1969

Mammalian Cell Genetics I. Selection and Characterization of Mutations Auxotrophic for L-Glutamine or Resistant to 8-Azaguanine in Chinese Hamster Cells IN VITRO

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MAMMALIAN CELL GENETICS I. SELECTION AND CHARACTERIZATION OF MUTATIONS AUXOTROPHIC FOR L-GLUTAMINE OR RESISTANT TO 8-AZAGUANINE IN CHINESE HAMSTER CELLS *IN VITRO*[1]

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Received September 27, 1968

BIOCHEMICAL mutants in somatic cell cultures have been obtained either by establishing cell cultures from mutants known in the whole organism that are also expressed at the cellular level or by means of various techniques to isolate clones of cells that have developed phenotypes different from those of the parental culture. The present report describes the isolation and cytogenetic characterization of two genetic variants, *viz.*, L-glutamine (gln) auxotrophy and 8-azaguanine (azg) resistance that had arisen spontaneously in the population of an established cell line of Chinese hamster. By the use of cell fusion technique, we showed that both mutations were recessive. Preliminary results of this work have been previously summarized (CHU, MERRIAM, and BRIMER 1967; CHU and BRIMER 1968).

MATERIALS AND METHODS

Cells: An aneuploid cell line (V79–122D1) derived originally from the lung of a male Chinese hamster (*Cricetulus griseus*, 2n=22) was used (FORD and YERGANIAN 1958). The stem-line cell consists of 23 chromosomes with several notable changes from the normal karyotype (YU and SINCLAIR 1964). Over 95% of the population remain at the diploid level, and the stem line karyotype was maintained by repeated cloning. Cloning was done by plating of single cells and picking of developing colonies with a capillary pipette which was cleansed and sterilized between colony-pickings in boiling distilled water.

Culture method: The cells were grown in EAGLE's minimal essential medium containing 15% undialyzed fetal calf serum. The experimental medium for testing nutritional requirements contained, instead, 15% fetal calf serum that was dialyzed 24 hrs against cold running tap water and 4 hrs against cold running distilled water. Unless otherwise indicated, the medium contained 2×10^{-3} Moles L-glutamine. In this medium and in a humid atmosphere of CO_2 and air at 37°C, the average generation time of these cells was about 12 hrs as determined by both cell growth and autoradiography (CHU, unpublished data). Determination of nutritional requirements was performed by inoculating an appropriate number of cells (50 to 5×10^5) into replicate 100 mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.) containing desired media. When colonies 1 to 3 mm in diameter appeared in 8 to 14 days, the cultures were fixed with methanol, stained with Giemsa, and the colonies were enumerated. Minute colonies containing fewer than 50 cells

[1] Research jointly sponsored by the National Cancer Institute and the U. S. Atomic Energy Commission under contract with Union Carbide Corporation.

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were not included. For each determination, 3 to 6 replicate cultures were used. The plating efficiency referred to is the percentage of single cells inoculated into a Petri dish which are able to undergo division and form macroscopic colonies. The standard error of the mean in present studies was usually less than 20% (see Table 3). The reliability of the plating method is indicated by the results shown in Table 4.

Selection for glutamine auxotrophs: The procedure for selecting glutamine auxotrophic mutants was essentially the thymineless death method, as outlined for HeLa cells by DeMars and Hooper (1960). The method utilizes the differential growth of prototrophs and amino acid auxotrophs during a state of thymine deficiency created by aminopterin. In the present study, 10^5 or 10^6 cells were inoculated to each Petri dish containing 20 ml of the Eagle's medium in which L-glutamine was replaced with 2×10^{-2} M monosodium L-glutamate. The level of glutamate was reduced to 10^{-3} M in the second selection and in subsequent experiments. After 2 days 4×10^{-7} M (3.2×10^{-6} M in later experiments) aminopterin, together with 10^{-5} M hypoxanthine and 10^{-4} M glycine, was included in the medium ("HAG" medium). After another 6 days, the cells were washed with Hanks' balanced saline and incubated in normal medium. Surviving colonies were isolated about 10 days later by direct picking with a capillary pipette.

Selection for drug resistant mutants and sensitive revertants. The parental cell population was sensitive to azg at concentrations higher than 1 μg/ml ($\sim6\times10^{-6}$ M). A subline resistant to azg was obtained by exposing mass cultures of hamster cells to increasingly higher concentrations of the drug (from 0.2 to 9 μg/ml). To maintain the azg concentration, it was necessary to feed the cultures every 2 or 3 days. In a selective "THAG" medium which contains thymidine (10^{-7} M), hypoxanthine (10^{-5} M), aminopterin (3.2×10^{-6} M), and glycine (10^{-4} M), the drug sensitive cells could grow, but the resistant cells could not (see Szybalski, Szybalska, and Ragni 1962). Aminopterin alone at 10^{-9} M without supplement is toxic to the hamster cells.

Direct assay for cellular uptake of hypoxanthine-8-^{14}C: 2×10^5 to 4×10^5 cells were inoculated in each 60 mm plastic Petri dish and incubated for 24 hrs. Hypoxanthine-8-^{14}C (48.4 μc per μmole at a final concentration of 0.5 μc/ml was added to the cultures, which were incubated at 37°C for various periods (1 to 6 hrs). The incorporated radioactivity was determined by liquid scintillation counting of cell extract on paper discs (Regan and Chu 1966).

Preparation of cell extract: About 10^7 cells in each Roux flask culture were rinsed twice with ice-cold 0.05 M phosphate buffer at pH 6.9. Cells were dislodged with a rubber policeman, collected by centrifugation at 1500 rpm at 4°C for 10 min, and suspended in 2 to 3 ml buffer. Then, the cells were disrupted by sonication for 30 sec and the resulting suspension was centrifuged at $17{,}000\times g$ at 4°C for 30 min. The supernatant fluid was collected, analyzed for protein content (Oyama and Eagle 1956), and stored at —10°C.

