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# A protease inhibitor blocks SOS functions in *Escherichia coli:* Antipain prevents λ repressor inactivation, ultraviolet mutagenesis, and filamentous growth

**(error-prone DNA repair/prophage induction/*tif-1* mutation/carcinogenesis)**

M. STEPHEN MEYN*, TOBY ROSSMAN†, AND WALTER TROLL†

† Department of Environmental Medicine and *Department of Pathology, New York University Medical Center, New York, N.Y. 10016

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**ABSTRACT** Inhibition of DNA synthesis in *E. coli* by treatment with carcinogenic and mutagenic agents results in the coordinate expression of a group of diverse functions (SOS functions) including λ prophage induction, filamentous growth, and an error-prone DNA repair activity (SOS repair) believed to be responsible for ultraviolet mutagenesis. It has been proposed that this SOS induction proceeds via irreversible proteolytic inactivation of repressor(s) for SOS functions. To test this hypothesis, we investigated the effect of a protease inhibitor, antipain [(1-carboxy-2-phenylethyl)carbamoyl-L-arginyl-L-valylargininal], on SOS induction. We found that 0.5 mM antipain (which has no effect on cell growth, overall RNA and protein synthesis, or induction of β-galactosidase) drastically decreases mutagenesis.

Antipain also blocks expression of thermally induced mutator activity (another manifestation of SOS repair) and filamentous growth in a *tif-1* mutant that expresses SOS functions at 42° without inhibition of DNA synthesis or detectable DNA damage. Furthermore, antipain inhibits thermal induction of λ prophage in the *tif-1* mutant without affecting the kinetics of thermal induction of λcI857 prophage. This λ mutant codes a temperature-sensitive repressor that is directly destroyed by heat and does not require the SOS induction pathway for inactivation at 42°. From our results we conclude that antipain inhibits λ prophage induction by blocking proteolytic inactivation of λ repressor and that it inhibits the induction or expression of SOS repair and filamentous growth. Our results suggest a role for proteolytic cleavage in the regulation of SOS functions.

Exposure of *Escherichia coli* to UV light or other agents that cause DNA damage results in induction of λ phage in bacterial lysogens (1), filamentous growth in strains containing the *lon*$^-$ mutation (2, 3), mutagenic reactivation of UV-irradiated phage (4, 5), production of protein X (6), and induction of an error-prone DNA repair activity (7). The same phenomena also can be observed after halting cellular DNA synthesis, either by thymine starvation (2, 8) or by incubating temperature-sensitive DNA synthesis mutants at nonpermissive temperatures (9, 10). It has been suggested that these and other coordinately controlled processes, known as SOS functions (11), are inducible (3, 11–13).

The only known repressor for an SOS function is that for λ prophage (14). Roberts and Roberts (15) have shown that the λ repressor is proteolytically cleaved when bacterial lysogens are induced by UV light or mitomycin C. On the basis of this finding, they and others (13, 16) have formulated models for the common regulation of SOS functions in which proteases play a key role. These models propose that genes responsible for these functions are normally repressed. Upon formation of an induction signal, which is common to all inducing treatments, these genes are coordinately expressed, possibly by irreversible proteolytic inactivation of their repressors (13, 15, 16). These models thus suggest that SOS induction may proceed via a mechanism that is different from the classical model of induction of the lactose operon (17), in which inactivation of the repressor is thought to occur through an allosteric alteration resulting from the reversible binding of a small inducing molecule.

Roberts and Roberts (15) recognized that their results do not eliminate the possibility that proteolytic cleavage of λ repressor could be a secondary consequence of inactivation of the repressor by another means. In order to help clarify the role of proteases in SOS regulation, we have determined the effect of the protease inhibitor antipain [(1-carboxy-2-phenylethyl) carbamoyl-L-arginyl-L-valyl-argininal] (18) on expression of three SOS functions. Antipain is one of a group of low-molecular-weight protease inhibitors isolated from actinomycetes (19). It was selected for its relative nontoxicity (19), small size (which allows it to permeate the bacterial cell membrane), and ability to inhibit the trypsin-like protease II found in *E. coli* (20). We found that antipain inhibits inactivation of λ repressor and expression or induction of two other SOS functions: error-prone DNA repair and filamentous growth. This inhibition occurred at concentrations that do not inhibit general RNA and protein synthesis, β-galactosidase induction, or cell growth.

