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Delayed Mutation in Chinese Hamster Cells

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Abstract—*The possibility was examined that mutational events can be delayed for more than one or two cell divisions following treatment of Chinese hamster cells with the DNA alkylating agent ethyl methane sulfonate. If mutations in mammalian cells are delayed, the proportion of mutant cells in colonies grown from single mutagen-treated cells will reflect the cell division at which the mutation is genetically fixed, i.e., a first division mutation yields a 1/2 mutant colony, a fifth division mutation produces a 1/32 mutant colony, etc. In the present study, replating of cells from single colonies grown for six to seven days after mutagen treatment resulted in the discrete ratios of glucose-6-phosphate dehydrogenase (G6PD) -deficient mutant to wild-type colonies expected for a delayed mutational process which produces mutations over at least 8–10 cell generations. Further, when cells from 7- to 10-day colonies, grown from ethyl methane sulfonate (EMS) -treated cells were replated into selective medium containing 6-thioguanine (6TG), the number of 6TG-resistant colonies obtained per flask was distributed over a very wide range, consistent with a mutational delay process. These results could not be explained by differences in the number of cells per colony or plating efficiency in selective medium. Assuming that the relative number of 6TG-resistant colonies per flask reflects the time of mutation, EMS treatment produced two groups of mutational events: one which occurred within the first five cell generations and another uniformly distributed over at least the next eight to nine divisions. These results support the conclusion that EMS induces mutants for at least 10–14 cell generations after treatment and raise the possibility that current methods to assess the mutagenic potential of an agent might lead to significant underestimation. The role of delayed mutation in the phenomenon of "mutation expression time" is also discussed.*

INTRODUCTION

The conversion of a DNA lesion into a stable genetic change after mutagen treatment of bacteria usually occurs within the first or second cell generation (1–5). This process has generally been assumed to be equally rapid in mammalian cells, and most methods for measuring mutation frequency using cultured cells are based on this assumption (6). Recently, we have found that treatment of G_1-synchronized single Chinese hamster ovary (CHO) cells with ethyl methane sulfonate (EMS) produced colonies containing both glucose-6-phosphate dehydrogenase (G6PD) enzyme-deficient mutant and wild-type cells (mosaic colony) when assayed by histochemical staining and replica plating (7). The G6PD enzyme is X-linked and functionally haploid in this cell line. About half of these mosaic colonies contained 1/2-mutant sectors and the remainder 1/4-

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and 1/8-mutant sectors. The relative proportion of mutant cells in these mosaic colonies primarily reflects the cell division at which the mutation was genetically fixed (8), and thus the appearance of 1/4- and 1/8-sectored colonies can be explained by a mutational fixation event which is delayed for one and two cell divisions, respectively. In principal, from the ratio of mutant and nonmutant cells generated in colonies after mutagen treatment of single cells, one should be able to determine the cell division at which the mutational event occured. However, as mutational events occur at successive cell divisions, a smaller proportion of the cells in a colony will be mutant, i.e., a fourth generation mutation will produce a 1/16 sector, an eighth division mutation will produce a 1/256 sector, etc. These low percentage sectors might not be detected by the direct staining of colonies for G6PD activity, especially if the mutant cells are not clustered within an unstained sector but are dispersed randomly throughout the colony or lie beneath wild-type staining cells. We have used various replating techniques to investigate this possibility, and we now report here that EMS induces mutational events in CHO cells which are distributed over at least 10–14 cell divisions.

MATERIALS AND METHODS

Culture and Mutagenesis of Cells

The CHO cell line, CHO-k1, used for these studies was routinely cultured in Ham's F12 medium supplemented with 5% fetal calf serum. Less than 2% of the cells were tetraploid when cultured in this medium. Mitotic cells were collected using the shake-off method of Terashima and Tolmach (9) and incubated for 0.5 h to bring the cells into G_1 as described previously (7). In this approach 90–97% of the cells are synchronized in G_1 (7, 8). Cells synchronized in G_1 were treated for 1.5 h with 667 μg/ml EMS, and dispersed into single cells for replating (8).

