*MAMMALIAN CELL GENETICS, II. CHEMICAL INDUCTION OF SPECIFIC LOCUS MUTATIONS IN CHINESE HAMSTER CELLS IN VITRO**

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Chemical induction of point mutations in mammalian tissue culture cells will make possible a study in these cells of genetic events at the molecular level similar to previous studies with microorganisms. Most reports on chemical induction of mutations in mammals describe studies of chromosome aberrations,[1] dominant lethals,[2, 3] variegated-type position effect,[4] and, just recently, a qualitative method to isolate nutritional mutants in tissue culture cells after treatment with chemical mutagens.[5]

The present report presents quantitative methods for measuring mutation frequency in chemically treated Chinese hamster cells in culture. Spontaneous point mutations that resulted in L-glutamine auxotrophy (gln^-) or 8-azaguanine resistance (azg^r) were observed earlier in cultures of Chinese hamster cells.[6] We have shown that the reverse-mutation frequency of gln^- and both the forward- and reverse-mutation frequencies of *azg* increased significantly over the spontaneous frequencies after treatment with ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), or N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). Factors such as inoculum size, expression time, and level of selective agent were found to have a profound influence on the mutation frequency. Some preliminary results of this study have been published elsewhere.[7]

Materials and Methods.—The origin, culture procedures, and properties of the Chinese hamster cell line V79-122D1 have been described previously.[6] The basic medium used in cell maintenance and plating was Eagle's minimal essential medium. After mutagenic treatment of the cells, selective agents were added to the cultures at various times. For recovering reversions from gln^- to gln^+, medium lacking glutamine but containing glutamic acid was used. For recovering the forward mutations from azaguanine sensitivity (azg^s) to resistance (azg^r), 5–30 μg/ml of the drug were added to the medium. For recovering reversions from azg^r to azg^s, the selective "THAG" medium contained thymidine (10^{-7} *M*), hypoxanthine (10^{-5} *M*), aminopterin (3.2×10^{-6} *M*), and glycine (10^{-4} *M*). Both the survival and the mutation frequencies were determined on the basis of the frequencies of colonies formed.

Any spontaneous mutations that might have accumulated were removed by subjecting the cell population to appropriate selection media before the induction experiments. To remove the spontaneous gln^+ revertants, the same "thymineless death" method originally used for selecting gln^- mutants was used.[8, 6] To remove the spontaneous azg^r mutants, the azaguanine-sensitive, wild-type population was maintained for 6–7 days in THAG medium before being returned to the standard medium.

The general experimental design used in mutagen treatment and in assaying mutants is depicted in Figure 1. The three chemical mutagens initially used were chosen because of their potent mutagenicity and their use in parallel experiments with *Neurospora*[9, 10] and mice[3] in this laboratory. EMS and MMS were obtained from Eastman Organic Chemicals, Rochester, New York; MNNG was from Koch-Light Laboratories, Colnbrook, Great Britain. EMS and MNNG were used directly, and 0.2 *M* of MMS in absolute methanol was prepared. Appropriate further dilutions were then made in

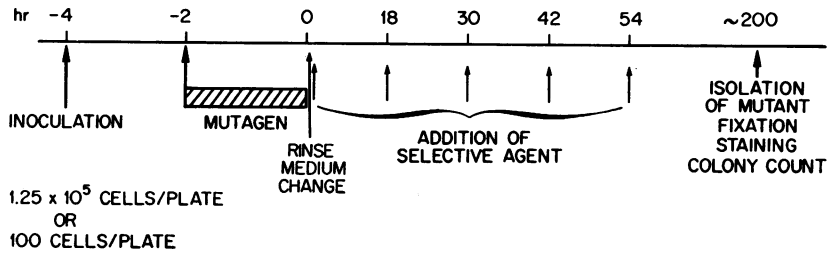

FIG. 1.—Experimental scheme for chemical induction of mutations in Chinese hamster cell cultures.