## MATERIALS AND METHODS

**Bacterial Strains.** The bacterial strains used in this study are described in Table 1.

**Preparation of Cultures.** Cultures were grown overnight at 37° in minimal broth Davis without glucose (MBD) (Difco) with 0.2% glucose added, supplemented with tryptophan, 10 μg/ml (MST). WP44$_s$ was grown at 30° in this medium supplemented with arginine, 20 μg/ml.

**Survival after UV Irradiation.** Logarithmic–phase cultures were prepared and irradiated as described previously (21). Cells were exposed to UV radiation in and serially diluted with MBD (Difco). Survival after irradiation was determined by plating onto "semi-enriched minimal medium" (MBD plus 0.2% glucose and 5%, vol/vol, Difco nutrient broth) (SEMM) agar plates (SEMM solidified with 1.5% Difco agar) with and without antipain, incubating at 37° for 2 days, and counting the resultant colonies. UV fluence was measured at 254 nm by an International light IL570 photometer.

**Mutagenesis.** UV-induced Trp$^+$ revertants were scored by irradiating cultures as described above and then plating the irradiated and unirradiated bacteria at various dilutions onto SEMM agar plates (22). Plates were incubated at 37° for 3 days and the resultant colonies were counted. On this medium, Trp$^+$

Abbreviations: antipain, (1-carboxy-2-phenylethyl)carbamoyl-L-arginyl-L-valylargininal; MBD, minimal broth Davis without glucose; MST, MBD supplemented with 0.2% glucose and tryptophan (10 μg/ml); SEMM, MBD supplemented with 0.2% glucose and 5% (vol/vol) nutrient broth.

Table 1. Bacterial strains used

| Strain | Genotype/phenotype | Source* |
|---|---|---|
| WP2 | *trp*⁻,*lon*⁻/Sfi⁻ | E.W. |
| $WP2_s$ | *trp*⁻,*uvrA*,*lon*⁻/Sfi⁻ | E.W. |
| WP5 | *trp*⁻,*lexA*,*lon*⁻/Sfi⁻ | F.M. |
| WP10 | *trp*⁻,*recA*,*lon*⁻/Sfi⁻ | F.M. |
| $WP44_s$ | *trp*⁻,*arg*⁻,*uvrA*,*tif-1*,*lon*⁻ | E.W. |
| $WP44_s$-NF | *trp*⁻,*uvrA*,*tif-1*,*lon*⁻/Sfi⁻ | E.W. |
| $WP44_s$-NF($\lambda^+$) | *trp*⁻,*uvrA*,*tif-1*,*lon*⁻/Sfi⁻ | E.W. |
| $WP44_s$-NF($\lambda c1857$) | *trp*⁻,*uvrA*,*tif-1*,*lon*⁻/Sfi⁻ | E.W. |

* E.W. = E. Witkin; F.M. = F. Mukai.

revertants produce large colonies against a limited lawn of Trp⁻ growth (22). Streptomycin-resistant mutants were scored by the method of Clarke and Hill (23). *tif-1*-induced Trp⁺ revertants were scored by growing $WP44_2$-NF to exponential phase (approximately $5 \times 10^8$ cells per ml) at 30° in MST medium and plating 0.1-ml suspensions of undiluted and serially diluted bacteria onto SEMM agar with and without 0.5 mM antipain. Plates were incubated at 30° and 42° for 4 days and scored for resulting Trp⁻ and Trp⁺ colonies.

**Filamentous Growth.** Strain $WP44_s$ was grown in MST medium at 30° and plated on SEMM agar with and without 0.5 mM antipain, without irradiation. The plates were incubated at 30° and 42° and examined by phase contrast microscopy for the presence or absence of long snake-like nonseptate filaments.

**β-Galactosidase Induction.** Logarithmic-phase cells grown MBD plus 0.2% glycerol and tryptophan (10 μg/ml) were induced and assayed for β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) as described previously (20).

**Incorporation of [*U*-$^{14}$C]Leucine and [2-$^{14}$C]Uridine.** A previously described (20) method was used except that the cells were grown in MST medium.

**λ Phase Induction.** Logarithmic-phase cultures of strains $WP44_s$-NF ($\lambda^+$) and $WP44_s$-NF ($\lambda c1857$) grown at 30° in MST medium were induced for phage by transferral to a water bath at 42°. Induction was monitored by measuring the decrease in culture turbidity at 650 nm with a Bausch and Lomb Model 20 spectrophotometer.