Assay for inosinic acid-guanylic acid pyrophosphorylase (IMPPase): The procedure used was a modification of that of Littlefield (1963). The incubation mixture (total volume 50 μl) contained about 2 μg of protein, 100 μg of crystalline bovine serum albumin, and the following at the concentrations of 10.3 mμ moles of hypoxanthine-8-^{14}C, 50 mμ moles of 5-phosphorylribose 1-pyrophosphate, 270 mμ moles of $MgCl_2$, and 180 mμ moles of tris (hydroxymethyl) aminomethane at pH 7.4. After incubation at 37°C for 15 to 120 min, 25 μl of the incubation mixture were transferred to Whatman diethylaminoethyl cellulose DE81 paper. A drop of acetone was added to the spot to stop the reaction. After all samples were applied, 30 μl of nonradioactive IMP solution (5 mg/ml H_2O) were applied to every spot. 0.5 N formic acid was allowed to ascend through the origin spot, the paper was dried, and the IMP marker-spot outlined under ultraviolet light was cut out and assayed for radioactivity by liquid scintillation counting. The specific activity of IMPPase referred to below represents counts per min of phosphorylated derivatives of hypoxanthine-8-^{14}C per μg of cell extract protein.

Somatic cell hybrid: Somatic cell fusion was encouraged by first plating together 5×10^5 cells of the glutamine auxotrophic and 5×10^5 azaguanine resistant cells per Petri dish containing glutamate (10^{-3} M) instead of glutamine. No virus was added. After one day at 37°C, a "THAG" mixture was added to the medium and the cultures were transferred to 29°C for two days before being returned to 37°C. After another week, during which most of the cells had died or dislodged from plates, surviving colonies were isolated for further tests.

RESULTS

Recovery of glutamine requiring variants: After the cells had been exposed for 6 days to aminopterin in the absence of thymidine (HAG medium), the survival frequency was 3.1×10^{-4}. A total of 48 colonies were isolated. Approximately one month after isolation, when enough cells had been obtained, each of these clones was tested for glutamine requirements by growing replicate 60 mm plates of 10^4 cells each, either in glutamine or glutamate-supplemented media. Growth was checked daily and the nutritional requirement of the cell line was determined on the basis of cell growth after 8 days. Thirty-eight clones could not grow in glutamine-free (but glutamate-supplemented) medium; the 10 remaining clones were glutamine independent.

In a repeat aminopterin selection experiment in which the glutamate level was adjusted to 10^{-3} M, the number of survivors was 2.8×10^{-3}. Among 20 clones isolated and tested, 19 showed absolute requirements for glutamine, whereas only one was glutamine independent.

The class of prototrophic isolates usually exhibited slower growth than the average rate of the parental population, a fact which may explain the reason for their surviving the selection procedure.

Chromosome analysis was made on 27 of the first group of 38 glutamine-dependent isolates. Seventeen clones had the same modal chromosome number of 23 as the parental population, 2 clones had 23 chromosomes and a minute fragment, and the remaining clones had 24 or even 26 chromosomes. One of the 23-chromosome lines had an unusually long chromosome as a marker.

Spontaneous revertability of the glutamine auxotrophic isolates: The plating efficiency of two isolates that required glutamine and two that did not was tested in media supplemented with varying concentrations of glutamate (Table 1). Several facts are evident: (1) The wild-type V79 cells did not require glutamine for growth, but showed a reduced plating efficiency in medium supplemented with glutamate. The feasible level of sodium glutamate was found to be 10^{-3} M; higher

TABLE 1

Plating efficiency in percent of clones of Chinese hamster cells in media supplemented with L-*glutamine or monosodium* L-*glutamate (Experiment 241)*

	Clonal cell line and putative genotype				
Glutamate concentration (M)	V79 *gln*$^+$	224G4 *gln*$^+$	224G12 *gln*$^+$	224G7 *gln*$^-$	224G11 *gln*$^-$
5×10^{-2}	0	0	0	0	0
1×10^{-2}	0.6	0.3	0	0	0
5×10^{-3}	18	5	10	0.6	0
1×10^{-3}	45	16	28	13	0.6
5×10^{-4}	44	17	35	12	2
Glutamine concentration (M)					
2×10^{-3}	93	75	94	84	46

concentrations were inhibitory. (2) The two *gln*$^+$ isolates, 224G4 and 224G12, had requirements similar to those of the parental cells. (3) The two *gln*$^-$ isolates, 224G7 and 224G11, which showed an absolute glutamine requirement when tested one month after isolation, exhibited some growth in glutamate-supplemented medium 4 months after their isolation when this particular experiment was done. The colonies that could grow on glutamine-deficient media are likely to be the descendants of spontaneous revertants. In other words, during this time of continuous *in vitro* maintenance of the clonal cell lines, gradual accumulation of revertants to glutamine prototrophy might have occurred. To test this possibility, some original isolates in frozen storage were thawed out and retested. The result shows that they exhibited the expected auxotrophic phenotype.

The same aminopterin selection technique (with HAG medium) was applied to the clonal line 224G11 in order to eliminate the accumulated revertants in the population. A fluctuation test (LURIA and DELBRÜCK 1943) was then performed in order to ascertain if this spontaneous reversion to prototrophy was due to a mutational event. Twelve new single-cell clonal derivatives from 224G11 were isolated and designated as 260–1 to 260–12. All 12 clonal lines were shown to be glutamine dependent and free from prototrophic revertants. The fluctuation test was performed 35 days after purification (removal from HAG medium) when enough cells had been obtained. All clonal lines were tested by inoculating 5×10^5 cells to each of the ten 100 mm plates containing 10 ml glutamate-supplemented medium. The results of this experiment are shown in Table 2. In the glutamine-free medium most of the cells began to deteriorate in about 6 to 14 days, leaving a few surviving colonies in certain plates. There were no survivors in cultures of three of the clonal lines: 260–3, 260–11, and 260–12. It appears, therefore, that reversion to glutamine prototrophy occurred randomly and was probably due to back mutation.

TABLE 2

Spontaneous reversion frequency in 12 new clonal isolates from a glutamine-requiring mutant cell line, 224G11 (Experiment 275)

Clonal cell line	Number of surviving colonies per plate*	Mean number of colonies per plate
260–1	0, 0, 0, 0, 1, 0, 1, 0, 0, 0	0.2
260–2	5, 6, 2, 4, 3, 3, 1, 13, 3, 2	4.2
260–3	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
260–4	0, 0, 0, 1, 0, 0, 0, 0, 0, 0	0.1
260–5	1, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.1
260–6	0, 2, 0, 0, 2, 0, 0, 0, 0, 0	0.4
260–7	0, 4, 1, 1, 1, 0, 2, 0, 0, 0	0.9
260–8	1, 2, 2, 0, 0, 0, 0, 0, 0, 0	0.5
260–9	7, 1, 2, 0, 2, 3, 9, 4, 7, 1	3.6
260–10	1, 3, 0, 0, 5, 0, 0, 0, 0, 0	0.9
260–11	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
260–12	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0

* 5×10^5 cells inoculated to each plate.

