

SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity

(*Salmonella* mutatest/phage inductest/carcinogen tests)

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ABSTRACT We present and evaluate the SOS chromotest, a bacterial test for detecting DNA-damaging agents. It is a colorimetric assay based on the induction by these agents of the SOS function *sfiA*, whose level of expression is monitored by means of a *sfiA::lacZ* operon fusion. The response is rapid (a few hours), and does not require survival of the tester strain. Dose-response curves for various chemicals include a linear region. The slope of this region is taken as a measure of the SOS inducing potency. Comparison for a number of substances of known genotoxicity of the SOS inducing potency determined in the SOS chromotest with the mutagenic potency determined in the *Salmonella* assay (mutatest) revealed a striking quantitative correlation over more than 7 orders of magnitude. The sensitivity of the SOS chromotest (lowest amount detected) is equal to that of the mutatest and generally 4–40 times higher than that of a phage induction assay (inductest). From a practical point of view our observations contribute to the validation of the SOS chromotest as a test for detecting genotoxins and in particular genotoxic carcinogens. From a theoretical standpoint the results suggest that mutagenic potency measured in the mutatest reflects the level of induction of an SOS function and that most genotoxins are inducers of the SOS response in bacteria.

Agents that interact with DNA *in vivo* have potential adverse effects on human health. In particular they may induce transmissible mutations and cancer. On the other hand, some of them are also used in cancer treatment. The detection and classification of these agents, as well as the elucidation of their largely unknown modes of action, are central problems in genetic toxicology.

Bacteria are widely used as indicator organisms in test systems for genetic toxins (1, 2). They offer practical advantages and may provide insights into the basic mechanisms of genotoxicity and of its consequences. One of the best known systems is the *Salmonella*/microsome assay (mutatest). This reversion assay, performed on *Salmonella* strains, provided strong indications that carcinogens were generally mutagens and vice versa (3–6). Many other bacterial tests have been described, including phage induction assays (inductest) (7–11). A number of test systems were recently the object of an international collaborative study (12).

In the present paper we describe another assay for genotoxic agents, which adds to our understanding of the molecular basis of bacterial tests and offers further practical advantages. It is based on the following rationale. Consequences of genotoxic action that are taken as end points in bacterial toxicology tests (such as mutagenesis or phage induction) are often not due to the primary action of the agent but rather, at least in part, to the responses of the cell to this action. Indeed, although bacteria

are the simplest DNA-containing cells, they possess elaborate mechanisms to respond to DNA-damaging agents (13). In *Escherichia coli* some of the responses induced by DNA-damaging treatments involve a set of functions known as the SOS responses (14–16) as well as “damage inducible” (*din*) genes (17).

We have taken advantage of an operon fusion placing *lacZ*, the structural gene for β -galactosidase, under control of the *sfiA* gene (18), an SOS function involved in cell division inhibition, to devise a simple and direct colorimetric assay of the SOS response to DNA damage. We call this assay the “SOS chromotest.” It is quantitative and provides a parameter, the SOS-inducing potency (SOSIP), which for most of the compounds examined is closely correlated with the mutagenic potency determined in the mutatest. The correlation with the mutatest provides a validation of the SOS chromotest as a test for genotoxins.

The results suggest in addition that the mutagenic potency measured in the mutatest reflects the level of induction of an SOS function and that most genotoxic agents are indeed inducers of the SOS response in bacteria.

MATERIALS AND METHODS

Bacterial Strain. The strain PQ37 used in this study has the genotype F^- *thr leu his-4 pyrD thi galE galK or galT lacΔU169 srl300::Tn10 rpoB rpsL uvrA rfa trp::Muc⁺ sfiA::Mud(Ap, lac)cts*. It is constitutive for alkaline phosphatase synthesis. This strain was derived by standard genetic techniques from a *sfiA::lacZ* fusion strain (18).

Media, Buffers, and Reaction Mixtures. Bacteria were cultured in LB medium (19) supplemented with ampicillin at 20 μ g/ml. Z buffer is as described by Miller (19). T buffer is 1 M Tris adjusted to pH 8.8 with HCl. S9 microsome fraction for activating test compounds was prepared from rats treated with Aroclor 1254 (5).