Hanks' balanced saline (HBSS) and applied to cells within an hour of preparation. All operations were conducted under strict light control at wavelengths longer than 700 mμ.

Plastic Petri dishes (60 or 100 mm, from Falcon Plastics, Los Angeles, California) were used for all plating experiments. Cells became attached to the surface of the plate usually within an hour after inoculation. After being exposed to a mutagen, the cells were rinsed twice with HBSS and transferred to the growth medium.

Results and Discussion.—Table 1 summarizes the results of inactivation experiments on V79 cells by the three mutagens. The two-hour treatment was chosen because of its facility of operation and the short half life of certain chemicals in dilute solution. Increased time of treatment at a given concentration reduced cell survival.

The spontaneous mutation frequencies of gln^- to gln^+ and azg^s to azg^r varied from experiment to experiment and increased with time between purification (for removal of pre-existing spontaneous mutations) and the mutation experiment.

To date, evidence has been obtained in the hamster cells for a significant increase in forward-mutation frequency from azg^s to azg^r and in reverse-mutation frequencies from gln^- to gln^+ and from azg^r to azg^s with EMS, MMS, and MNNG. It has been found that the recovery of the mutants after chemical treatment depends on the conditions of the experiment. Therefore, for quantitative studies it is necessary to define these conditions in order to maximize this recovery. The

TABLE 1. *Cytotoxicity of three chemical mutagens to Chinese hamster cells in culture.*

Mutagen treatment*		No. of cells plated	No. of Colonies per Plate		Per Cent Survival	
			Expt. A	Expt. B	Expt. A	Expt. B
None		100	86	117	100	100
		200		190		100
EMS	10^{-3} *M*	100	85	142	99	121
	5×10^{-3} *M*	100	75	105	87	89
		200		195		103
	10^{-2} *M*	100	65	47	47	40
		200		103		54
MMS	1×10^{-3} *M*	100	7	14	6	14
	2×10^{-3} *M*	100	0		0	
MNNG	5×10^{-6} *M*	100		43		37
	10^{-5} *M*	100	58		67	
	5×10^{-5} *M*	100	1	0	1	0
	10^{-4} *M*	100	0		0	

* Two hours at 37°C, followed by two rinses with Hanks' balanced saline and medium change.

following factors have been shown to influence the mutation frequency after treatment of these cells with chemical mutagens.

Inoculum size: Harris[11] has reported that the spontaneous mutation frequency to puromycin resistance in a line of pig kidney cells increases with the population density in culture. He attributed this phenomenon to the "feeder-layer effect," which *enhanced* the growth and recovery of the newly emerged mutants. Since mutagens at the concentrations used in the present study appreciably reduced the cell survival (e.g., 30–40 per cent killed), thus creating a disparity in population density between the treated and untreated cells, the relation between the size of the cell inoculum and the induced mutation frequency was investigated. The results of one experiment are shown in Table 2. Since

TABLE 2. *The relationship between inoculum size and the frequency of mutation (from azaguanine sensitivity to resistance) in Chinese hamster cells in culture.*

No. of cells per plate	Mutagen treatment	Mutation frequency per 10^5 survivors	Induced: spontaneous ratio
2.5×10^5	None	2.6	4.3
	EMS	11.3	
5×10^5	None	1.2	3.6
	EMS	4.2	
10×10^5	None	0.3	1.3
	EMS	0.4	

Expt. 333, V79, EMS: 10^{-2} *M*, 2 hr; survival = 69%. Azaguanine: 9 μg/ml. Expression time: 18 hr.

a maximum plating efficiency of 100 per cent has been achieved for this line of hamster cells,[6] crowded conditions in culture apparently did not provide the feeder-layer effect; instead, crowding *impaired* the recovery of mutations. This result was confirmed and extended to lower cell numbers in another experiment. It was then decided that an inoculum size at 1.25×10^5 cells per 100-mm plate would be suitable, since further reduction in cell inoculum would necessarily increase the number of plates per experimental series and thus reduce efficiency.