The data from the fluctuation test may be used to estimate mutation rate, using the methods of LEA and COULSON (1949). The probability of no mutational event, $P_0 = e^{-m}$, where m is the mean number of mutations per culture, is equal to 3 per 12, or 0.25. On the basis of the number of cells inoculated in all plates, the estimated mutation rate can thus be calculated as 1.4×10^{-7} mutations per cell per generation. The calculation was made as follows: mutation rate $= \frac{1.4}{2 \times 10 \times 5 \times 10^5} = 1.4 \times 10^{-7}$; $e^{-1.4} = 0.25$ = fraction of cultures with no mutant; 5×10^5 = cells per plate; 10 = number of plates per clonal cell line. The factor of 2 is used because the number of cell generations is approximately twice the final number of cells.

Selection for cells resistant to 8-azaguanine: The wild-type V79 cells were intolerant of azg in concentrations above 1 μg/ml. A resistant cell line (225–1) was obtained by exposing bottle cultures to increasingly higher concentrations of the drug over a period of 25 days. Cells that survived and multiplied in the drug-containing medium were subcultured when necessary. Resistant cells selected at 9 μg/ml azg exhibited resistance also to 1 mg/ml. Cytological examination of a sample revealed that all the cells had an identical karyotype of 23 chromosomes that differ slightly from those of the parental stem cells. This karyotypic uniformity of the 225–1 cell population, in contrast to the variability in the parental population, probably indicates a single cell origin of the resistant cells in spite of the fact that mass selection procedure was practiced.

A subline, 225–1a, was separated from 225–1 and maintained continuously in the standard medium in the absence of the drug. This subline possessed the same karyotype as its sister 225–1 line when examined 2 months after their separation. After another year, the karyotypes of cells, as well as chromosome number distributions in both lines, appeared to have been altered somewhat. Although 23 chromosome cells were still present in both lines, the modal chromosome number had become 22.

On these two occasions (2 and 14 months) the two resistant cell lines, as well as the drug-sensitive parental cells, were tested in various media. The results of the two experiments are virtually the same, and only those of the experiment done at 14 months are presented in Figure 1 and Table 3. All three lines showed maximum plating efficiency in normal medium. In the presence of the drug, only the resistant cells survived. It is apparent, therefore, that after prolonged maintenance in drug-free medium, the resistant 225–1a cells retained the same phenotype, thus indicating that resistance is not due to adaptation.

In THAG medium, the wild-type cells showed unaffected plating efficiency, although the colony size was smaller than that in normal medium (Figure 1). This slower growth of cells may be attributed to undetermined effects of aminopterin that had not been completely alleviated by the addition of thymidine, hypoxanthine, and glycine. It should be noted here that the optimal concentrations of thymidine, hypoxanthine, and glycine in the THAG medium had been experimentally determined for the V79 cells.

The resistant cells, on the other hand, failed to grow in THAG medium, pre-

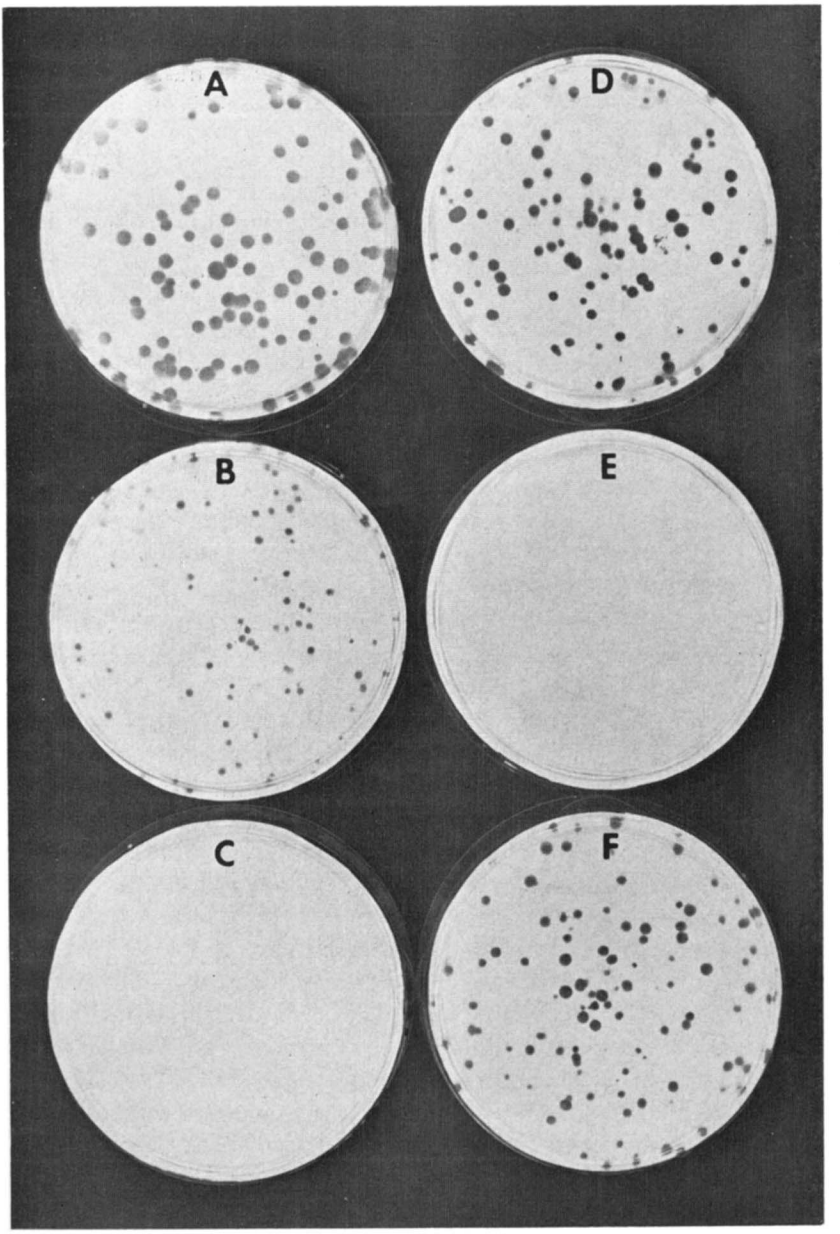

FIGURE 1.—Difference in growth response of V79–4 and 225–1a cells in various experimental media. One hundred cells were plated in each case and all plates were incubated 9 days. A. V79–4 cells, standard medium; B. V79–4 cells, THAG medium; C. V79–4 cells, 30 μg/ml azaguanine; D. 225–1a cells, standard medium; E. 225–1a cells, THAG medium; F. 225–1a cells, 30 μg/ml azaguanine.