Mutation Assays

G6PD Locus. G6PD activity in individual colonies was assayed by incubating 6 to 8-day colonies for 1 h at 37°C in a solution containing 0.14 M NaCl, 0.33 mg/ml nitroblue tetrazolium, 0.17 mg/ml phenazine methosulfate, 0.17 mg/ml NADP, 2.5 mg/ml glucose-6-phosphate adjusted to pH 6.8, 0.012% (w/w) Triton X-100 detergent (7, 8, 10). Using a replica-plating procedure, we have previously shown (7) that cells containing wild-type levels of enzyme stain dark blue, whereas cells deficient in enzyme activity have little or no staining; this phenotype is stable. Metabolic cooperation at the G6PD locus does not seem to be significant since the proportion of mutant to wild-type cells in a mosaic colony is maintained upon replating (7).

G6PD-negative cells which occur at low frequency in individual colonies were detected by replating the cells from single 5 to 7-day colonies and staining the resulting colonies for G6PD activity as described above. Specifically, EMS-treated G_1-synchronized cells were plated at a density of 1.5 cells per well into 48 96-well plates and the plates scanned for wells containing single colonies after five days of culture. During this and the next two days of growth, cells from wells containing single colonies were replated into 100-mm plates; after six to seven days of growth, the resulting colonies were stained for G6PD activity and the ratio of wild-type to G6PD-negative colonies determined.

Hypoxanthine Guanine Phosphoribosyltransferase (HGPRT) Locus. EMS-treated G_1-synchronized and parallel sets of untreated cells were plated into 100–200 25-cm^2 flasks yielding 80–190 colonies per flask. By dividing the cells into many small subpopulations, either no or only one mutational event will occur in the majority of flasks. Plating efficiencies for EMS-treated and untreated cells were 40–62% and 65–80%, respectively. After 7–10 days of culture, the number of 6-thioguanine-resistant cells per flask was

determined by replating the cells from each flask into two to six 100-mm plates at a density of 5–8 $\times$ 10^5 cells per plate in MEM supplemented with 5% fetal calf serum and containing 10 μM 6-thioguanine (6TG) or, in some experiments, F12 growth medium containing 100 μM 6TG (11). The two selective media yielded similar results. After 10–14 days of growth, the surviving colonies were fixed, stained, and the number of 6TG-resistant colonies produced per flask determined.

In some experiments, 6TG-resistant colonies were isolated from parallel plates and the growth rate in F12 medium and plating efficiency in 6TG-containing medium determined. From these data the apparent time of mutation (T) for 6TG-resistant cells obtained from a particular flask was calculated by subtracting the number of cell generations (Nr) required to produce the observed number of 6TG-resistant cells ($Nm/PE = 2^{Nr}$) from the total number of cell generations that occur before replating (Gt/Dt). The following formula expresses this relationship:

$$T = Gt/Dt - (\log Nm/PE)/0.301$$

where Gt is the time (period) of growth in hours before replating, Dt is the doubling time (hours) for the particular colony, Nm is the observed number of 6TG-resistant colonies, and PE is the plating efficiency in selective medium.

The distribution of the number of viable cells in individual colonies was determined by plating EMS-treated cells into 96-well plates at a density of 1.5 cells per well, culturing eight days, replating the cells from wells containing single colonies, and the counting the number of resulting colonies after fixing and staining.