Test Procedures. SOS chromotest. The test consists of colorimetric assays of enzymatic activities after incubating the tester strain in the presence of various amounts of compound. A detailed procedure will be published elsewhere. Briefly, an exponential-phase culture grown to $OD_{600} = 0.4$ in LB medium plus ampicillin at 37°C is diluted 1:10 into either activation mix (5) or fresh medium. Fractions (0.6 ml) are distributed into glass test tubes containing 20 μ l of the compound to be tested. After 2-hr incubation at 37°C with shaking, β -galactosidase and alkaline phosphatase activities are assayed.

Mutatest and inductest. The mutatest (5) and inductest (8) were performed as described.

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Abbreviations: SOSIP, SOS inducing potency; 4NQO, 4-nitroquinoline 1-oxide.

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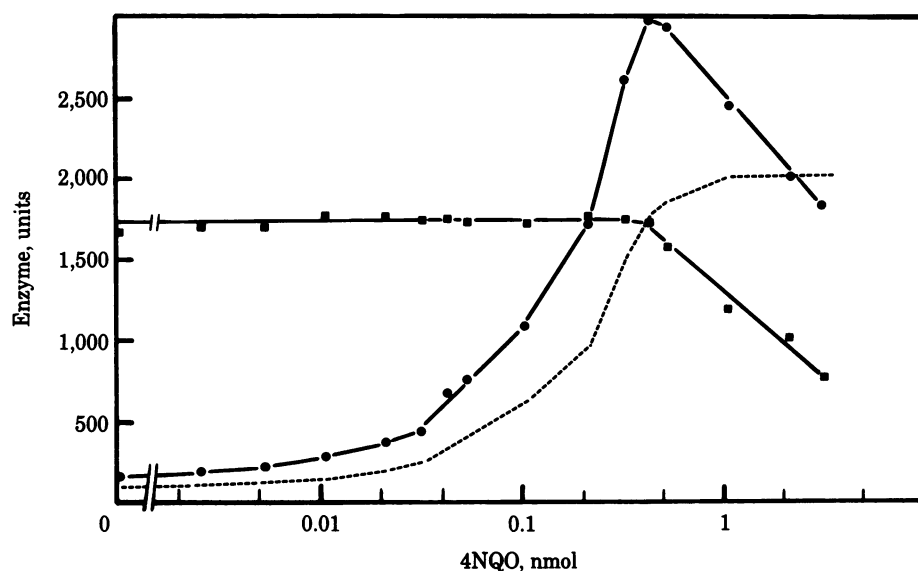


FIG. 1. SOS chromotest: dose-response relationships obtained with 4NQO. Assays were performed without metabolic activation. The abscissa represents the amount of compound per assay (0.3 ml). The amount per ml is thus 3.33 times higher. The ordinate represents β -galactosidase activity (β) (●) and alkaline phosphatase activity (p) (■), both in units per assay. The broken line represents the ratio $R(c) = \beta/p$. The induction factor at concentration C is defined as $I(c) = R(c)/R(0)$, in which $R(0)$ is the $R(c)$ without added DNA-damaging agent.

Enzyme Assays. Alkaline phosphatase. T buffer (2.7 ml) is added to 0.3 ml of cell culture. Cell membranes are disrupted by adding 0.1 ml of 0.1% sodium dodecyl sulfate solution and 0.15 ml of chloroform and mixing vigorously. Tubes are equilibrated at 28°C. The reaction is started by addition of 0.6 ml of *p*-nitrophenyl phosphate solution (4 mg/ml in T buffer) and stopped by addition of 1 ml of 2 M HCl. After 5 min, 1 ml of 2 M Tris is added to restore the color, which is measured spectrophotometrically at 420 nm. Enzyme units are calculated as for β -galactosidase (19).

β -Galactosidase. The assay is as described by Miller (19). The protocol is the same as for alkaline phosphatase except that Z buffer replaces T buffer, *o*-nitrophenyl galactoside replaces *p*-nitrophenyl phosphate, and the reaction is stopped with 2 ml of 1 M Na_2CO_3 .

RESULTS

The tester strain carries a *sfiA::lacZ* operon fusion and has a deletion for the normal *lac* region so that β -galactosidase activity is strictly dependent on *sfiA* expression. To increase the response to certain DNA-damaging agents the strain was made deficient in excision repair (*uvrA*) (13), and to allow better dif-

fusion of chemicals into the cell it was made lipopolysaccharide deficient (*rfa*) (5). The assay consists of incubating the tester strain with various concentrations of the agent to be tested. After a time for protein synthesis, β -galactosidase activity is assayed. The classical microsomal activation preparation (5) can be included in the incubation mixture.