TABLE 3

Growth response of Chinese hamster cells sensitive or resistant to 8-azaguanine (Experiment 381)

Cell line*	Number of cells plated	Plating efficiency (percent)		
		Normal medium	Medium with azaguanine†	Medium with THAG‡
225–1	100	98.3 ± 4.9§	98.2 ± 6.5	0
225–1a	100	108.3 ± 3.2	101.6 ± 5.7	0
V79–4	100	110.3 ± 5.0	0	98.7 ± 0.1

* Both 225–1 and 225–1a were at 62nd passage 14 months after original splitting into 2 sublines. V79–4 was a new clone isolated from V79.
† 30 μg/ml.
‡ Thymidine, hypoxanthine, aminopterin, and glycine.
§ Standard error of the mean.

sumably because of the loss or reduced activity of IMPPase. This test medium, therefore, can be utilized to eliminate the preexisting resistant cells in a sensitive cell population and to recover possible revertants from drug resistance to sensitivity.

Random occurrence of phenotypic change to azaguanine resistance: The persistence of the resistant phenotype in 225–1a cells in the absence of the drug seems to indicate that resistance is a relatively stable, heritable character. The line of evidence in further support of mutational origin of drug resistance comes from results of fluctuation tests.

To determine whether plating of the V79 cells yields fluctuations larger than those ascribed to random sampling, parallel platings were made from single cultures both in the presence and absence of azg (Table 4). Part A of the table summarizes the results of 4 plating experiments in which a relatively small number of cells were plated. The number of colonies represents the number of surviving cells as a fraction of the number of cells plated. Thus a homogeneity test of binomial samples according to the Brandt-Snedecor Chi-square formula (FISHER 1948) was performed. The results show that there was no significant difference in the proportion of colonies observed in different samples that were obtained from the same cell culture. Part B summarizes the results of six experiments, showing the frequencies of surviving azg-resistant colonies per 5×10^5 V79 cells inoculated to each plate. Another drug-sensitive revertant cell line (265–8, see below) was similarly tested in one experiment. Here we can see that the agreement obtained between variance and the mean indicates Poisson distributions which have been confirmed by application of the Chi-square test. Hence, it is unlikely that large errors of unknown origin occurred.

Two fluctuation experiments (Table 5) were performed by using V79, as well as a drug-sensitive revertant cell line (265–8, see below). The cell populations were purified in the THAG medium and shown to be free of resistant cells (in more than 10^6 cells tested). One hundred cells were inoculated in drug-free medium to each of a large number of 16-ounce prescription bottles and allowed to multiply independently. After 40 and 52 days, respectively, cells in each bottle

TABLE 4

Number of colonies in different samples from the same culture
Part A. Plating small number of cells (binomial distribution)

	Experiment No.					
Sample No.	243	255	266	381	381	381
1	260/400(3)	168/200	80	107	96	106
2	292/400(3)	157/200	72	116	99	112
3	264/400(3)	145/200	86	108	100	107
4	137/200(3)	107/100	96	99†	95(9)	107(9)
5	133/200(3)	95/100	80	98†	88(9)	91(9)
6	71/100(3)	105/100	84	99†	87(9)	109(9)
7	68/100(3)	43/50	..	..	100(30)	102(30)
8	51/100(3)	59/50	..	..	102(30)	107(30)
9	26/50(3)	44/50	..	..	91(30)	104(30)
10	30/50(3)	..	..	..	106(30)	92(30)
11	34/50(3)	..	..	..	92(30)	103(30)
12	248/400(9)	..	..	..	..	..
13	288/400(9)	..	..	..	..	..
14	304/400(9)	..	..	..	..	..
Cell line	225–1	V79	V79	V79–4	225–1	225–1a
Number of cells plated	*	*	100	100	100	100
Azaguanine μg/ml	‡	0	0	0	‡	‡
Mean	65.2	92.8	83.0	104.5	96.0	103.6
Variance	3.8	23.4	45.1	10.8	9.5	11.6
χ^2	0.7	2.0	2.7	0.5	0.9	1.1
P	>0.99	0.99	0.75	0.99	>0.99	>0.99

* The denominator indicates the number of cells plated.
† In THAG medium.
‡ The figures in parentheses indicate azaguanine concentration.

were trypsinized, counted, reinoculated at 1×10^6 or 2.5×10^6 cells per bottle, and exposed to 9 μg/ml of azg. Eight days later, some cultures showed no resistant cells, others had a few resistant colonies, and still others contained innumerable colonies. These results of high variance among cultures clearly indicate the random occurrence of resistant cells and possibly represent spontaneous mutations. Furthermore, the mutational events leading to azg resistance in the cell population were not dependent upon the presence of analog. The spontaneous mutation rate from azg sensitivity to resistance was estimated in the two experiments to be 1.5×10^{-8} and 1.6×10^{-8} per cell per generation.

Spontaneous reversions to azaguanine sensitivity: Strains 225–1 and 225–1a cells, inoculated 10^6 per plate, were incubated in THAG medium for 14 days with medium renewal twice weekly. The great majority of the cells degenerated in 2 or 3 days; however, a few discrete colonies survived at a frequency of 5.5×10^{-7} in 225–1 cultures and 2.5×10^{-7} in 225–1a cultures respectively. Eight such clones (265–1 to 265–8) that we isolated were expected to be azg-sensitive. Upon testing, however, it was revealed that only one (265–8) of the 8 isolates was sensi-

TABLE 4—Continued

Part B. Survival of azaguanine-resistant cells in large populations (Poisson distribution)

Sample No.	Experiment No. 316	320	321	323	332	333
1	98	77	2	5	23	5
2	105	78	1	2	9	3
3	122	83	0	3	9	6
4	101	81	2	4	16	7
5	138	78	1	2	12	6
6	134	59	2	6	11	3
7	94	55	1	2	33	5
8	113	81	1	0	13	11
9	103	84	..	2	29	7
10	118	80	..	..	..	6
Cell line*	V79	265–8	V79	V79	V79	V79
Number of cells plated	5×10^5	5×10^5	5×10^5	5×10^5	5×10^5	5×10^5
Azaguanine μg/ml	9	9	9	9	9	9
Mean	112.6	75.6	1.2	2.9	17.2	5.9
Variance	229.3	102.9	0.5	3.4	80.2	5.2
χ^2	18.3	12.3	2.9	9.3	37.3	7.9
P	0.05	0.25	0.90	0.50	<0.005	0.75