RESULTS

To increase the probability of detecting mosaic colonies with a low percentage of mutant cells, 100 EMS-treated G_1-synchronized CHO cells were plated into each well of a series of 24-well plates, cultured three days, and the cells in each well replated into a 100-mm dish. The resulting colonies (average of 279 per plate) were stained for G6PD activity and the number of G6PD-negative colonies in each plate scored. At the end of the

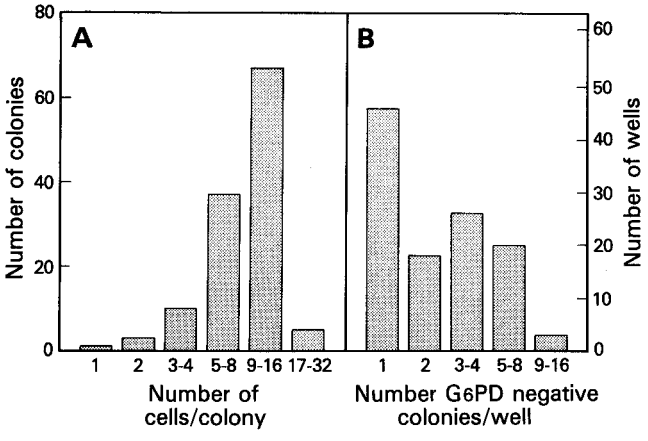

Fig. 1. (A) Effect of replating on the detection of G6PD-negative colonies. EMS-treated, G_1-synchronized cells were plated into a series of wells (100 cells per well). After three days of growth, one set of wells was fixed and stained, the number of cells in colonies determined, and a histogram constructed. The average number of colonies per well determined after six days of growth was 39. (B) Cells in the remaining wells (655) were replated, the resulting colonies (279 per plate) stained for G6PD activity, and a histogram of the number of G6PD-negative colonies per well constructed.

initial three-day period of growth, the average colony contained 10 cells and 89% of the cells had doubled more than two times (yielding five or more cells) and 59% more than three times (Fig. 1A). Thus, if one cell in a colony became G6PD-deficient during the initial three-day growth period, it should yield a pure G6PD-negative colony upon replating that would be easily detected. Similarly, the earlier the mutational event occurs after mutagen treatment, the larger the expected yield of G6PD-negative colonies after replating. Figure 1B shows the distribution of the number of G6PD-negative colonies obtained after replating 655 wells. If most mutational events occur within the first or second cell division after mutagen treatment, the distribution in Fig. 1B should resemble the distribution of the number of cells per colony in Fig. 1A with a single major peak at three to four or five to eight G6PD-negative colonies per well, after correcting for plating efficiency (72%).

In actual fact, the largest peak (46) is observed at one G6PD-negative colony per well. It is unlikely that these single G6PD-negative colonies are derived from spontaneous mutants since the frequency of G6PD-negative colonies in untreated control experiments is less than one in 10^5. Also, the appearance of these single G6PD colonies is most likely not due to preferential division lag of G6PD mutant cells after EMS treatment since we have previously observed that G6PD-negative colonies produced after EMS treatment have the same average diameter as wild-type colonies (8). Further, of the 46 G6PD-negative colonies in this group, 21 were mosaic, and of these, four were 1/8 mutant, three 1/4 mutant, eleven 1/2 mutant and three 3/4 mutant. The appearance of these mixed phenotype (mosaic) colonies suggests that mutations occurred after replating and is consistent with a mutational process which produces mutant events over at least the first five cell generations following EMS treatment.

In the above replating experiment, 113 wells out of a total of 655 gave one or more mutant colonies, which yields an EMS-induced mutation frequency of about one per 200 cells ($1/39 \ln 542/655 = 4.9 \times 10^{-3}$). With this high mutation frequency, it was feasible to determine in individual colonies the cell division at which the mutational event occurred. By replating cells from single colonies after five to seven days of growth and staining the resulting colonies for G6PD activity, colonies which contain a low frequency of G6PD-negative cells should be detected. In theory, a mutation occurring at the nth cell generation after mutagen treatment will produce a colony containing $1/2^n$ mutant cells. Since G6PD-deficient cells grow at the same rate as wild-type cells and have similar plating efficiencies (7, 8), the ratio of G6PD-negative to wild-type cells in colonies obtained after

Table 1. Distribution of G6PD Mosaic Colonies Derived from Individual Replated Colonies

