA degradation by multiple DNases which include the *recBC* DNase. The previous failure to identify the *recBC* DNase as an active participant in the induction process is probably due to the involvement of more than one DNase in the induction process. The delay in induction, as seen in *recB*$^-$ strains with our ASase assay, would not be detected in a plaque assay system. We have observed an absolute requirement for the presence of the *recBC* DNase to obtain induction triggered by DNA degradation at the replication fork in a plasmolyzed, permeable cell preparation (unpublished data). Our results on DNA degradation are likely related to the observations by Gudas and Pardee (3) who demonstrated that X-protein synthesis is triggered by bleomycin or nalidixic acid treatment. If prophage induction is dependent on or coordinated with the synthesis of X protein, then our results are in good agreement with their model for triggering X-protein synthesis.

PROPOSED MODEL

These results encourage some speculation on the nature of the early molecular events in the induction of prophage and other SOS functions. The involvement of specific DNases such as *recBC* DNase plus the correlation between each inducing treatment's specific type of initial effect on DNA and the resultant kinetics of repressor inactivation suggest the existence of a final, possibly common, predegradative DNA structure(s) upon which the specific DNases act. Differences in time needed to complete the inactivation of the repressor molecules by the various inducing treatments may reflect differences in time required to reach such a final predegradative DNA form. The long lag period observed for appearance of DNA degradation and the subsequent prophage repressor inactivation after thymine deprivation or temperature shift-up with chain elongation dna_{ts} mutants, imply that the resulting chromosome structure, consisting of DNA with no physical damage but with an immobilized replication fork, is comparatively resistant to the attack of the DNases, and time-consuming modification steps may be necessary before the chromosome becomes susceptible to DNase attack. Less-time-consuming modifications may be necessary to bring a UV- or mitomycin C-damaged chromosome to the final predegradative state (20-min lag). In the case of novobiocin, the chromosome structure produced must be stable and not convertible to the DNase-susceptible predegradative form even after prolonged incubation. Similarly, the failure to induce prophage in DNA initiation temperature-sensitive mutants at nonpermissive temperatures is probably related to a resulting stable chromosome structure, presumably a completed chromosome without a replicating fork (23, 24).

One can speculate further about the nature of a hypothetical final predegradative DNA structure that acts as a substrate for the DNases such as the *recBC* DNase and whose degradation product(s) serves as a signal for the initiation of induction. It seems reasonable to suggest that the final predegradative structure is similar to or possibly identical with the DNA structure produced by bleomycin, streptonigrin, or colicin E2 treatment. In the case of bleomycin (and possibly streptonigrin), current evidence indicates that the final product, double-strand breaks, are produced as a result of single-strand scissions (11–14). Double-strand breaks are a direct product of colicin E2 activity (16). It should be noted that *in vitro* studies showed that the most efficient DNA substrate for purified *recBC* DNase is linear double-stranded DNA and that the enzyme did not act on circular DNA molecules, even if nicks were present (25–27). Based on these observations, we suggest that the most efficient final predegradative DNA structure capable of providing an induction signal is probably double-stranded DNA with scissions

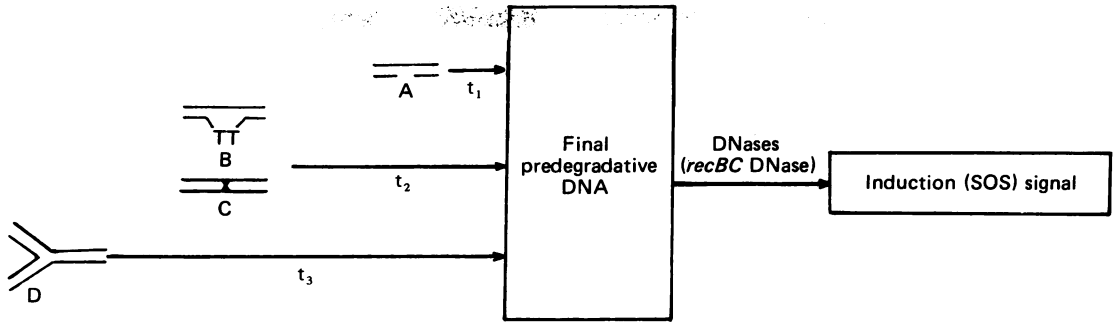

FIG. 6. Diagram of the model for induction of prophage and SOS functions. The DNA structures shown represent some examples of initial modifications produced by different inducing treatments: DNA with scissions (A), DNA with pyrimidine dimers (B), alkylated DNA (C), and DNA at an immobilized replication fork (D). The arrows t_1, t_2, and t_3 represent the different lengths of time required for conversion of the initially modified DNA to a final predegradative DNA structure. The differences in arrow lengths are arbitrary and are not accurate representations of the relative lengths of the time periods.

(possibly double-stranded scissions) or a related structure(s). Specific DNases such as *recBC* DNase then act on this structure and the degradation products serve as a signal for induction of prophage and other SOS functions. Fig. 6 shows a diagram of our proposed model. Studies of a large number of inducing agents have generated reports indicating a great variety of potencies. This may simply reflect differences in cellular efficiency for converting the variously modified DNA structures initially produced by these agents to final degradative DNase-susceptible DNA structures.

Of course these experimental results do not rule out the induction of SOS functions by mechanisms that do not depend on strand scissions, especially the presumably lethal double-strand scissions. In fact, induction of all SOS functions cannot depend solely on signals generated by lethal damage because some of the consequences of induction, such as recombination events and mutations, are detected in survivors of inducing treatments. The SOS functions elicited by different types of damage to DNA may vary quantitatively or qualitatively. In our experiments, the UV doses used were designed to elicit the maximum response and the most efficient induction signal. Consequently, our optimal dose (2500 ergs/mm^2) was much higher than that generally used for *in vivo* studies of UV mutagenesis or UV induction of prophages. We observed approximately 0.01% survival of the treated cells at the optimal UV dose. We observed similarly low survival after mitomycin C (5 μg/ml for 30 min) and bleomycin (30 μg/ml for 30 min) treatments—0.10% and 0.21%, respectively.

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