Mutation expression time: The next problem was to decide when the selective agent should be added. The "time of mutation expression" refers here to the interval between quenching the mutagen (rinsing with HBSS and medium change) and adding the selective agent.

At various times after removal of mutagen, the number of cells per colony was determined by direct microscopic observation of living cultures or by counting cells in fixed preparations. Fifty colonies per time period, in either treated or untreated plates, were counted. The results in three experiments were in full agreement; data from one such experiment on the number of cells per colony at various times are shown in Figure 2. Only the results in the untreated controls are illustrated, since they did not differ significantly from those in the mutagen-treated series.

The average time for cell doubling in this cell line is shown to be approximately 12 hours, which agrees with previous determinations arrived at through autoradiographic techniques under our laboratory conditions (Chu, unpublished data), as well as with the results of Sinclair.[12] At 18 hours after mutagen re-

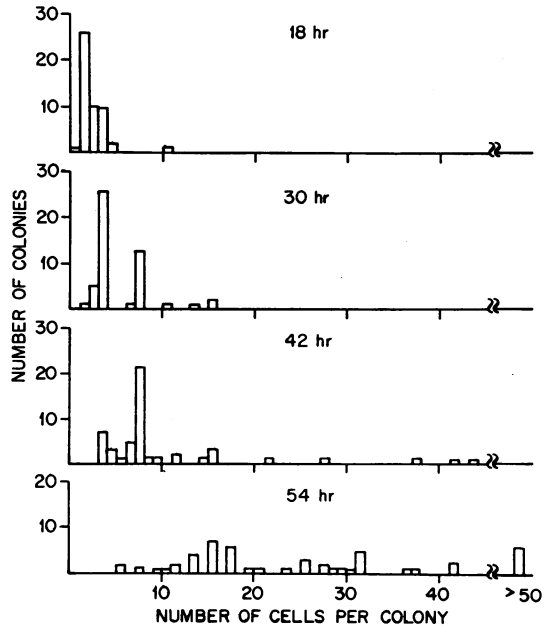

FIG. 2.—Number distribution of colonies, each consisting of varying numbers of hamster cells after different periods of growth at 37°C. All were untreated except sham operations of a rinsing with balanced saline and medium change at time zero (expt. 326).

moval, most cells divided once, and a small proportion of them divided twice. This initial mitotic delay observed in both treated and untreated cultures could have been the result of handling.

In three experiments, when the selective agent, azaguanine in this case, was added at the zero time immediately after mutagen quenching, we found no increase in mutation frequency in the treated series, in comparison to the control. However, when the addition of the selective agent was delayed, thus permitting cells to grow and divide, the mutation frequency seemed to increase with time up to 42 hours. The relationship between the time of mutation expression and mutation frequency observed in one of the two repeat experiments is shown in Table 3 and graphically depicted in Figure 3. It is clear that, under experimental conditions, a maximum number of mutations could be recovered 42 hours after the removal of the mutagen—a time interval permitting approximately three cell divisions to occur. At 54 hours, a decline in the frequencies of both

TABLE 3. *The relationship between time of mutation expression and mutation frequency in Chinese hamster cells in culture.*

Expression time (hr)	Mutagen treatment	Plating efficiency	Per cent survival	Mutation frequency per 10^5 survivors
18	None	143	100	36.6
	EMS	106	74	81.2
30	EMS	117	82	130.1
42	None	132	92	17.4
	EMS	112	78	170.8
54	None	138	97	15.3
	EMS	110	77	120.9

Expt. 345, V79*, 1.25×10^5 cells/plate. EMS: 10^{-2} *M*, 2 hr. Azaguanine: 30 μg/ml.

