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Genetics

Quantitative forward mutation assay in *Salmonella typhimurium* using 8-azaguanine resistance as a genetic marker

(genetic toxicology/mutagenesis/screening assay)

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ABSTRACT **We have developed a quantitative forward mutation assay using *Salmonella typhimurium*, in which resistance to the purine analog 8-azaguanine is used as a genetic marker. We present the assay protocol, the concentration-dependent toxicity and mutagenicity of five known mutagens (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ICR-191, 9-aminoacridine, dimethylnitrosamine, and benzo[*a*]pyrene), and reconstruction experiments testing the assay for possible bias. The relative merits of forward versus reverse mutation assays are discussed.**

Bacterial mutation assays are inexpensive, rapid, and easy to perform. Ames and his colleagues have developed a set of *Salmonella typhimurium* strains that are permeable to a wide range of chemicals and also are partially deficient in DNA repair (1). Both of these characteristics have been demonstrated to increase the sensitivity of the tester strains to chemical mutagens. The Ames tester strains commonly applied in screening for potential mutagens carry *in toto* three specific point mutations in the histidine operon, which must be reverted or suppressed in order to produce a histidine prototroph. This set of mutations includes one unknown base-pair substitution (hisG46) and two frameshift mutations (hisC3076 and hisD3052) for which the DNA sequence changes required have been determined (1). Use of data from independent assays with several strains permits classification of a chemical mutagen with regard to its ability to cause the particular base substitution or either of the two frameshift mutations. Such classification is important in understanding the mechanisms by which chemicals cause genetic change.

A practical point must be emphasized, however, about the toxicologic application of these specific reversion assays: any set of tester strains containing a small number of revertable point mutations may not encompass the entire set of possible mutagenic lesions in DNA caused by environmental chemicals. For instance, the unknown base-pair substitution mutation (his G46) used in Ames' strains TA1535 and TA100 may represent only one of the six possible base substitution mutations (A·T → T·A, G·C, C·G; G·C → C·G, A·T, T·A). Similarly, addition and deletion (frameshift) mutations have been shown to require specific DNA sequences at the site of mutation. A good example is 9-aminoacridine (9-AA), to which hisC3076 (TA1537) is sensitive but hisD3052 (TA1538) is not (2).

Some increase in the generality of response of specific reversion assays may be expected from suppressor mutations. However, the appearance of the many slowly growing *his*$^+$ colonies over several days after scoring (48 hr) of an assay by the published protocols (1) suggests to us that suppressor revertants may not be numerically significant. Quantitative data regarding the frequency of such events for the Ames strains are not yet available, and thus it is not now possible to ascertain whether a strain can be reverted by events other than direct back-mutation. For example, for his G46, it is not explicitly known how many of the six possible base-pair interconversions could lead to revertants.

Therefore, we arrive at the conclusion that the present set of tester strains may not contain sufficient specific genetic lesions to justify the expectation that all possible mutational mechanisms (and thus all possible mutagens) are detectable.

There is, however, a simple genetic solution to this problem of mutagen-mutation specificity—forward mutation assays based on inability of mutants to convert enzymatically a drug to a toxic metabolite. In such assays, a large portion of a structural gene (and possibly control genes) is theoretically the target for chemical mutagens. Base-pair substitutions affecting amino acids at the catalytic site or at key structural positions would be observed as a phenotypic change to drug resistance. Frame-shift mutations occurring over most of a structural gene, except perhaps for the terminal codons, would similarly be expressed.

A number of quantitative forward mutation assays based on drug resistance have been devised for bacteria (3), neurospora (4), rodents (5), and human cells (6). Here we report our recent development of a quantitative assay that combines desirable physiologic characteristics (diminished repair capacity, increased permeability) of the Ames strains with a simple selection system for forward mutation. Selection is based on the ability of mutants to form colonies in the presence of 8-azaguanine (8-AG). Resistance in *S. typhimurium* is apparently related to the activity of the enzyme xanthine phosphoribosyltransferase (5-phospho-α-D-1-diphosphate:xanthine phosphoribosyltransferase, EC 2.4.2.22) which both transports and phosphoribosylates 8-AG at the cell membrane (7–10). The nucleotide product of this enzymatic activity is further metabolized and incorporated into nucleic acids, ostensibly exerting its toxic action at this level.