* All purified in THAG medium prior to each experiment.

tive (Table 6). It can be seen from the table that the revertant 265–8 cells which resembled the original sensitive V79 cells could tolerate the drug at 1 μg/ml, but were killed at 5 μg/ml. Both cell lines were sensitive at 3 μg/ml in another experi-

TABLE 5

Estimated forward spontaneous mutation rate from azaguanine sensitivity to resistance in Chinese hamster cells in culture

Experiment No.	Sensitive cells used	Days after purification	Number of cells inoculated per culture	Number of cultures	Number of surviving colonies per culture	Mutation rate*
298	V79	40	2.5×10^6	40	0(2)† 1–10(4) ~100(1) ~300(1) ~1000(14) monolayer (18)	1.5×10^{-8}
309	265–8	52	1×10^6	79	0(6) 10–20(6) 100–300(5) monolayer (62)	1.6×10^{-8}

* Number of mutations per cell per generation.
† Figures in parentheses are the number of cultures.

TABLE 6

Growth response of Chinese hamster cell lines to various concentrations of 8-azaguanine (Experiment 290)

Cell line	Number of cells plated	Plating efficiency (percent) in various concentrations of azaguanine (μg/ml)					
		0.5	1	5	10	50	100
V79*	100	110	120	0	0	0	0
225–1	100	NT†	NT	83	86	93	NT
265–1	100	NT	148	173	153	NT	NT
265–2	100	NT	111	111	105	NT	NT
265–3	100	NT	168	172	170	NT	NT
265–4	100	NT	86	86	81	NT	NT
265–5	100	NT	93	99	99	NT	NT
265–6	100	NT	75	68	82	NT	NT
265–7	100	NT	134	140	NT	NT	142
265–8*	100	88	99	0	0	0	0

* Purified prior to experiment. In this experiment, in medium containing azaguanine higher than 5 μg/ml, the mean plating efficiency of V79 cells was 0.004%, but no surviving colony was found in more than 3×10^4 265–8 cells inoculated.

† NT = not tested.

ment. In contrast, clones 265–1 through 265–7, like 225–1, were resistant to 10 μg/ml or higher levels of azg.

A direct assay was made for the cellular uptake of ^{14}C-hypoxanthine in various cell lines. In Figure 2 it can be seen that in V79 cells the incorporation of ^{14}C-hypoxanthine into trichloroacetic acid-insoluble material, after an initial lag, increases linearly with time during the first 6-hr incubation. The rate of incorporation in 265–8 cells was similar to that in V79 cells. On the other hand, 265–1 and 265–2 cells behaved like 225–1 cells in that there was no detectable incorporation of the radioactive precursor. These results would appear to agree with the azg cytotoxicity data presented above. Nevertheless, in another hypoxanthine-incor-

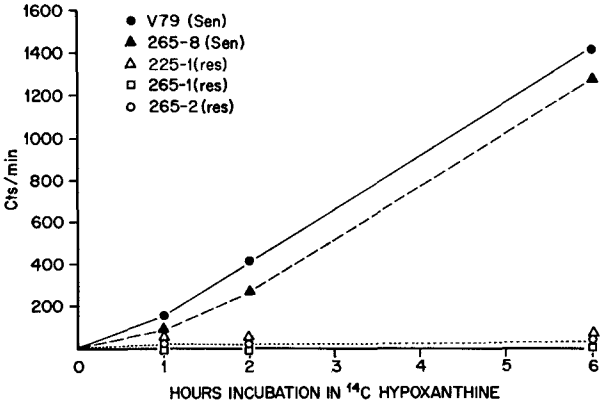

FIGURE 2.—Incorporation of hypoxanthine-8-^{14}C as a function of time at 37°C into various Chinese hamster cell lines sensitive or resistant to 8-azaguanine.

TABLE 7

Incorporation of ^{14}C-hypoxanthine in 8-azaguanine resistant or sensitive Chinese hamster cells in the presence or absence of aminopterin (Experiment 285)

Cells	Counts per minute*	
	+TAG†	—TAG
V79	5611	5035
225–1	17	18
265–1	1344	219
265–2	866	81
265–8	3963	2736

* Total radioactivity of acid-insoluble materials per culture (average of triplicate) following 6-hr incubation with ^{14}C-hypoxanthine.
† Thymidine, aminopterin, glycine.

poration experiment in which the biosynthesis of purines was blocked by the action of aminopterin, the following pattern began to emerge (Table 7): (1) The sensitive V79 and 265–8 cells were fully capable of utilizing the exogenous preformed hypoxanthine, whether or not *de novo* purine synthesis was blocked by aminopterin; (2) the resistant 225–1 cells seemed to have lost the ability of incorporating preformed purines; and (3) the phenotypically resistant revertants tested, namely 265–1 and 265–2, were constitutively capable but seemingly unlikely to incorporate a significant amount of the preformed purines unless the cellular *de novo* synthetic pathway of purines was impaired.

The pattern of purine utilization was further demonstrated in a cell growth experiment in which azg-sensitive or resistant cells were plated in THAG medium with varying concentrations of hypoxanthine. No growth was observed in some of the resistant revertants (265–1, 265–3, 265–6 and 265–7) but other resistant revertants (265–2, 265–4 and 265–5) could indeed utilize exogenous hypoxanthine, thereby enabling a small proportion of them to survive and proliferate (Table 8). This finding could explain the recovery of these "partial revertants" in THAG medium in the first place, as well as their apparent resistance

TABLE 8

Relative capacity of preformed purine utilization in 8-azaguanine resistant or sensitive cells following blocking of de novo *purine synthesis with aminopterin (Experiment 291)*

Cells	Plating efficiency (percent) in various concentrations of hypoxanthine (M)			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
V79*	15	76	0	0
225–1	0	0	0	0
265–2	43	9	0	0
265–4	3	0	0	0
265–5	1	0	0	0
265–8*	92	95	23	4

* Purified prior to experiment.

to the guanine analog. It could be further inferred that these partial revertant cells might be able to synthesize either a reduced amount of IMPPase or a defective enzyme.