**Antipain.** Antipain was obtained from the U.S.–Japan Cooperative Cancer Research Program. Sterile stock solutions of antipain at 100 mM were prepared by dissolving antipain in distilled water and then filtering the solution through 0.22-μm Millipore filters. Agar plates containing antipain were prepared by adding sterile antipain to previously autoclaved agar held at 50° to avoid heat inactivation of the antipain.

## RESULTS

**Effect of Antipain on Survival after UV Irradiation.** Bacteria carrying the mutation *uvrA* lack excision repair. All remaining DNA repair is dependent on the *recA* gene (24). Fig. 1 shows the survival of UV-irradiated $WP2_s$ (*uvrA*) in the presence and absence of 0.5 mM antipain. Addition of antipain to the plating medium increased the UV sensitivity of $WP2_2$ bacteria without affecting survival of unirradiated controls. Antipain also increased the UV sensitivity of the Uvr⁺ parent strain, WP2, which possesses a full complement of repair genes. Antipain had no effect on UV-induced killing of closely related strains WP10 and WP5, which are defective in postreplication repair and carry the *recA-1* and *lexA-102* mutations, respectively. Thus, it would appear that antipain specifically inhibits DNA repair that is dependent on *recA*⁺ and *lexA*⁺.

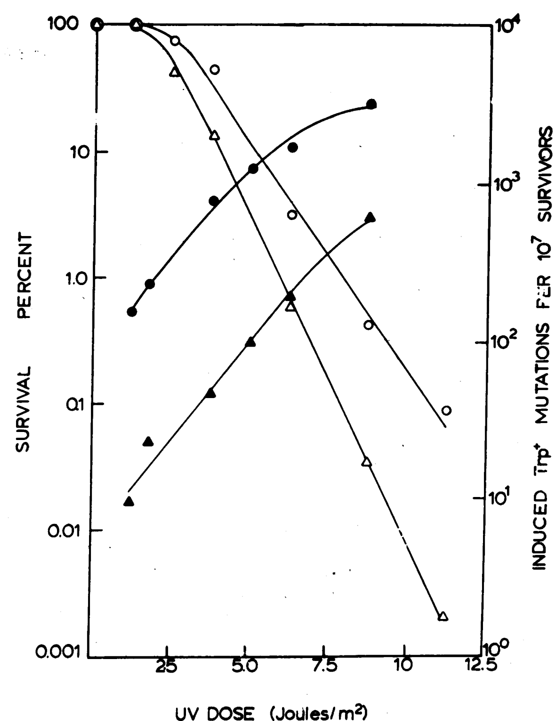


FIG. 1. Survival and mutability after UV irradiation, strain $WP2_s$ (*uvrA*) plated in the presence and absence of 0.5 mM antipain. ○, survival, absence of antipain; △, survival, presence of antipain; ●, induced Trp⁺ revertants, absence of antipain; ▲, induced Trp⁺ revertants, presence of antipain.

**Inhibition of UV-Induced Mutagenesis by Antipain.** Evidence has accumulated that UV-induced mutagenesis in *E. coli* is dependent on an inducible process dependent on *recA*⁺ and *lexA*⁺ (11, 13, 25). The frequency of the UV-induced mutation Trp⁻ to Trp⁺ for *uvrA* bacteria in the presence and absence of 0.5 mM antipain is shown in Fig. 1 and Table 2. Antipain caused a 70–90% reduction in the frequency of UV-induced Trp⁺ revertants/$10^7$ bacteria (Fig. 1). Table 2 shows the decreased yield of Trp⁺ revertants in the presence of antipain at sublethal UV fluences. This concentration of antipain does not affect the rate of growth, plating efficiency of 3 Trp⁺ revertant, or number of spontaneous Trp⁺ revertants detectable. Antipain had a similar effect on the frequency of UV-induced mutations to streptomycin resistance (data not shown). To eliminate the possibility that antipain inhibits expression of Trp⁺ revertants, *uvrA* bacteria were irradiated, incubated at 37° for 2 hr in MST medium in the presence or absence of 0.5 mM antipain, and then plated onto SEMM selective agar. Antipain was present only during the first 2 hr after irradiation, and selection of Trp⁺ revertants took place in the absence of antipain. In this experiment, antipain caused a 90% decrease in the number of Trp⁺ revertants.

**Differential Sensitivity of Cellular Functions to Antipain.** Although the above data indicate that 0.5 mM antipain inhibits expression of an SOS function (error-prone DNA repair), it is possible that this effects results from general effects on cellular metabolism. To investigate this possibility, [*U*-$^{14}$C]leucine incorporation, [2-$^{14}$C]uracil incorporation, β-galactosidase induction, growth rate, UV-induced mutagenesis, and survival after UV irradiation were measured in the presence of varying concentrations of antipain. The results of these experiments are shown in Fig. 2.