The chemicals tested may at certain concentrations inhibit protein synthesis, which would lead to an underestimate of β -galactosidase induction. To correct for this, we estimate general protein synthesis during the incubation period. The strain was made constitutive for alkaline phosphatase synthesis (20). This enzyme, noninducible by DNA-damaging agents, is assayed in parallel with β -galactosidase. The ratio of the two activities (β -galactosidase to alkaline phosphatase) is taken as a measure of the specific activity of β -galactosidase.

Because different compounds could vary in their rate of metabolism, penetration, or triggering of the SOS response, it was first necessary to study the kinetics of induction. Results with a series of known genotoxins (data not shown) established that the activity ratio of β -galactosidase to alkaline phosphatase rises to a plateau in 70–100 min. This plateau was generally maintained for more than 2 hr. In the remainder of this work we take

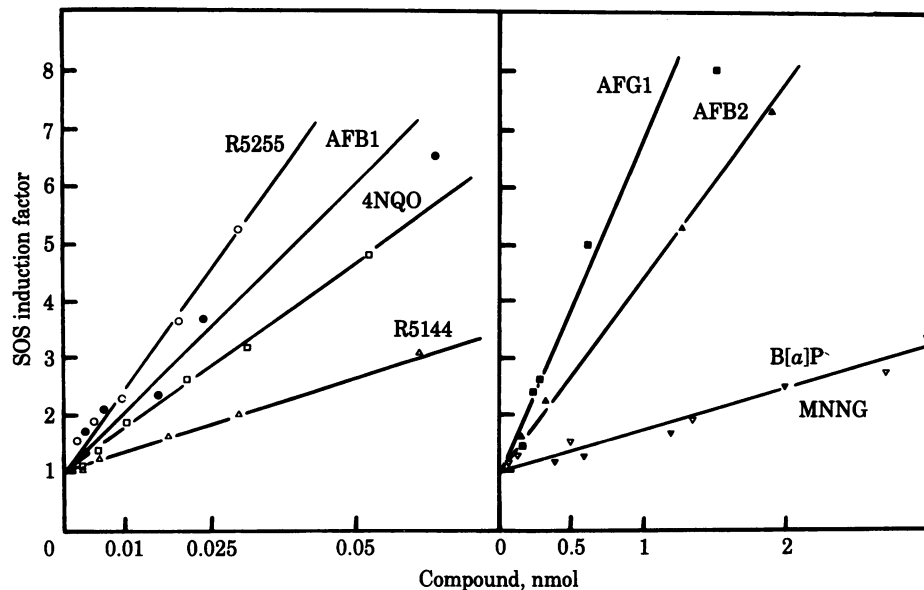


FIG. 2. SOSIP for a series of compounds. The variation of the induction factor $I(c)$ with the amount of compound has a linear portion that allows us to define the SOSIP of the compound as $\Delta I/\Delta C = \Delta R/R(0)\Delta C = \text{SOSIP}$, in which R is the ratio β/p . Closed symbols, compounds tested with activation; open symbols, compounds tested without activation. SOSIP values are given in Table 2. Abbreviations for compounds are defined in the legend of Fig. 3.

the ratio measured after 2-hr incubation as representative of the plateau value.

To compare different experiments and different compounds, we normalize this activity ratio to its value in the absence of test compound. This is especially important when comparing experiments with and without activating preparation, which influences alkaline phosphatase activity (20). The normalized value is called the induction factor, *I*.

The dose-response curve for the well-known carcinogen and mutagen 4-nitroquinoline 1-oxide (4NQO) shows that the induction factor rises to a maximum, then remains constant, even at concentrations that inhibit general protein synthesis (Fig. 1).

It is remarkable that for all active compounds tested the plot of the induction factor versus concentration includes a linear portion (Fig. 2). We call its slope the SOSIP. Thus the SOS chromotest defines a simple parameter, the SOSIP, that for

each substance is a quantitative expression of its capacity to induce the *sfia* response in *E. coli*.

We have compared the responses obtained in the SOS chromotest and in two other tests, the mutatest (5) and an inductest (8), with a wide range of agents, for most of which carcinogenicity data are available (Table 1). The results indicate that compounds active in the mutatest (including certain esters and nitrosamines) are active in the SOS chromotest, whereas compounds inactive in the mutatest (dimethyl sulfoxide, NaCl, caffeine, and aspirin) are inactive in the chromotest.