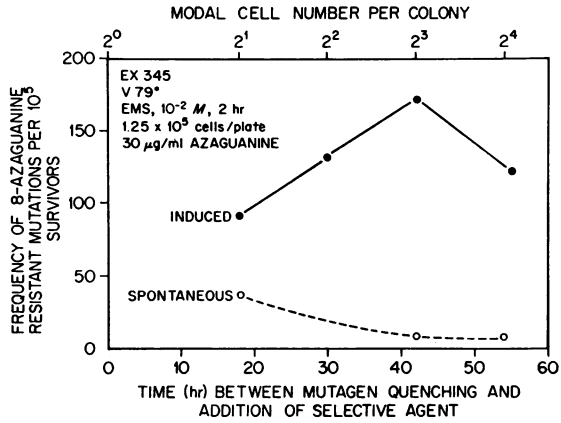

FIG. 3.—Time of mutation expression in relation to mutation frequency in cultured hamster cells.

spontaneous and induced mutations occurred. This was probably due to the crowding effect, as discussed earlier.

Biochemical evidence indicates that alkylating agents such as MMS and EMS react with the four bases of DNA in the following decreasing order: guanine (G) > adenine (A) > cytosine (C).[13] The predominant mutagenic effect of alkylation is probably due to base-pair transition from G:C to A:T.[14] The pairing-error theory[14] requires that, before the induced mutant phenotype can appear in the population, at least two rounds of DNA replication occur after alkylation. Although we have no chemical data on alkylation of DNA in these cells, our data on time of mutation expression seem to be consistent with this requirement.

Another possible explanation for the delayed expression of the mutant phenotype is the presence in the cytoplasm of the mutated cell or its immediate progeny of sufficient quantities of the wild-type gene messenger or products. Several cell divisions might be required before such a messenger or products became diluted or nonfunctional.

Concentration of selective agent: Table 4 summarizes the result of an experiment in which the influence of the concentration of azaguanine on the frequency of forward mutation from *azg*^{s} to *azg*^{r} was studied. We used a newly isolated clonal line, V79-4, which showed 100 per cent plating efficiency and which was azaguanine-sensitive. The optimal experimental conditions for recovery of mutations were provided to obtain a maximum frequency of mutations. It can be seen in this table that any one of the three chemical mutagens led to a highly significant increase in mutation (*azg*^{s} to *azg*^{r}) frequency, in comparison to the untreated control. Since the survival in this experiment was 52.5 per cent in EMS-treated and 41.1 per cent in MNNG-treated cells, these increases in the mutation frequency of the treated populations over the untreated controls could not have been due to selective killing of the wild-type parental cells, but rather to experimental induction at this locus. Although the cell survival in MMS-treated series in this particular experiment was low, the highly significant in-

TABLE 4. *Frequency of spontaneous and chemically induced mutations in Chinese hamster cells resistant to various levels of 8-azaguanine.*

Mutagen treatment	Concentration of 8-azaguanine (μg/ml)	Mutation Frequency per 10^5 Survivors: Definitive colonies	All colonies
EMS 10^{-2} *M*, 2 hr (*S* = 52.5%)	5	150.4	198.7
	10	89.6	114.4
	30	27.3	34.4
MMS 10^{-3} *M*, 2 hr (*S* = 5.8%)	5	157.2	657.9
	10	24.8	53.8
	30	0.8	13.8
MNNG 10^{-5} *M*, 2 hr (*S* = 41.1%)	5	181.4	256.4
	10	90.9	132.9
	30	36.6	58.4
None (*S* = 100%)	5	2.6	14.1
	10	2.2	7.0
	30	1.9	1.9

Expt. 382, V79-4, 1.25×10^5 cells/plate. Expression time: 42 hr. *S* = survival.

crease in the frequency of the induced mutation clearly renders improbable the possibility of selective killing.