UV-induced mutagenesis was inhibited by antipain at concentrations as low as 0.1 mM; the 50% inhibitory dose was 0.19

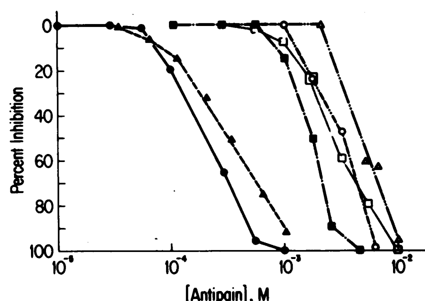


FIG. 2. Effect of antipain concentration on cellular functions in strain $WP2_s$ (*uvrA*). ●, $Trp^+$ revertants induced by UV irradiation, 5 $J/m^2$; ▲, survival after UV irradiation, 11.2 $J/m^2$; ■, induction of $\beta$-galactosidase; □, growth in MST medium; ○, [$U$-$^{14}C$]leucine incorporation into protein; △, [2-$^{14}C$]uridine incorporation into RNA.

mM. In contrast to this sensitivity, to begin 1 mM antipain was needed to inhibit $\beta$-galactosidase induction and growth. Total [$U$-$^{14}C$]leucine incorporation into protein and [2-$^{14}C$]uridine incorporation into RNA were even more resistant to inhibition by antipain. Because 0.5 mM antipain does not inhibit total RNA or protein synthesis, growth, or induction of an unrelated operon ($\beta$-galactosidase), it is unlikely that its almost total inhibition of UV-induced error-prone DNA repair is due to nonspecific effects on cellular metabolism. For further characterization of the antipain sensitive event(s) in SOS regulation we examined the effect of antipain on expression of three SOS functions in a *tif-1* mutant which expresses SOS functions at elevated temperatures.

**Inhibition of *tif-1* Mutator Activity by Antipain.** At 42° *tif-1* mutants induce λ prophage (26), grow in filaments (27), and exhibit increased spontaneous and UV-induced mutator activity (22, 28). *tif-1* maps in or near the *recA* gene (28) and may affect the *recA* gene product (13). The effect of antipain on mutator activity mediated by *tif-1* was determined by incubating strain $WP44_s$-NF at 30° and 42° for 4 days on SEM agar with and without 0.5 mM antipain and counting the resultant $Trp^+$ and $Trp^-$ colonies. Incubation at 42° for 4 days increased the frequency of $Trp^+$ mutants approximately 9-fold over the frequency at 30° (Table 2). This effect is believed to be due to the expression of error-prone DNA repair by *tif-1* mutants at 42° (13, 28). Antipain prevented the appearance of more than 85% of the thermally induced $Trp^+$ revertants without affecting the number of $Trp^+$ revertants on plates incubated at 30°.

*tif-1*-mediated filamentous growth in the presence of antipain was determined by incubating strain $WP44_s$ at 30° and 42° for 4 hr on SEMM agar containing arginine (20 μg/ml) with and without 0.5 mM antipain. Plates without antipain incubated at 42° contained snake-like filamentous cells whereas those with antipain contained cells and microcolonies similar in appearance to those on plates incubated at 30°. The results of these two experiments indicate that antipain blocks *tif-1*-mediated expression of two SOS functions.

**Effect of Antipain on λ Phage Induction.** The λ repressor is normally inactivated by SOS induction, and *tif-1* mutants that carry wild-type λ phage will induce prophage when heated at 42°. This induction is dependent on the *recA*$^+$, *tif-1*, and *lexA*$^+$ gene products. The effect of antipain on *tif-1*-mediated thermal induction of λ phage was determined by incubation of strain $WP44_s$-NF($\lambda^+$) at 42° in the presence of various concentrations of antipain. As shown in Fig. 3*a*, antipain delayed phage induction at concentrations as low as 0.177 mM. At 1 mM, antipain almost completely blocked the decrease in culture turbidity due to phage induction. This inhibition could be the result of antipain interfering with a step in prophage induction that occurs subsequent to inactivation of the λ repressor. In order to eliminate this possibility, the effect of antipain on thermal induction of a λ*c*I857 lysogen was determined.

