

Altered Enzymes in Drug-Resistant Variants of Mammalian Tissue Culture Cells

(mouse L cells/somatic cell genetics/structural gene mutants/hypoxanthine phosphoribosyl transferase/
8-azaguanine resistance)

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ABSTRACT Two selective procedures are compared in an effort to isolate variants of mouse L cells containing structural gene mutations. Among the resulting variant cloned cell lines are found two types of alterations in the enzyme hypoxanthine phosphoribosyl transferase (EC 2.4.2.8.) (1): enzyme with altered kinetic constants causing *in vivo* and *in vitro* resistance to 8-azaguanine; and (2) enzyme with altered heat sensitivity *in vitro*. These results support the view that tissue culture cell variants can arise from structural gene mutations.

Many phenotypic variants† have been isolated from mammalian tissue culture cell lines, including auxotrophic (1, 2), drug-resistant (1, 3), and temperature-sensitive variants (4, 5). Some of the properties of such variants are compatible with the hypothesis that they arise as a result of point mutations. For example: (1) Luria-Delbrück fluctuation analysis, when applied, indicates that the variants arise spontaneously (1, 6, 7); (2) variant phenotypes can be maintained for many generations in the absence of a selective medium; and (3) frequencies with which variants arise may be increased by treating the parental cell lines with chemical mutagens (8). On the other hand, certain observations bring into question the genetic origin of such variants. First, the frequencies with which one can isolate variants of different phenotypes are often extremely high (spontaneous frequencies as high as 10^{-3}) (3, 6). The reversion frequencies are also of the same order of magnitude (see Fig. 2). Second, the frequencies with which recessive variants arise are unaffected by changes in the number of chromosomal complements present in the parental cell line. Haploid cultured amphibian cells have been reported to yield BrdU-resistant variants at rates similar to diploid cells, rather than at much higher rates (9). This lack of correlation with chromosome number has also been reported among diploid, tetraploid, and octaploid Chinese hamster cells, screened for resistance to thermal shock or to 8-azaguanine (8AG) (10).

In view of these observations, it seemed necessary to prove

that variants can be isolated which contain gene products with altered physical properties. Such evidence would lend strong support to the hypothesis that variants can arise as a result of point mutations. In the course of screening a large number of mouse L cell variants for the presence of a nonsense suppressor gene, we have found a number of variants containing an altered enzyme. One variant has proven to contain an enzyme with altered kinetic constants, while several others have enzymes with increased heat sensitivity *in vitro*. Such changes are most readily explained in terms of altered amino-acid sequences in the enzymes resulting from mutated base sequences in the corresponding structural genes.

Selection of variants

The target enzyme selected for these studies was hypoxanthine phosphoribosyl transferase (HPRT, EC 2.4.2.8). This enzyme converts hypoxanthine (Hyp) or guanine to their corresponding nucleotides, IMP or GMP, and thus channels these purines into nucleic acid synthesis. This enzyme was chosen because it is readily assayed directly in cell-free extracts or indirectly in intact cells, and also because of the availability of selective methods for isolating variants having lost or regained this enzymatic activity. Purine analogs such as 8-azaguanine (8AG), 6-thioguanine (6TG), and 6-mercaptopurine kill cells containing HPRT and select for resistant variants, many of which have reduced HPRT activity (3). Such variants remain viable because purine nucleotides can be synthesized by *de novo* pathways in the absence of HPRT. Revertants, which have regained HPRT activity, can be selected by blocking *de novo* purine biosynthesis with aminopterin and simultaneously supplying Hyp. This renders the cells dependent on HPRT for synthesis of purine nucleotides from Hyp. The medium used for selecting HPRT⁺ revertants, called HAT medium (7), contains hypoxanthine, aminopterin, thymidine, and glycine, the latter two compounds compensating for the lack of reduced folate in the cells.

The parental mouse L cell line (obtained from J. Littlefield in 1971) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as described by Chu and Malling (8), allowing 4-6 days after treatment before addition of the selective agents. This procedure increased the frequency of clones resistant to guanine analogs about 100-fold over the spontaneous frequency. Two sets of such clones were isolated by different selective procedures. The first, set I, was selected for resistance to high levels of 8AG (3 μ g/ml) (3). The spontaneous frequency at which such clones arose was about

Abbreviations: HPRT, hypoxanthine phosphoribosyl transferase; 8AG, 8-azaguanine; Hyp, hypoxanthine; 6TG, 6-thioguanine; HAT, hypoxanthine-aminopterin-thymidine-containing selective medium; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PRibPP, phosphoribosyl pyrophosphate.