At the roughly comparable levels of cell survival and mutation yield and on an equimolar basis, MNNG is the most efficient one of the three mutagens used in mutation induction. MMS is more cytotoxic but also more mutagenic than EMS.

This experiment also shows that, at different concentrations of the selective agent 8-azaguanine, significantly different numbers of mutations might be recovered. Figure 4 illustrates the number and morphology of colonies in the EMS-treated and untreated cultures. It can be seen that the colony size varies, thus reflecting the rate of cell multiplication during the same period of incubation. The number of large, definitive colonies and that of all colonies are listed in Table 4. Some of the EMS-induced mutants from a similar experiment were isolated and further tested. Only two of the nine isolates found resistant to 5 μg/ml of azaguanine were likewise resistant to 30 μg/ml. A preliminary test on C^{14}-hypoxanthine incorporation[6] into each of these nine resistant mutants indicated that the incorporated radioactivity per cell was variable. This last finding suggests that independent forward-mutational events had occurred, each presumably representing a different isoallele with a different level of enzymatic activity controlled by the *azg* locus. Further work on the possible induction of "leaky" mutations by chemicals and on mutagenic specificity is in progress.

Summary.—Quantitative measurements of the frequencies of gene mutations induced in mammalian somatic cells in culture have not been previously done. We have isolated from populations of Chinese hamster cells mutant clones which are either auxotrophic for L-glutamine (*gln*$^-$) or resistant to 8-azaguanine (*azg*^{r}). The present experiments clearly demonstrate that ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) significantly increase the mutation frequency at both loci. Furthermore, we have found that the following factors affect the mutation yield:

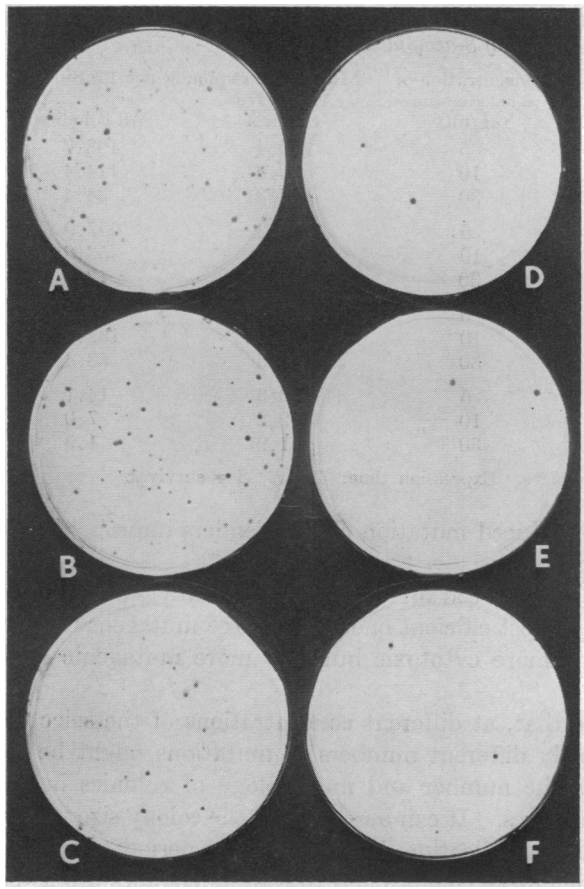

FIG. 4.—Surviving mutant colonies of hamster cells, either spontaneous or induced, in the presence of various concentrations of 8-azaguanine. To each plate, 1.25×10^5 drug-sensitive V79-4 cells were inoculated. All plates were incubated for 8 days. (*A–C*) Ethyl methanesulfonate-treated. (*D–F*) Untreated controls. Concentrations of 8-azaguanine (μg/ml) are as follows: 5 in *A* and *D*, 10 in *B* and *E*, 30 in *C* and *F* (expt. 382).

(1) inoculum size, (2) time period between mutagen quenching and the addition of a selective agent (mutation expression time), and (3) concentration of the selective agent.

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