λ phage carrying the *c*I857 mutation can be thermally induced at 42°. This is due to a temperature-sensitive repressor that fails to bind to its operator site at 42°, thereby allowing transcription of the phage genome and subsequent development of progeny and lysis of the cell (29). This induction does not require functioning *recA* and *lexA* gene products from the host cell (13). If antipain affects only SOS induction of λ, then it should have no effect on induction of λ*c*I857. When strain $WP44_s$-NF(λ*c*I857) was induced for phage by incubation at 42° in the presence of various concentrations of antipain, cell lysis due to phage induction was observed approximately 20 min after transfer at 42° and antipain in concentrations up to 1 mM had no effect on the observed decrease in culture turbidity due

Table 2. Effect of antipain on UV mutagenesis and *tif-1* mutator activity

| Strain | Antipain in medium | UV fluence ($J/m^2$) | Incubation temperature (°C) | No. of bacteria × $10^{-7}$: Screened per plate | No. of bacteria × $10^{-7}$: Total screened | No. of $trp^+$ revertants scored | Induced $trp^+$ revertants per $10^7$ bacteria plated |
|---|---|---|---|---|---|---|---|
| $WP2_s$* | 0 | 0 | 37 | 6.14 | 61.4 | 60 | — |
| | | 0.8 | 37 | 0.507 | 2.53 | 143 | 55.8 ± 4.7‡ |
| | | 1.8 | 37 | 0.507 | 2.53 | 609 | 241 ± 27 |
| | 0.5 mM† | 0 | 37 | 6.14 | 61.4 | 63 | — |
| | | 0.8 | 37 | 0.507 | 2.53 | 33 | 12.9 ± 3.1 |
| | | 1.8 | 37 | 0.507 | 2.53 | 80 | 31.6 ± 5.5 |
| $WP44_s$-NF | 0 | 0 | 30 | 0.781 | 4.68 | 52 | — |
| | | 0 | 42 | 0.781 | 4.68 | 440 | 82.3 ± 19.5 |
| | 0.5 mM | 0 | 30 | 0.781 | 4.68 | 47 | — |
| | | 0 | 42 | 0.781 | 4.68 | 83 | 10.1 ± 3.0 |

* UV fluences up to 1.8 $J/m^2$ are sublethal to strain $WP2_s$ resulting in 100% survival of irradiated cells when compared to unirradiated controls.
† The presence or absence of 0.5 mM antipain did not affect survival of irradiated cells at UV fluences shown on this table.
‡ Ninety-five percent confidence interval.

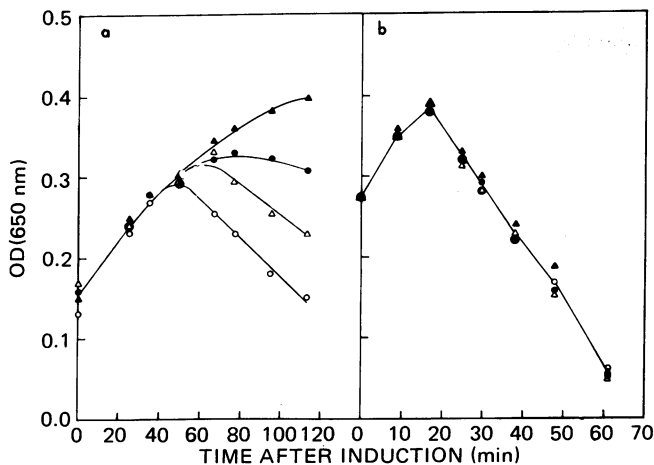


FIG. 3. Effect of antipain on thermal induction of λ phage in strain WP44$_s$-NF. Phage were induced by transferring exponential phase cultures to a 42° water bath. Induction was monitored by measuring the decrease in culture turbidity due to cell lysis by phage. *a*. Thermal induction of λ$^+$: ○, control; △, 0.177 mM antipain; ●, 0.5 mM antipain; ▲, 1 mM antipain. *b*. Thermal induction of λcI857: ○, control; △, 0.177 mM antipain; ●, 0.5 mM antipain; ▲, 1 mM antipain.

to phage induction (Fig. 3*b*). Thus, antipain has no apparent effect on the events leading to cell lysis that occur subsequent to inactivation of λ repressor.

## DISCUSSION

Antipain, a protease inhibitor, blocks UV mutagenesis, and enhances UV killing of bacteria that possess both the *recA*$^+$ and *lexA*$^+$ genes. Antipain also inhibits *tif-1*-mediated induction of wild-type λ prophage, *tif-1*-mediated expression of filamentous growth, and the mutator effect of the *tif-1* allele at 42°. From these findings we conclude that antipain inhibits expression of three different SOS functions: error-prone DNA repair, filamentous growth, and λ prophage induction. This inhibition is probably due to a specific effect on SOS induction or expression because, at concentrations required to inhibit SOS functions, antipain has no effect on cell growth, RNA and protein synthesis, or the induction of an unrelated operon (β-galactosidase).