Total number of colonies screened	Mutant clone	Number of mutant and wild-type colonies after replating individual colonies		Observed ratio of mutant–wild-type colonies	Theoretical ratio of mutant–wild-type for mutation occurring at cell division (n)
		G6PD$^-$	Wild-type		
1037 (EMS treated)	a	361	249	1:0.7	1:1 (1)
	b	189	94	1:0.5	1:1 (1)
	c	1	1130	1:1130	1:1024 (10)
	d	16	4068	1:254	1:256 (8)
	e	126	3893	1:31	1:32 (5)
526 (no treatment)	No mutants observed				

replating should be a direct indication of the cell generation at which the mutational event occurred in a particular colony.

A total of 1037 colonies grown from EMS-treated cells and 526 untreated colonies were examined for G6PD mutants in two separate experiments. After replating, an average of 1000 and 1200 colonies per individual replated colony was obtained in the EMS-treated and control groups, respectively. Five colonies (a, b, c, d, e) from the treated population yielded G6PD-negative colonies upon replating; no mutant cells were obtained from the untreated colonies (Table 1). The observed ratio of mutant to wild-type colonies is in close agreement with the discrete ratios expected for mutations occuring at the first (a and b), fifth (e), eighth (d), and tenth (c) cell divisions after EMS exposure. It is extremely unlikely that these ratios could have been obtained by differences in growth rates or plating efficiency between mutant and wild-type cells. Furthermore, G6PD-negative cells isolated from the fifth generation mutant (clone e) are genuine G6PD-negative mutants, based on the criteria of genetic stability and undetectable enzyme activity when assayed in cell extracts (8). Thus, EMS treatment of CHO cells induces mutations at the *G6PD* locus which occur over a period of at least 8–10 cell generations.

To determine whether mutational delay is a general phenomenon, we also investigated mutations at the *X*-linked *HGPRT* locus. Cells containing an enzyme-inactivating mutation at this locus are resistant to the toxic purine analog 6TG and can be identified as colonies surviving in medium containing this drug (11). As observed at the *G6PD* locus, delayed mutations predict that the proportion of 6TG-resistant cells in a colony depends upon when, after mutagen treatment, the mutation occurs. Thus, the number of 6TG-resistant colonies obtained upon replating should reflect the cell division at which the mutational event occurs. If mutational events are distributed over a series of cell generations, the number of 6TG-resistant colonies obtained after replating should also vary over a wide range. In contrast, if all mutations occur within one or two cell divisions after mutagen treatment, the distribution of 6TG-resistant colonies should be similar to the distribution of viable cells per colony.

EMS-treated cells were plated into a series of flasks, cultured eight days to allow for expression of mutations, and the cells in