METHODS AND RESULTS

Chemicals. ICR-191 was the generous gift of H. J. Creech of the Cancer Institute (Fox Chase, PA). 9-AA, benzo[*a*]pyrene (BP), glucose 6-phosphate (Glc-6-*P*), and Glc-6-*P* dehydrogenase were purchased from Sigma Chemical Co. (Saint Louis, MO). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 8-AG were obtained from K & K Laboratories (Plainview, NY). Dimethylnitrosoamine (DMN) was purchased from Eastman

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Abbreviations: 9-AA, 9-aminoacridine; 8-AG, 8-azaguanine; BP, benzo[*a*]pyrene; Glc-6-*P*, glucose 6-phosphate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMN, dimethylnitrosamine; P_i/NaCl, phosphate-buffered saline; PMS, post-mitochondrial supernatant; 8-AG^R, 8-azaguanine-resistant; 8-AG^S, 8-azaguanine sensitive.

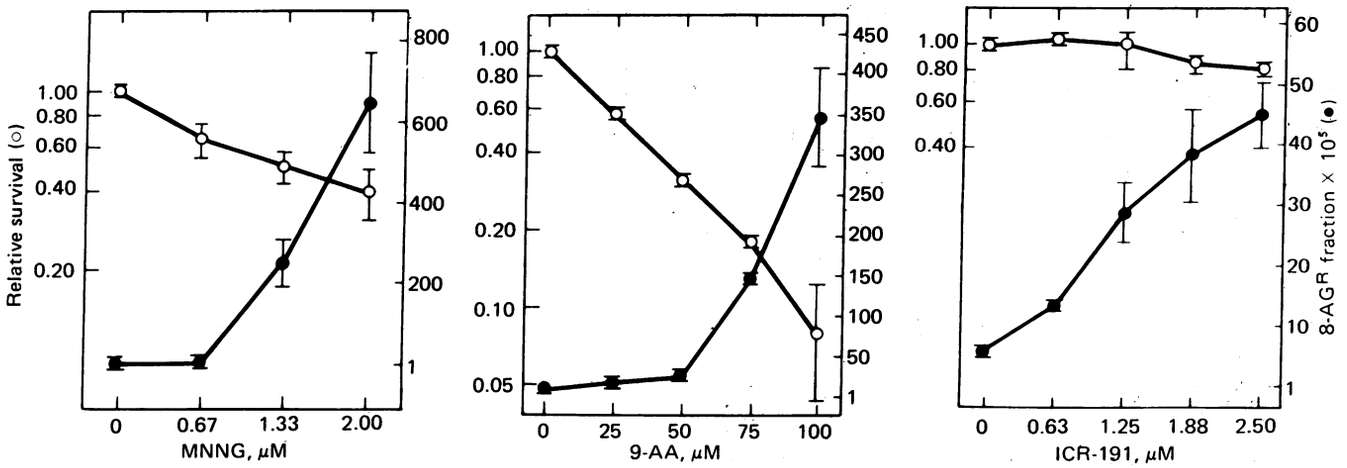

FIG. 1. Concentration-dependent toxicity and mutagenicity of MNNG, 9-AA, and ICR-191 to TM35 in a 1-hr exposure. (Error bars = 1 SD.)

Kodak Co. (Rochester, NY). DHOM Products (North Hollywood, CA) supplied the [8-^{14}C]8-AG. Aroclor 1254 was purchased from Analabs, Inc. (North Haven, CT).

Bacterial Strain. The bacterial strain TM35 used in these studies was a spontaneous histidine prototrophic revertant of *S. typhimurium* strain TA1535, generously provided by Bruce Ames. Strain TM35 originated from a clone chosen randomly from spontaneous revertant colonies isolated on a minimal agar plate containing 0.05 mM biotin without histidine.

Cells were grown in brain heart infusion (Difco) at 37° to a density of approximately 8×10^8/ml and then divided into 0.5-ml aliquots. Each aliquot received 55.5 μl of dimethyl sulfoxide and was then immediately frozen and stored at −80°. Upon thawing, each frozen aliquot contained approximately 1.25×10^8 viable cells. The use of identical frozen aliquots is important for reproducibility among experiments performed on different days.