Our results provide indirect evidence that antipain may not inhibit expression of a fourth SOS function, control of DNA degradation by exonuclease V (30, 31). The UV sensitivity of antipain-treated *uvrA* bacteria is similar to that of *uvrA*-(*lexA*)*rmn*$^-$ mutants. *rmn*$^-$ mutants are *lexA* derivatives that lack error-prone DNA repair but have regained control of exonuclease V activity, resulting in their being more resistant to UV radiation than their *lexA* parent (32). Because antipain-treated bacteria appear to be *rmn*$^-$ phenocopies with respect to UV sensivity and mutability, antipain may not inhibit expression of exonuclease V control.

It has been proposed that the diverse group of treatments and genetic backgrounds that induce SOS functions operate by generation of common nucleic acid intermediates that act as induction signals (3, 16, 26, 27). Thermal induction in the *tif-1* mutant apparently does not require these induction signals because, at 42°, the *tif-1* mutant expresses SOS functions without detectable DNA damage or disturbance of DNA synthesis (33). Since antipain inhibits expression of SOS functions in the *tif-1* mutant, it must inhibit a step in expression that occurs subsequent to production of any inducing signals. Furthermore, for λ prophage induction, the antipain-sensitive event must occur during *tif-1*-mediated SOS inactivation of λ repressor since antipain does not affect SOS-independent λ prophage induction. Therefore, the antipain-sensitive event is most probably proteolytic inactivation of the λ repressor itself. The antipain-sensitive proteolytic activity appears to be constitutive, since antipain inhibits *tif-1*-mediated SOS induction, which requires no *de novo* protein synthesis (34). Thus, λ prophage induction may be regulated by control of proteolytic cleavage of λ repressor, possibly through allosteric conformational changes in either repressor or protease. This type of regulation has been demonstrated in the case of protease II cleavage of aspartase I in *E. coli*. In this case, the conformational state of the allosteric enzyme aspartase I determines its susceptibility to proteolysis (35).

Our results support the hypothesis of Roberts and Roberts (15) that λ repressor is normally inactivated by irreversible proteolytic action and provide evidence suggesting that proteases play a key role in the induction of SOS functions, as proposed by Roberts and Roberts (15), Gudas and Pardee (16), and Witkin (13). Proteases are known to be involved in control mechanisms in a wide variety of organisms (36, 37). A system similar to SOS induction in *E. coli* may exist in mammalian cells. Pretreatment of host cells with UV light or x-radiation before infection has been shown to result in Weigle reactivation of UV-irradiated herpes simplex and simian virus 40 (38, 39). In addition, D'Ambrosio and Setlow (40) have shown that Chinese hamster cells exhibit enhanced rates of postreplication repair after treatment with small doses of UV light or the carcinogen *N*-acetoxyacetylaminofluorene. This enhancement requires *de novo* protein synthesis (40), suggesting that inducible DNA repair exists in mammalian cells. There is reason to believe that mammalian DNA repair plays a role in chemical carcinogenesis. Most carcinogens are both mutagens (41) and inducers of SOS functions (13, 42). The human genetic disease xeroderma pigmentosum, characterized by extreme sensitivity to UV light and susceptibility to multiple epidermal carcinomas, is associated with defects in DNA repair (43). Furthermore, fibroblasts from xeroderma pigmentosum patients express two SOS-like functions—Weigle reactivation and UV-induced mutagenesis—at lower UV fluences than do normal human fibroblasts (39, 44). Because protease inhibitors have been shown to block tumorigenesis by chemical carcinogens (45, 47), it is conceivable that, if an SOS-like repair function is responsible for carcinogenesis, it may require a proteolytic cleavage for induction or expression.

**Note Added in Proof.** Experiments on the effect of antipain on the thermal induction of λ have been carried out 20 times. The magnitude of the inhibition of λ$^+$ induction varies somewhat, depending on growth conditions. Antipain has a greater effect when glycerol is used as a carbon source, compared to glucose. Increased aeration also tends to decrease the effect of antipain. In general, the faster the growth rate, the more antipain is needed to inhibit induction. The effect of antipain can also be partially blocked by adenine, which causes a faster thermal induction of λ$^+$ in *tif-1*.

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