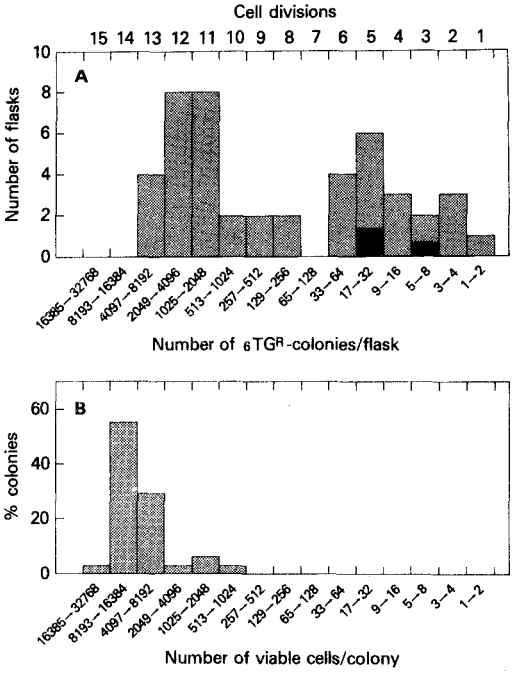

Fig. 2. Distribution of 6TG-resistant colonies after replating vs. the distribution of viable cells per colony. (A) Three hundred EMS-treated and 200 untreated G_1-synchronized cells were plated into 143 and 147 flasks yielding, after eight days, an average of 185 and 153 colonies per flask and 13,000 and 20,000 cells per colony, respectively. Cells from each flask were replated into four 100-mm plates containing selective medium, the number of 6TG-resistant colonies obtained per flask scored, and a histogram constructed. With this plating protocol, less than eight of the 45 flasks containing 6TG-resistant colonies would be expected to have mutants derived from more than one mutational event. Stippled bars represent the number of 6TG-resistant colonies; shaded bars, the number of spontaneous 6TG-resistant colonies. (B) Cells from 63 single colonies grown eight days from EMS-treated cells were replated and the total number of viable cells per colony determined as described in Materials and Methods.

each flask replated into 100-mm plates containing selective medium. Out of a total of 143 flasks, 45 produced 6TG-resistant colonies. The number of 6TG-resistant colonies obtained from these flasks was compared to the number of viable cells in the colonies at the time of replating (Fig. 2). The number of 6TG-resistant colonies obtained per flask varied over a wide range from within a factor of two of the average number of cells per colony (13,000) at the time of replating to only one to two per flask. Over half of the 45 flasks contained less than 512 6TG-resistant colonies, which is almost five cell generations less than the number of generations (13.7) required to yield the average number of cells per colony (13,000). These results are inconsistent with the concept that all mutations induced by EMS occur soon after treatment. Rather, they are in accord with the results at the *G6PD* locus that mutational events are distributed over many cell generations.

Figure 3A shows the temporal distribution of EMS-induced mutational events. The time of mutation was estimated using the average number of cells per colony to establish the mutant–wild-type ratio. Flasks producing 6TG-resistant colonies within a factor of two (1–1/2) of the average number of cells per colony were designated first generation mutants; those within a factor of four (1/2–1/4) were considered second generation mutants, etc. Using this approach, the results of five separate experiments having 3000–22,500 average cells per colony at the time of replating were combined (79,000 total cells, Fig. 3). By this analysis, about half the EMS-induced mutational events occurred within the first five cell divisions following treatment, with the remainder being uniformly distributed over the next eight to nine generations. From the cumulative frequency distributions (Fig. 3A), the first group of mutants is produced at a rate of 2×10^{-4} (± 0.2 *SE*) per mutagen-treated cell per division compared to 1×10^{-4} (± 0.2 *SE*) for the later-appearing group, and 0.2×10^{-4} (± 0.08 *SE*) for the spontaneous mutation rate.

To assess the effect of variations in cell growth rate and plating efficiency on the reliability of the above method for estimating mutation times, 6TG-resistant colonies were isolated from flasks which produced various numbers of mutants. From the growth rate of these cells in nonselective medium and plating efficiency in selective medium, the time of mutation in cell divisions required to produce the observed yield of mutants was calculated

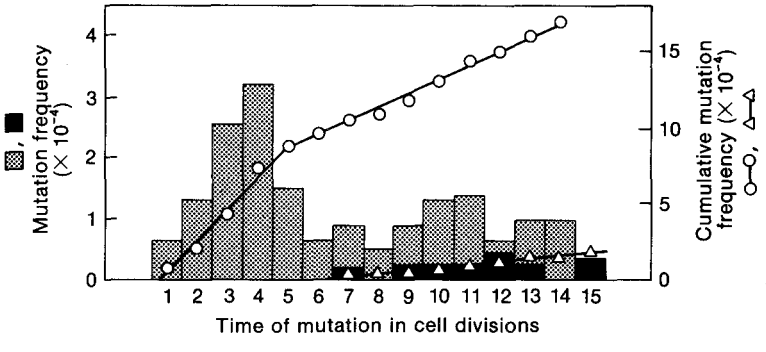

Fig. 3. Temporal distribution of EMS-induced mutational events at the *HGPRT* locus. The distribution of the number of 6TG-resistant colonies obtained per flask from five replating experiments (3000–22,500 cells per colony) were combined by grouping the data in intervals differing by negative powers of 2, setting the first interval at 1–½ of the average number of cells per colony at the time of replating, and designating it as a first generation mutation. The frequency of mutational events occurring at a particular cell division was calculated by dividing the number of flasks producing the number of 6TG-resistant colonies expected at that cell division by the total number of colonies in all flasks. Stippled bars represent the frequency of induced 6TG-resistant colonies. Shaded bars, frequency of spontaneous mutants; O and △, cumulative frequency of induced and spontaneous 6TG-resistant colonies, respectively.