Preparation of Post-Mitochondrial Supernatant. Male Sprague–Dawley rats weighing 100–150 g were fed a semisynthetic rat chow and given tap water. The animals received a single intraperitoneal injection of aroclor (400 mg/kg) 4 days prior to sacrifice by decapitation. The livers were perfused *in situ* with cold pH 7.0 phosphate-buffered saline (P_i/NaCl) containing, in mg/ml: NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.15; and KH_2PO_4, 0.2, removed from the body, and rinsed. Fresh ice-cold P_i/NaCl was added to yield a final concentration of 25% (w/vol). The livers were minced with scissors and homogenized in a glass Dounce homogenizer, with six strokes with a loose-fitting pestle. The homogenate was centrifuged at 9000 × *g* for 30 min at 4°. The post-mitochondrial supernatant (PMS) was then carefully decanted.

The PMS was filtered through a 0.8-μm Millipore filter (25 mm size) with a prefilter and then was sterilized by passage through a sterile 0.3-μm Millipore filter.

Mutation Assay. For each mutation experiment, two frozen aliquots of TM35 were quickly thawed in a 37° water bath and added to 49 ml of minimal Eagle's medium ($MgSO_4 \cdot 7H_2O$, 0.2 mg/ml; citric acid·H_2O, 2.0 mg/ml; K_2HPO_4, 10 mg/ml; $NaNH_4HPO_4 \cdot 4H_2O$, 3.5 mg/ml; glucose, 20.0 mg/ml; 0.05 mM biotin; pH 7.0) in a 100-ml screw-cap bottle. The culture was incubated for 1 hr at 37° in a shaking water bath (200 rpm). Doubling time was approximately 40 min.

For compounds not requiring metabolic activation, 4.95-ml samples of the culture were then placed in 20-ml screw-cap vials. The test compound was delivered to each vial in 50 μl of dimethyl sulfoxide. The vials were returned to the 37° shaking water bath and incubated for 1 or 2 hr (time of incubation is an experimental variable).

For compounds requiring activation, 4.0-ml samples of the culture were placed in 25-cm^2 plastic tissue culture flasks (Falcon). Each flask then received 0.5 ml of sterile PMS, 2 units of Glc-6-*P* dehydrogenase (delivered in 50 μl of 5 mM citrate), and 0.5 ml of minimal medium containing 5 mg of Glc-6-*P*, 5 mg of $NADP^+$, and 33.5 mg of $MgCl_2$. Controls received 0.5 ml of P_i/NaCl and 0.5 ml of minimal medium instead of the PMS and cofactors, respectively. The flasks were then placed in a 37° dry-air incubator and incubated for 1–3 hr.

After the treatment period, the cultures were transferred to plastic centrifuge tubes and centrifuged at 1000 × *g* for 15 min. The cultures were resuspended at room temperature in P_i/NaCl and diluted or concentrated (depending upon the toxicity of treatment) to a viable cell concentration of 1.0×10^7/ml.

To determine the number of 8-AG-resistant (8-AG^R) cells in each sample, 0.4 ml of the P_i/NaCl culture and 175 μl of a solution of 20 mg of 8-AG per ml in dimethyl sulfoxide were added to 9.4 ml of liquid (42°) top agar (0.6% Difco agar/0.6% NaCl/0.05 mM biotin), and 2.5 ml of this mixture was layered over each of three 100-mm plastic petri dishes containing 15 ml of minimal medium (pH 6.5) solidified with 0.6% agar. Therefore, about 1×10^6 cells were plated in each dish with a final 8-AG concentration of 50 μg/ml.

To determine toxicity, and hence the number of viable cells on the 8-AG plates, a 10^{-4} dilution was made of the original P_i/NaCl culture, 0.4 ml of this was added to 3.6 ml of liquid top agar, and 1 ml of the mixture was then layered over each of three 60-mm petri dishes, which contained 8 ml of minimal medium (pH 6.5) solidified with 0.6% agar.

Clones arising on the mutation and toxicity plates were counted with an automatic colony counter (Artek Systems) after a 36-hr incubation at 37° in a dry-air incubator. The fraction of 8-AG mutants after treatment was calculated as follows:

$$8\text{-AG}^R \text{ fraction} = \frac{\text{number of clones on 8-AG-containing plates}}{\text{number of clones on plates without 8-AG} \times 10^{-4}}$$

The dose-dependent toxicity and mutagenicity of three representative compounds not requiring activation (MNNG, ICR-191, and 9-AA) are presented in Fig. 1 and for two compounds requiring activation (DMN, BP), in Fig. 2.