Moreover, a comparison of mutagenic potency, measured by the mutatest, and SOSIP, measured by the SOS chromotest, revealed a striking quantitative correlation over seven orders of magnitude (Fig. 3).

Among the compounds tested two known carcinogens were positive in the SOS chromotest but negative in the mutatest.

Table 1. Responses in three different bacterial tests and carcinogenicity data

Compounds	Potency			Sensitivity, nmol			Carcinogenicity
	SOS chromotest	Inductest	Mutatest	SOS chromotest	Inductest	Mutatest	
Benzofurans and naphthofurans*							
R7000	26,000	1,200	200,000	0.00001	0.0004	0.0004	+ [†]
R6998	18,000	62	170,000	0.00001	0.0004	0.0004	+ [†]
R7100	11,000	83	45,000	0.00001	0.0004	0.0004	
R6597	9,000	36	20,000	0.00003	0.0009	0.004	+ [†]
R5255	130	2.8	630	0.002	0.03	0.3	
R5144	33	0.4	350	0.002	0.06	0.3	
R7216	0.03		0.1	3		60	
R7187	Neg		Neg	Neg		Neg	
Fungal toxins and antibiotics							
AFB1	75	0.5	12,000	0.005	0.06	0.006	+ (22)
AFG1	6.4	0.03	920	0.05	1.5	0.3	+ (22)
AFB2	3.3	0.03	690	0.1	1.6	0.3	+ (22)
AFG2	Neg	Neg	0.2	Neg	Neg	150	+ questionable (22)
MMC	70	0.5	Neg [†]	0.005	0.03	Neg [†]	Carcinostatic (23)
NCS	390	0.02	Neg (24)	0.0002	0.00009	Neg (24)	Carcinostatic (24)
Esters							
MMS	0.02	Neg	0.2	19	Neg	640	+ (25)
EMS	0.0004	Neg	0.03	490	Neg	24,000	+ (25)
DMS	0.04	Neg [§]	0.1	2	Neg [§]	550	+ (25)
DES	0.008	Neg	0.05	30	Neg	12,000	+ (25)
β-Propiolactone	0.1	0.00006	1.6	<20	340	75	+ (25)
Propane sultone	0.03		2.8	<8		<110	+ (25)
Nitrosamines							
DMN	0.002	Neg [¶]	0.002	40	Neg [¶]	130,000	+ (25)
DEN	0.03	Neg [¶]	0.005	60	Neg [¶]	49,000	+ (25)
MNNG	0.9	0.007	44	0.15	6.8	0.7	+ (25)
Miscellaneous							
B[a]P	0.8	0.1	100	0.7	0.4	4	+ (25)
4NQO	71	0.9	2,100	0.006	0.05	0.05	+ (25)
Dimethyl sulfoxide	Neg	Neg	Neg	Neg	Neg	Neg	Neg (25)
NaCl	Neg			Neg			?
Caffeine	Neg		Neg (25)	Neg		Neg (25)	Questionable (25)
Aspirin	Neg		Neg (25)	Neg		Neg (25)	Neg (25)

For each bacterial test, the inducing or mutagenic potency and the sensitivity of the test (lowest amount detected = lowest point at which the response is systematically over twice the background) are indicated. Neg, compound had only background activity. SOS chromotest: potency is induction factor per nmol per assay (SOSIP); sensitivity is amount of compound in nmol per assay. Inductest: potency is inverted value of amount of compound in nmol per ml for half-maximal induction; sensitivity is amount of compound in nmol per assay. Mutatest: potency is histidine-independent revertants per nmol per plate; sensitivity is amount of compound in nmol per plate. Data are from experiments performed in parallel (see legend of Fig. 3) except when otherwise indicated. Numbers in parentheses are references. Abbreviations are defined in the legend of Fig. 3.

* Data for the mutatest and inductest are from ref. 21.

† F. Zajdela, personal communication.

‡ Mitomycin C (MMC) is mutagenic in the *uvr*⁺ strain.

§ Dimethyl sulfate (DMS) is a weak inducer detected only in spot test.

¶ The inductest was performed independently from the two other tests.

The first, neocarcinostatin, is known to be toxic to bacteria but is detected in tests that do not require the survival of the tester strain. The second, mitomycin C, is detectably mutagenic only in *uvr*⁺ derivatives of the *Salmonella* used in the mutatest (6). A single compound, aflatoxin G2, was negative in the SOS chromotest and weakly positive in the mutatest. This compound is a questionable carcinogen (Table 1).