In order to verify this assumption, a cell-free assay that is specific for the IMPPase activity was performed. Preliminary experiments established the optimal conditions for the assay method. These experiments further established that the amount of hypoxanthine-^{14}C incorporated into IMP was proportional to the concentration of the enzyme, thus validating the assaying procedure. The results of the enzyme determinations in various cell lines are summarized in Figure 3. It can be seen that, under the experimental conditions, the enzyme activity per unit amount of total cell protein in 265–8 cells seemed to exceed that in the wild type V79 cells, even though the rates of hypoxanthine utilization in these two cell lines were similar (Figure 2). Different clonal isolates, which survived the THAG selection and were superficially azg-resistant, are shown to exhibit varying degrees of IMPPase activity. Finally, the enzyme activity was not present in 225–1 cells or in one of the clonal isolates, 265–6.

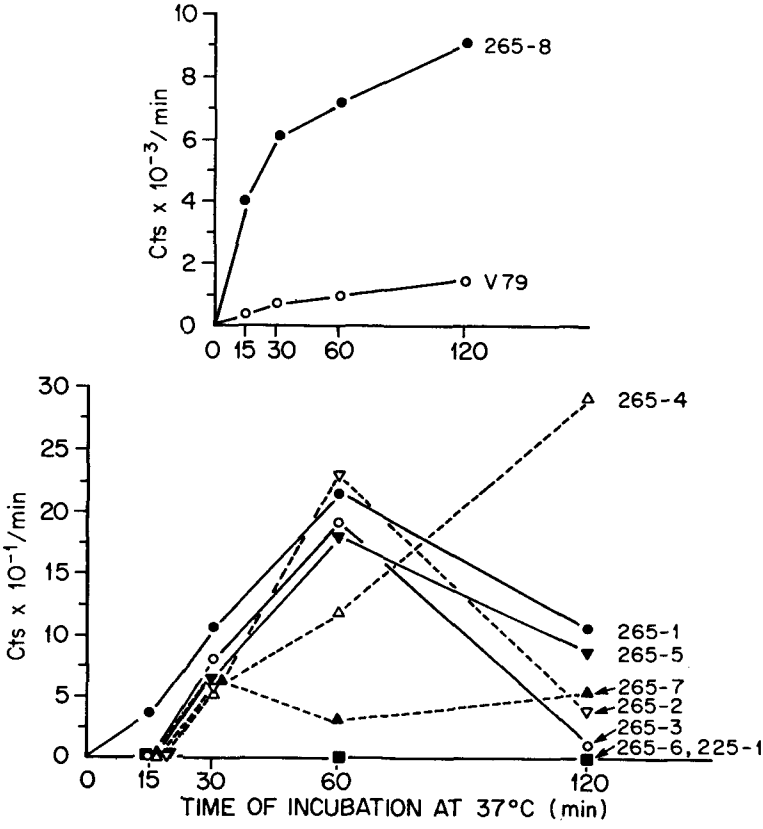

FIGURE 3.—The activity of inosinic acid pyrophosphorylase in various Chinese hamster cell lines, as indicated by the relative amounts of acid-insoluble, phosphorylated derivatives of hypoxanthine-8-^{14}C per μg of cell protein.

Formation of a cell hybrid between glutamine auxotrophic and azaguanine resistant mutants: Following cocultivation of 224G11 and 225–1 cells and selection in glutamine-free THAG medium, the majority of cells in the mixed culture began to die and detach from the plate. A number of morphologically distinct colonies of large cells survived and 5 were isolated. One of the isolates, 236–2–5, appeared to be not an autotetraploid of either 224G11 or 225–1 cells, but a combination of chromosomes from both parents (Figure 4). An autotetraploid cell is larger in size than its diploid counterpart and possesses an exact doubling of the diploid chromosome complement. The 236–2–5 cells differed from an autotetraploid in the latter respect and could thus be the progeny of a cell hybrid resulting from the fusion of a 224G11 and a 225–1 cells. It is also implicit that the hybrid cell could undergo apparently normal cell divisions. To identify an intraspecific cell hybrid such as the present instance on the cytological evidence alone is however, by no means certain, because of the similarities of the karyotypes of both parents and the possible karyotypic alterations that might have occurred both before and after cell fusion.

The suggestion that 236–2–5 cells might be the progeny of a cell hybrid was supported by a functional test, the results of which are summarized in Table 9. Results from this and other experiments indicate that 224G11 cells were glutamine requiring and azg-sensitive and that 225–1 cells were azg resistant and glutamine independent. The glutamine-free THAG medium could not sustain the growth of either parent. On the other hand, the presumed hybrid cell apparently survived well in glutamine-free THAG medium. The conclusion that can be drawn from this experiment is that both glutamine auxotrophy and azaguanine resistance appear to be recessive characters. These two mutants have thus been designated as gln^- and azg^r *vs.* gln^+ for glutamine prototrophy and azg^s for azaguanine sensitivity.

DISCUSSION

Selection of genetic variants: Genetic variations such as chromosomal aberrations and gene mutations have been shown to occur in mammalian cell cultures

TABLE 9

Growth response of mutants and somatic-cell hybrid in various experimental media

Medium	Cell line			
	V79 azg^s; gln^+ 2n = 23	225–1 azg^r 2n = 23	224G11 gln^- 2n = 23	236–2–5 HYBRID 4n = 46
Normal	+	+	+	+
+ Azaguanine	—	+	—	—
— Glutamine	+	+	—	+
+ THAG*	+	—	+	+
+ THAG, — Glutamine	+	—	—	+

* Thymidine, hypoxanthine, aminopterin, glycine.
+ = growth; — = no growth.