Phenotypic Expression. In the mutation assay protocols outlined above, cells were plated under selective conditions

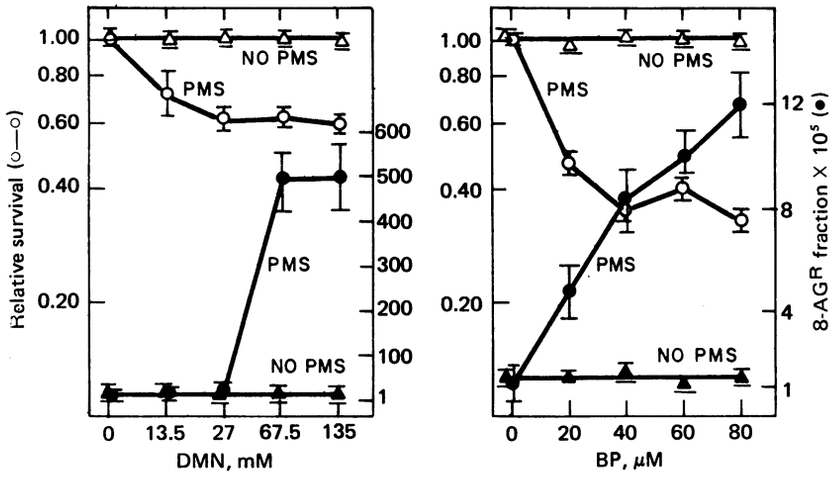

FIG. 2. Concentration-dependent toxicity and mutagenicity of BP and DMN to TM35 in a 2-hr exposure. (Error bars = 1 SD.)

within 30 min after treatment with the mutagen. Cultures were grown in minimal medium after treatment to ascertain the time required for full phenotypic expression of the mutation. The treatment procedure was the same as described above for the mutation assay without activation. After treatment with ICR-191 or MNNG, cultures were incubated in minimal medium, and hourly samples were plated to determine 8-AG^R mutant fraction.

The amount of residual growth that occurred on the 8-AG plates was sufficient for full phenotypic expression, even when the cultures were plated immediately after treatment (Fig. 3). In fact, the mutant fraction decreased significantly and then stabilized at a constant level when the cultures were allowed to grow before plating. This phenomenon probably results from the fact that rapidly growing bacteria possess multiple copies of their DNA (11). A mutation in one of the copies of DNA present in a given bacterium will be "diluted out" if the bacterium is allowed to divide instead of being plated immediately. The same phenomenon occurred with identical kinetics in the Ames reversion assay (data not shown).

Fluctuation Test for 8-AG Resistance. In order to test the hypothesis that 8-AG^R mutants arise spontaneously by a process independent of the application of selective conditions, we performed the fluctuation test of Luria and Delbrück (12). An 8G sensitive (8-AG^S) culture (TM35, 3×10^6/ml) was sampled and plated in quintuplicate to determine the 8-AG^R background fraction. The culture was then diluted 10^{-6} with minimal medium, and 15 independent 1-ml cultures were initiated (~3 cells per culture). These were allowed to grow for 36 hr at 37°. Each culture was then diluted to 10^7/ml and plated in triplicate to determine the 8-AG^R fraction.

Analysis of variance, performed to compare the variation among replicate samples from the original culture and the variation among the 15 subcultures, showed that the variation in the 8-AG^R fraction among different samples taken from the original population was the same as the variation associated with the determination of each mutant fraction ($P = 0.2$). However, the variation in the 8-AG^R fraction among the 15 subcultures initiated from small inocula was significantly different from the variation associated with the determination of each mutant fraction ($P \ll 0.01$). These data demonstrate that resistance to 8-AG arises from random events in the population and not from adaptation to selective conditions.

Uptake of [^{14}C]8-AG by 8-AG^S and 8-AG^R Cells. In order to begin the biochemical characterization of the mutation assay, we compared the ability of 8-AG^R and 8-AG^S cells to incorporate radioactive 8-AG. Twenty-five 8-AG^R mutant clones were isolated from 8-AG-containing plates, purified, and stored in frozen aliquots as described above. Five were spontaneous mutants; five each were induced by ICR-191, 9-AA, MNNG, and β-propiolactone. The 8-AG^R clones were shown to maintain their 8-AG resistance when grown in the absence of 8-AG (no change detectable through 100 generations). A 0.45-ml minimal medium culture of each mutant strain and a wild-type control were prepared (~1×10^8 cells per ml). After a 1 hr preincubation at 37°, 0.01 μmol of [^{14}C]8-AG (22 μCi/μmol) was delivered to each culture in 50 μl of 0.01 M NaOH. A 0.2-ml sample was taken from each at the time the [^{14}C]8-AG was added and again after 2-hr incubation at 37°. Each 0.2-ml sample was filtered through a 0.45-μm Millipore filter under vacuum and quickly washed with 50 ml of P_i/NaCl. Each filter was dried, added to 10 ml of Aquasol (New England Nuclear), and assayed for radioactivity in a liquid scintillation counter.