It should be noted that compounds that require metabolic activation, with the exception of dimethylnitrosamine and diethylnitrosamine, yield a relatively weaker response in the SOS chromotest than in the mutatest (Fig. 3). For such compounds the agar medium used in the mutatest is believed to stabilize the activation enzymes (5). The fact that aflatoxin G2 is not detected in the SOS chromotest may thus be due to poor activation. In the case of diethyl- and dimethylnitrosamines, it has been shown that activation is more effective in liquid than on plates (5).

We also found a generally good correlation between the SOSIP measured in our test and the inducing potency determined in the inductest. This is in agreement with previous findings describing a correlation between the responses in the mutatest and the inductest and is not surprising because both the SOS chromotest and the inductest are known to depend on induction of the SOS response (21, 26).

In series of esters and nitrosamines, however, several compounds, although positive in the SOS chromotest, were either negative (methyl and ethyl methanesulfonate, dimethyl and diethyl sulfate, and dimethyl- and diethylnitrosamine) or weakly positive (β -propiolactone) in the inductest. In fact all of these compounds have low SOSIP. Thus the inductest is less sensitive than the SOS chromotest in its detection capacity, giving a positive response only for compounds whose SOSIP is above a certain threshold.

Another measure of sensitivity is the lowest detectable amount of an active substance. We define this from the dose-response curve, taking the lowest point at which the response is systematically at least twice background. It can be seen (Table 1) that the SOS chromotest is more sensitive than the inductest, generally detecting 1/4th to 1/40th the amounts of compounds active in both tests. Similarly, the SOS chromotest is comparable to or more sensitive than the mutatest for the substances screened (Table 1).

DISCUSSION

We describe here the SOS chromotest, a test for detecting DNA-damaging agents. It is based on the induction by these agents of the SOS function *sfiA*, whose level of expression is monitored by means of a *sfiA::lac* operon fusion. We further present a preliminary characterization of the SOS chromotest, measuring its response to a number of compounds and comparing these responses to those of the classical mutatest (5) and inductest (8).

The SOS chromotest has several practical advantages. It is easy to perform, requiring only a single strain and simple colorimetric enzyme assays: β -galactosidase and alkaline phosphatase. In qualitative determination only the β -galactosidase assay is required. It is rapid, giving a response within several hours. Furthermore, it does not require survival of the tester strain, thus permitting detection of toxic substances (such as neocarcinostatin) that are negative in the mutatest. The SOS chromotest gives quantitative responses, which, remarkably, are a linear function of the concentration of compound tested in the low dose range. The slope of this linear region defines a single parameter, the SOSIP, representing the increase in induction factor per nmol of compound tested. It is striking that the SOSIP varies over a 60-million-fold range for the substances tested.