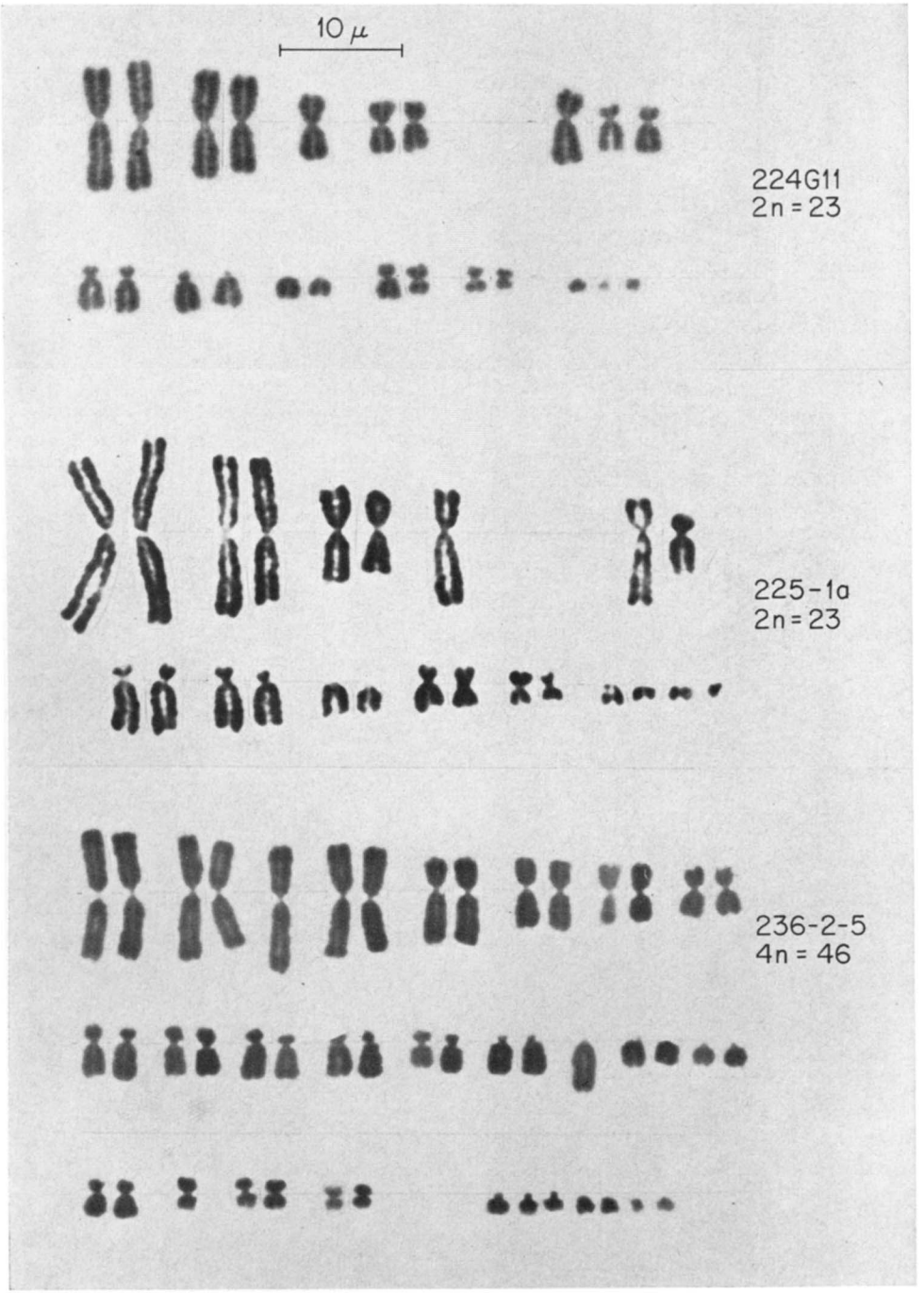

FIGURE 4.—The karyotypes of 224G11 and 225–1a cells and the karyotype of the presumed cell hybrid resulting from the fusion of the two parental cells.

originated from several species (for reviews see HARRIS 1964; DAVIDSON 1964; KROOTH 1965; GARTLER and PIOUS 1966). With the exception of numerous instances of chromosome modifications that were induced by physical, chemical, or biological means, almost all the reported genic mutations that have occurred in cell populations in the course of *in vitro* cultivation were presumably spontaneous in origin or were inadvertently induced. For example, a spontaneously arisen proline auxotrophic mutant has been isolated in another cell line of Chinese hamster (KAO and PUCK 1967). There is no clear evidence as to the genetic basis, such as nuclear or cytoplasmic, of all these mutants developed *in vitro*.

In a number of instances the mutants have been shown to be stable; that is, the selected character of the cell line is retained after a number of cell generations in the absence of the selecting agent. Where fluctuation tests have been applied, the results indicate that the mutants arise independently of the selecting agent. Several of these mutant lines have been characterized by specific enzyme changes. The two mutants, glutamine auxotrophy and azaguanine resistance, isolated in the present studies also exhibited some of these characteristics. Furthermore, we have shown that both forward and reverse mutations at both *gln* and *azg* loci can occur spontaneously (this report) and can be induced by chemical mutagens (CHU and MALLING 1968a,b). These assumptions are at least compatible with nuclear genetic control of such mutants (GARTLER and PIOUS 1966), although definitive proof for such an assertion is lacking.

If both mutations studied were recessive in character, as shown by the analysis of a somatic hybrid between two cells, each carrying a parental trait, the question arises as to how easily a recessive mutation in a diploid cell can be recovered. Theoretically, several possibilities exist: (1) An autosomal recessive could have existed either in homozygous or heterozygous state. In a heterozygote, mutation of the dominant allele to recessiveness will change a cell's phenotype. (2) Repeat mutational events at both alleles of a locus or mitotic disjunctional error in a heterozygote could lead to homozygosis of a recessive character. (3) Since the hamster cells under study were aneuploid, hemizygous condition at either locus could have existed, either before or after the occurrence of mutation. (4) Since V79 cells originated from a male hamster, sex-linked recessives in these cells would, of course, be more readily detected, but we have no evidence that both loci are sex-linked. It is interesting to note, however, that in Lesch-Nyhan syndrome in man the control of inosinic acid-guanylic acid pyrophosphorylase activity is apparently X-linked (SEEGMILLER, ROSENBLOOM and KELLEY 1967).