The 8-AG incorporation rate by the wild-type control was $4.3 \pm 1.3 \times 10^{-18}$ mol/cell per hr (95% confidence limits). Uptake of [^{14}C]8-AG in the control was linear through the 2-hr

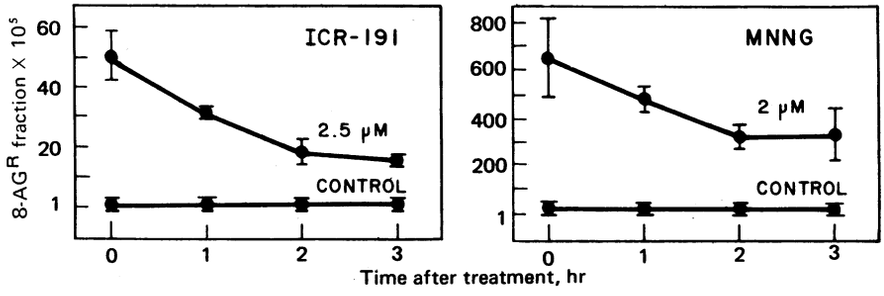

FIG. 3. Decrease in observed mutant fraction during growth after treatment. (Error bars = 1 SD.)

Table 1. Plating efficiency of 8-AG^R clones in the presence of 8-AG and 8-AG^S cells

Clone	Control	8-AG	8-AG plus cells
S-1	1.00 ± 0.15	0.93 ± 0.05	0.81 ± 0.07
S-2	1.00 ± 0.02	1.16 ± 0.03	1.04 ± 0.03
S-3	1.00 ± 0.04	0.95 ± 0.03	0.87 ± 0.08*
S-4	1.00 ± 0.07	0.98 ± 0.01	0.83 ± 0.03*
S-5	1.00 ± 0.08	0.92 ± 0.01	0.73 ± 0.08*

S-1, S-2, S-3, S-4, and S-5 were randomly chosen spontaneous 8-AG^R clones, plated on minimal medium agar alone (control), with 8-AG at 50 μg/ml, or with 8-AG at 50 μg/ml plus 10^6 8-AG^S cells. Values shown are means ± SD.
* For difference from control, $P < 0.05$ (t test).

incubation, and the concentration of 8-AG used was saturating. Of the mutants tested, 19 had no detectable (<5%) activity and the other 6 had partial activity (5–20%).

These results demonstrate only that all mutants examined had decreased ability to take up 8-AG. Whether this was due to decreased transport, phosphoribosylation, or other biochemical deficiencies is not yet known.

Reconstruction Experiments. In order to test for possible bias in quantitative determination of induced 8-AG^R mutation, three reconstruction experiments were performed. In the first experiment, the plating efficiency of five spontaneous 8-AG^R mutants was determined in the presence of 8-AG and 8-AG^S cells. Each 8-AG^R mutant was subjected to three plating conditions: (*i*) no 8-AG; (*ii*) 8-AG at 50 μg/ml; and (*iii*) 8-AG at 50 μg/ml plus 1×10^6 8-AG^S cells per plate. Approximately 200 8-AG^R cells were plated on each dish. 8-AG alone had no effect, (Table 1). However, the presence of 8-AG with 8-AG^S cells did decrease the plating efficiency of four of the mutants by approximately 20%. This might be the result of crossfeeding of toxic products between 8-AG^S and 8-AG^R cells.

In the second reconstruction experiment, a population containing a known high fraction of 8-AG^R cells was exposed to the same treatment protocol of the mutation assay to test for any possible quantitative effect of the protocol on the relative abilities of 8-AG^R and 8-AG^S cells to grow and form colonies. A mixed population of 8-AG^S and 8-AG^R cells was prepared (8-AG^R/8-AG^S cells, 1:2). This population was treated for 2 hr with 0, 0.16, and 1.60 mM β-propiolactone. After treatment and resuspension in P_i/NaCl, the culture was diluted and plated with and without 8-AG to determine the relative survival of the mixed population and the ratio of 8-AG^R to 8-AG^S cells. Approximately 300 viable cells were plated on each dish. The experiment was performed twice with two different spontaneous 8-AG^R mutants. The high concentration of β-propiolactone killed a significant portion of the mixed population