for each colony and plotted against the time of mutation determined from the approach in Fig. 3 (see Fig. 4). This latter approach assumes that wild-type and 6TG-resistant cells have similar growth rates in nonselective medium and all 6TG-resistant cells have similar plating efficiencies in selective medium. Thus, if reduced cell growth rate and/or plating efficiency is the sole explanation for the distribution of 6TG-resistant colonies in Fig. 3 and all mutations actually occur within one or two cell divisions after mutagen treatment, then the data points in Fig. 4 should cluster about a line parallel to the x axis (y intercept of 1–2) with a correlation coefficient of 0. Instead, the points cluster about a 45° line expected of a 1:1 correlation (0.91 correlation coefficient), suggesting that the time of mutation determined from the approach used in Fig. 3 is a reasonable estimate of the true time of mutation. These results provide further evidence that differences in cell growth rate and plating efficiency are not the explanation for the distribution of 6TG-resistant colonies observed in Figs. 2 and 3 but are consistent with a delayed mutational mechanism.

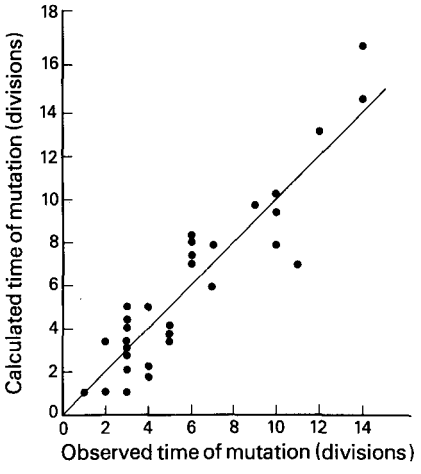

Fig. 4. Influence of growth rate and plating efficiency on observed time of mutation: correlation of observed and calculated time of mutation. Growth rates in nonselective medium and plating efficiency in selective medium were determined for 6TG-resistant colonies isolated from flasks yielding varying numbers of mutants. For each colony, time of mutation in cell divisions was calculated as follows:

$$T = (Gt/Dt) - (\log Nm/PE)/0.301$$

where Gt is the time (period) of growth in hours before replating, Dt is the doubling time (hours) for the particular colony, Nm is the observed number of 6TG-resistant colonies, and PE is the plating efficiency in selective medium. These values are plotted against the time of mutation in cell division obtained from Fig. 3A. This determination of time of mutation assumes that wild-type and 6TG-resistant cells have similar growth rates in nonselective medium and all 6TG-resistant cells have similar plating efficiencies in selective medium. The solid line represents the best fit line obtained by linear regression analysis of the data points.

DISCUSSION

We originally observed that single EMS-treated cells produced mosaic colonies containing both G6PD enzyme-deficient mutant and wild-type cells in various sectored patterns and relative proportions: 1/2, 1/4, 1/8 (7). We then found that the observed variation in the relative sizes of the mutant sectors could be explained by a delay in the fixation of the mutation for one or two cell generations following treatment with the mutagen (8). In this report, we have presented three lines of evidence which support the conclusion that, in the CHO cell line, mutational events are distributed over at least 10–14 cell generations after treatment with the DNA alkylating agent EMS. First, when small populations of EMS-treated cells were replated after three days of culture, a significant fraction of the G6PD$^-$ mutants were sectored colonies, suggesting that a mutational event occurred after replating. Second, the relative number of G6PD$^-$ to wild-type cells in single colonies occurred in the ratios expected for a delayed mutational event. Third, at the *HGPRT* locus, the number of 6TG-resistant colonies obtained after replating mutagen-treated cell populations was distributed over a very wide range, as predicted for a mutational delay process, and does not seem to be explainable by variations in the number of viable cells per colony or differences in plating efficiency.