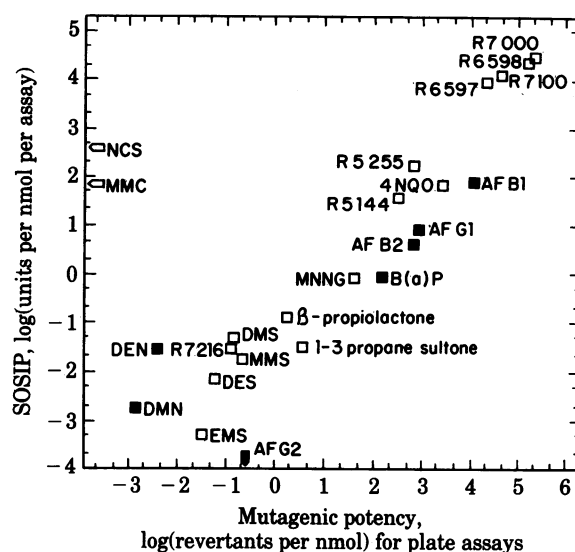


FIG. 3. Correlation between the SOSIP and the mutagenic potency in the mutatest for a series of genotoxins. For each compound [except for the benzo and naphthofurans (21)] experiments were performed in parallel to determine the SOSIP and the mutagenic potency in the mutatest (strain TA100). The same stock solutions and, when needed, the same activation mixtures were used. Independent determinations varied by less than a factor of 3. Closed symbols, compounds tested with activation. Open symbols, compounds tested without activation. Arrows, compounds that were not detected in one of the tests. Chemicals: abbreviations and origins. Aflatoxin (AF) B1, G1, B2, and G2, diethylnitrosamine (DEN), 1,3-propane sulfone, and β -propiolactone were from Serva; ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) were from Eastman; benzo[*a*]pyrene (B[a]P), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), dimethyl sulfate (DMS), and diethyl sulfate (DES) were from Aldrich; dimethylnitrosamine (DMN) was from Ega; 4NQO was from Fluka; caffeine and mitomycin C (MMC) were from Sigma; dimethyl sulfoxide and sodium chloride were from Merck; aspirin was from Upsa; neocarcinostatin (NCS) was from Kagaku Antibiotics Research; and 2-nitrobenzofuran (R5144), 2-nitro-5-methoxybenzofuran (R5255), 2-nitronaphtho(2-1-*b*)furan (R6597), nitro-2-methoxy-8-naphtho(2-1-*b*)furan (R6598 (shown on the plot as R6598 by error)), 2-nitro-7-methoxynaphtho(2-1-*b*)furan (R7000), nitro-2-methoxynaphtho(2-1-*b*)furan (R7100), 7-methoxynaphtho(2-1-*b*)furan (R7187), and naphtho(2-1-*b*)furan (R7216) were from R. Royer.

In bacteria, DNA damage is known to induce the SOS responses, a series of manifestations including increased repair and mutagenic activities, prophage induction, and cell division arrest. Induction of all these functions depends on the activation of the *recA*⁺ product to a protease able to cleave the *lexA*⁺ product, the general repressor of SOS function, and the λ C⁺ product, the repressor of phage λ (16).

The striking quantitative correlation over 7 orders of magnitude and the comparable sensitivities of the responses in the mutatest and the SOS chromotest suggest that the mutatest may in fact essentially detect SOS mutagenesis. The mutagenic potency, like the SOSIP, would directly reflect the level of expression of some SOS function under *lexA* control. A likely candidate is the *umuC*—or its counterpart *mucC* (27) carried on plasmid pKM101 gene product—known to be indispensable for SOS mutagenesis (28), under *lexA* control, and inducible by DNA-damaging agents (29).

As early as 1953 Lwoff (30) proposed λ induction as an assay for carcinogens and carcinostatic agents. It is known now that λ induction involves cleavage of the λ repressor by *recA* protease (31) and is thus an SOS manifestation. On the other hand, a number of mutagens and known genotoxins are negative in the inductest. We have examined some of these mutagens and found them to be positive in the SOS chromotest (Table 1),

showing that they are in fact inducers of the *sfiA-lacZ* fusion. The compounds positive in the SOS chromotest but negative in the inductest all had low SOSIP. This greater sensitivity of the SOS chromotest may reflect an underlying molecular difference. All inductests assay SOS induction through the expression of functions repressed by the λ CI protein: phage production (7, 8) or synthesis of endolysin (11), β -galactosidase (10), or galactokinase (9). The SOS chromotest, on the other hand, is based on induction of *sfiA* expression, repressed by the *lexA* protein (18). Although both λ CI and *lexA* repressors are cleaved by *recA* protease (31, 32), the cleavage rate is at least 10-fold greater for the *lexA* protein than for the λ repressor (32, 33). This could explain the greater sensitivity of the SOS chromotest, which reveals activity with compounds of low SOSIP that are inactive in the inductest and detects 1/4th to 1/40th the amounts of active compounds. Such an explanation is supported by the fact that an inductest, in which a phage with a mutant repressor (*λ*CI857*ind*⁺) is used in conditions in which the repressor is destabilized (35°C), reaches the same range of sensitivity as the SOS chromotest (11). However in this case the conditions of the assay as well as quantitation are more difficult than in the SOS chromotest.

Two different genetic phenomena have been causally associated with cancer induction: mutations (22, 23) and chromosomal rearrangements (34). Functions encoded by the SOS genes are important both for mutagenesis (*umuC*) and for transposition (*himA*, *himB*) (35), which may lead to genetic rearrangements. If a similar inducible pathway through which genotoxic agents could influence mutagenesis and chromosomal rearrangements exists in higher cells (36) it would help account for the variable propensity of different genotoxic agents to induce gene mutation, chromosomal rearrangements, and cancer (37). We would thus be closer to a unified view of chemical carcinogenesis.

We have recently examined the response to UV light (260 nm) of the SOS chromotest. We found that a doubling of β -galactosidase specific activity was obtained at about 0.05 J/m² or less. This, according to Boyle and Setlow (38), suggests that very few UV lesions per chromosome are enough to give a detectable signal in the test.

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