Table 2. Relative toxicity of β-propiolactone to 8-AG^S and 8-AG^R cells in a mixed population

	Clone S-3		Clone S-4	
β-Propiolactone, mM	Relative survival of mixed population	8-AG^R mutant fraction	Relative survival of mixed population	8-AG^R mutant fraction
0.00	1.00 ± 0.07	0.30 ± 0.03	1.00 ± 0.04	0.32 ± 0.03
0.16	0.70 ± 0.07	0.36 ± 0.03	0.51 ± 0.04	0.29 ± 0.02
1.60	0.06 ± 0.003	0.35 ± 0.06	0.03 ± 0.005	0.27 ± 0.06

S-3 and S-4 were randomly chosen 8-AG^R mutants. Values are the means of triplicate points ± SD; t tests showed no significant differences ($P > 0.01$) between mutant fractions of treated and control cultures.

Table 3. Survival of treated 8-AG^R cells in the presence of 8-AG and a high density of 8-AG^S cells

MMS, mM	8-AG^S cells, no./plate	Treated 8-AG^R cells, no./plate	Clones, no.	Survival of treated 8-AG^R cells, %
0	10^6	0	30 ± 2	—
0	0	300	273 ± 19	100 ± 7
0	10^6	300	308 ± 7	102 ± 2
10	0	3,000	328 ± 6	12.0 ± 0.2
10	10^6	3,000	367 ± 15	12.3 ± 0.5
20	0	30,000	165 ± 20	0.60 ± 0.07
20	10^6	30,000	216 ± 5	0.68 ± 0.02

8-AG^R population was treated with methylmethanesulfonate (MMS) for 1 hr. In calculating survival of treated 8-AG^R cells plated in the presence of 8-AG^S cells, spontaneous mutants (background = 30) were subtracted. Values are means ± SEM ($n = 3$).

(Table 2). However, the ratio of 8-AG^R cells to 8-AG^S cells in the treated population did not differ significantly, demonstrating that 8-AG^R cells are under no selective pressure during the treatment period.

The third reconstruction experiment tested the possibility that treated 8-AG^R cells might recover from toxic treatment and clone with a higher efficiency on plates with a high density of 8-AG^S cells than on plates without 8-AG^S cells. A mixed population of 8-AG^R mutants (25 strains) was treated with methylmethanesulfonate at concentrations that were expected to yield 100, 10, and 1% survival. After treatment, each culture was plated under selective conditions in the presence or absence of 10^6 8-AG^S cells. The results (Table 3) indicate that there was no cell density-dependent difference in the cloning efficiency of treated mutants.

DISCUSSION

Reverse mutation assays are an invaluable tool for investigating the mechanism of action of a particular mutagen. For example, 9-AA reverts TA1537, but not TA1538. This strongly suggests that the

-G-G-G-G-
-C-C-C-C-

sequence of TA1537 (13), but not the

-C-G-C-G-C-G-
-G-C-G-C-G-C-

sequence of TA1538 (14), is a 9-AA-mutable site. This sort of specificity, however, is what led us to wonder about the use of reversion assays for general screening purposes in which many different classes of chemical mutagens would be encountered. Could there be frameshift mutagens that specifically recognize sequences other than those found in the three commonly used Ames tester strains? Could there be mutagens that cause only base-pair substitutions which do not revert his G46? Could a less specific but equally sensitive forward mutation assay obviate the need to develop enough revertant frameshift strains to cover all theoretical possibilities? It seems reasonable to expect that a forward assay would respond to all six possible base-pair interconversions, a large proportion of possible frameshift mutations, and large deletions.

From a toxicological testing standpoint, a single forward mutation assay seems to hold an advantage over a series of reversion assays. For screening purposes, it is not necessary to distinguish whether a mutagen causes base-pair substitutions, frameshifts, or both.

Of course, bacterial cells differ from human cells in terms of chromosome structure, metabolism, and DNA repair, and a response in one system will not necessarily predict the response in the other (15). However, the anticipated regulatory requirement for testing large numbers of environmental chemicals involves immediate application of state-of-the-art bioassay technology. Thus, bacterial assays are now an important aid in assessing the mutagenicity of a chemical. We suggest that a forward assay may be better suited to this purpose than a small set of reversion assays, and we offer a simple method for testing our hypothesis in *S. typhimurium*.

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