In Chinese hamster cells, DNA alkylating agents such as EMS produce a number of DNA lesions (O^6-alkylguanine and alkylphosphotriesters) which are only slowly removed from DNA (12–19). The persistence of these DNA lesions might account for the delayed appearance of mutations by increasing the probability of base mispairing during successive DNA replication cycles or by errors introduced during repair by a slow-acting or inducible repair process. EMS treatment appears to produce two groups of mutational events: one which occurred within the first five cell generations and another uniformly distributed over at least the next eight to nine divisions. The biphasic nature of this distribution could be explained by the existence of two classes of mutagenic lesions, one that is relatively stable and has a fixed probability of producing a mutation during each replication cycle, and another that is initially present in a greater quantity in the DNA, or is much less stable than the first class and has a higher (twofold) probability of inducing a mutation. Alternatively, the observed biphasic distribution could reflect the kinetics of induction and removal of a single class of DNA lesion. A third possibility is that a portion of the mutations which occur at late division times do not have sufficient time for dilution of the *HGPRT* enzyme before 6TG-selection and thus are preferentially eliminated from the population. The above findings raise the possibility that long-lived DNA lesions which have the potential of producing mutations many cell generations after their introduction into the DNA may be an important factor in the process of carcinogenesis.

The concept of delayed mutation also has implications for assessments of the mutagenic potential of an agent by currently used methods. Usually, relatively large populations of cells are treated with the mutagenic agent, cultured for 5–10 days with periodic replating to allow for expression of the mutant phenotype, and assayed for mutants by drug-selective techniques such as 6TG. This method is based on the assumption that mutations occur within one or two cell divisions following mutagen exposure, and thus the average number of mutants in the total cell population can be assumed to be a reasonable estimate of the number of mutation events. However, mutational events that occur at successive cell generations will be diluted by the early-appearing mutations or lost during subculture and will thus be underrepresented in the total population. To estimate the potential error in this approach, we compared the mutation frequency obtained in one delayed mutation experiment (Figs. 2 and 3) with that obtained by the population average method using the same EMS-treated population. EMS-induced mutation frequency by the replating approach was calculated by dividing number of flasks producing 6TG-resistant colonies by the total number of cells surviving EMS treatment (number of replated colonies) and subtracting the spontaneous mutation frequency calculated in a similar manner. Using this approach the mutation frequency determined by the replating method was fivefold greater than that obtained by the population average method (11×10^{-4} vs. 2×10^{-4}). Clearly, serious errors in estimating the true mutagenic potential of an agent can result when delayed mutational effects are ignored.

Delayed mutation might explain, in part, the phenomenon of "mutation expression time." To obtain the maximum frequency of mutation using the above population average approach, cells are usually cultured 5–10 days after mutagen treatment before the selective drug is applied. If mutational events occur over a series of cell divisions and requirements for enzyme dilution are assumed to be negligible, then the average frequency of mutants in the test population n doublings after mutagen treatment is given by the expression $(1/PT)$ $[N1(1) + N2(1/2) + N3(1/4) \ldots Ni (1/2^{ni})]$, where $N1$ is the number of first generation mutants, $N2$ the number of second generation mutants, etc., and Pt is the total number of cells surviving the mutagen treat-

ment. Applying this expression to the temporal distribution of mutant events in Fig. 3, the average frequency of EMS-induced 6TG-resistant mutants in the total cell population will appear to reach a plateau in five to six population doublings even though a significant number of mutant events occur at later generations. Thus, it would seem that delayed production of mutant events as well as enzyme dilution are involved in the "expression time" phenomenon.

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