In principle and practice, there are at least three different methods for mutation selection in mammalian cell cultures: (1) Mass selection procedure has been conveniently and successfully used for selection of mutations leading to resistance to extrinsic agents or to certain altered nutritional requirements (see HSU and KELLOG 1959). The parental cells are usually nonviable in the selective environment, permitting the isolation of the surviving mutants. (2) The second selective method creates a situation in which the parental cells die and the mutant cells do not grow but are able to keep alive in the selective environment. The thymine starvation method, first proposed and practiced in mammalian cell cultures by

DEMARS and HOOPER (1960), is an example. These investigators obtained a glutamine-requiring mutant line from HeLa cells. We have also shown the isolations in two repeat experiments of a number of glutamine auxotrophic mutants in Chinese hamster cells. The method should be equally applicable to other mammalian cells as well, although the optimal concentrations of various chemicals may be different for different cells, as we have demonstrated here. (3) The third type of selective technique results in either differential growth between the mutant and parental cells or selective killing of the parental cells. Thus PUCK and KAO (1967) have proposed the use of 5-bromodeoxyuridine and visible light for selective killing of wild-type cells and for isolation of nutritionally deficient mutants. This and other similar procedures have been used in our laboratory (CHU, unpublished), utilizing agents such as colchicine and hydroxyurea for selective killing of cells at specific cell stages. It should be pointed out that whereas the thymine starvation method has been applied for screening auxotrophs of nonessential amino acids, the selective killing methods, which are analogous to the penicillin method for selecting biochemical mutants in bacteria (DAVIS 1948; LEDERBERG and ZINDER 1948), are feasible to recover any mutants which interfere with the normal growth of the cells.

Cytology of the mutants: Chromosome changes have been observed in both parental populations and various clonal isolates in the present study. Karyotypic characteristics in eukaryotes are potentially useful for cytogenetic analysis such as cytological mapping of genes. Several chromosome markers observed in our study served for the identification of certain clonal lines and for monitoring the purity and changes of the clonal populations. We have, however, no evidence that these chromosomal modifications were associated with the specific gene mutations in question. Additionally, karyotypic analysis has proved to be valuable in the identification of the somatic cell hybrid.

Reversion studies: Reversions at both loci have been shown to occur and were probably due to reverse mutational changes. The reverse mutation rate has been estimated for glutamine dependence but not for azaguanine resistance (only frequency). At present, it is not certain whether the reversions were due to back mutation at respecitve loci or to suppressors. In any event, both reversions appeared to be single-step changes. LIEBERMAN and OVE (1959) reported two-step mutational changes with greater resistance to puromycin in AMK-2-2 cells, which were possibly derivatives from the mouse L strain. SZYBALSKI *et al.* (1962) observed in a human bone marrow cell line, D98, sequential changes to different levels of resistance to azg. LITTLEFIELD (1963) isolated several lines of mouse L cells with a small degree of resistance to azg and suggested that these partially resistant cells may be heterozygous for their ability to synthesize active IMPPase. MORROW (1964) also concluded that there are two levels of azg resistance in L cells. In the present work, partial reversions at the azg locus apparently occurred. One possible interpretation is that they represent single-step, isoallelic mutational changes.

By use of the selective THAG medium, revertants which seemingly had equivalent degrees of resistance to azg were shown to possess varying IMPPase activities.

The kinetics of enzymes isolated in these revertants is under further study. It is already clear, however, that each revertant possesses a different enzymatic activity, either quantitatively or qualitatively, or both. One of the revertants, 265–8, even surpassed the wild-type cells in enzyme activity. The finding on partial reversions representing an isoallelic series is analogous to those reversions found in other organisms, for example, Neurospora (GILES 1955).

Somatic cell hybrid: Finally, we have obtained a somatic hybrid that possibly represents the result of cell fusion between a gln^- cell and an azg^r cell, as indicated by both karyotypic analysis and growth response in test media. The modes of inheritance of glutamine auxotrophy and azg resistance as recessive characters were thus inferred. The cell hybrid apparently could undergo an indefinite number of cell divisions. Hybridization techniques should be very useful for genetic analysis of such phenomena as gene action, transfer of genetic material, recombination, and segregation, as reported and discussed by EPHRUSSI and his coworkers and by others (see EPHRUSSI, SCALETTA, STENCHEVER, and YOSHIDA 1964; GARTLER and PIOUS 1966; SILAGI 1967; WEISS and GREEN 1967; and WEISS, EPHRUSSI, and SCALETTA 1968).

The authors wish to thank DR. WARREN SINCLAIR, Argonne National Laboratory, for the gift of V79 Cells; DR. M. A. KASTENBAUM, Director's Division, ORNL for advice on statistics; and DRS. ROY CURTISS III, H. V. MALLING, and C. M. STEINBERG for discussions and reading of the manuscript.

SUMMARY

Two selective techniques have been employed to isolate genetic variants in an established cell line (V79–122D1) of Chinese hamster (*Cricetulus griseus*, 2n = 22). The parental cells had an aneuploid (2n = 23) constitution, an average generation time of 12 hrs, and maximal plating efficiency.—The first selective technique makes use of the differential growth of prototrophs and amino acid auxotrophs during a state of thymine starvation created by aminopterin. A number of clones have been recovered which, unlike the parental cells, were no longer capable of utilizing L-glutamic acid but required L-glutamine for growth. Results of a fluctuation test indicate that reverse mutation to gln^+ occurred randomly. The rate of reverse mutation was estimated as 1.4×10^{-7} per cell per generation. —Cytological examination established the clonal nature of these mutational isolates. Several mutants were found to possess characteristic chromosome marker(s), which might or might not be related to the mutational event.—The hamster cells were sensitive to 8-azaguanine at concentrations greater than 1 μg/ml. By mass selection, a derivative subline exhibited a 1000-fold increase in resistance to the drug as compared with the parental cells. The resistant subline consists of a single, uniform karyotype. Further experimental evidence supports the conclusion that resistance is due to a randomly occurring, spontaneous mutational event and not to adaptation. Resistance arises from a loss or reduction of the inosinic acid pyrophosphorylase activity with resultant inability of the cell to utilize preformed purines or their analogs. At this locus, both forward and reverse mutation rates occurred when cells were grown in appropriate media. The spon-

taneous forward mutation rate to drug resistance was 1.5×10^{-8} per cell per generation and the reversion frequency was 2 to 5×10^{-7}. Furthermore, there is evidence for the occurrence of a series of partial revertants with varying capacities of purine utilization. This last finding was confirmed by cell-free assay of the enzyme activity in parental as well as mutant cells. The partial revertants were interpreted as representing single-step, isoallelic mutations.—By plating the glutamine-requiring cells and azaguanine-resistant cells together in an experimental medium in which neither parental cell could survive alone, we obtained a cell hybrid through fusion. A number of morphologically distinct colonies were observed. One such isolated colony contained the combined chromosome complements from both parents. This tetraploid hybrid clone could be propagated through apparently normal mitosis. Functional tests indicate that both glutamine auxotrophy and azaguanine resistance behaved as recessive characters in the hybrid cell.

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