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† The term "variant" is used here to mean any cell line having an altered phenotype, relative to the parental cell line, that can be maintained on repeated recloning in the absence of a selective agent.

10^{-6} . After mutagenesis with MNNG or ethylmethanesulfonate this frequency increased to about 10^{-4} . As observed in Fig. 1 (*upper half*), independently isolated, cloned cell lines resistant to high levels of 8AG contained different amounts of HPRT activity. Such heterogeneity of HPRT content in 8AG-resistant cell lines has been observed by others (3, 11, 12). For the experiment shown in Fig. 1, HPRT activity was measured in the intact cell as the level of [^3H]-Hyp incorporated into nucleic acid. We have confirmed that this indirect *in vivo* measurement of HPRT activity correlates well, with occasional exceptions, with direct *in vitro* measurements of HPRT activity (11). A tendency of the *in vivo* measurements to estimate slightly higher HPRT specific activities than *in vitro* measurements is observed. As seen in Fig. 1, about 50% of the variant cell lines contain less than 10% of the HPRT activity present in the parental cell line. However, a considerable number of the variants contain HPRT activities comparable to the wild-type level. The

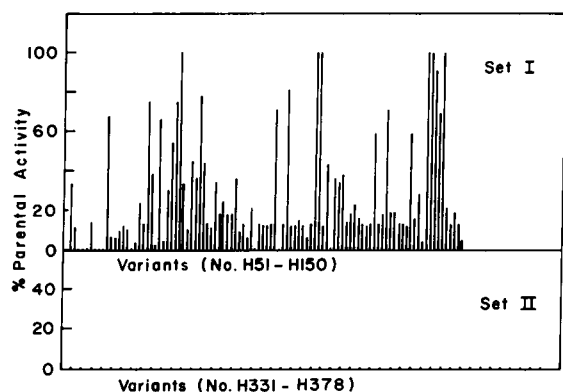


FIG. 1. Relative HPRT activity for two sets of mouse L cell variants, set I and set II, that were isolated as independent clones resistant to 8AG and 8AG + 6TG respectively. The level of HPRT activity in each variant cell line was determined by measuring the level of *in vivo* [^3H]hypoxanthine incorporated into trichloroacetic acid-precipitable product. The cells were grown in 15-mm wells (Linbro multitrays) containing Earle's minimal essential medium with 4 times normal levels of amino acids and vitamins (GIBCO), supplemented with 10% fetal-calf serum (Microbiological), penicillin (250 $\mu\text{g}/\text{ml}$), and streptomycin (62.5 $\mu\text{g}/\text{ml}$). At zero time, when each well contained approximately 3×10^3 cells, the above medium was removed and fresh supplemented medium plus nM [^3H]hypoxanthine (23.5 Ci/mmol) was added. The cells were incubated at 37° in a 5% CO_2 atmosphere for an additional 12 hr. The cells in each well were then washed twice with phosphate-buffered saline solution, trypsinized with four drops of trypsin solution (0.05%), and incubated an additional 15 min at 37° . Trypsinization was stopped by the addition of one drop of serum, and 1 ml of phosphate-buffered saline was added. The cells were then collected on Millipore filters; the cells were lysed, and the nucleic acid precipitated with 7% trichloroacetic acid. After three washes with trichloroacetic acid, the radioactive product was quantitated by scintillation counting. This procedure proved to be extremely reproducible. The parental L cell line under these conditions incorporated 200,000 cpm per 5×10^3 cells. Each number (H51 through H150, and H331 through H378) designates an independently isolated, cloned cell line derived from the same parental L cell line after MNNG treatment. The conditions of mutagenesis (incubation at 37° for 2.5 hr with 3 $\mu\text{g}/\text{ml}$ of MNNG) were identical for set I and set II. Approximately 20% of the parental cells survive this MNNG treatment.

mechanism by which these strains have achieved resistance to 8AG has not yet been determined, but it is interesting that the high levels of HPRT often decreased during prolonged passage in 8AG medium (i.e., months).

In an effort to isolate clones with uniformly low levels of HPRT activity, the combination of 8AG (3 $\mu\text{g}/\text{ml}$) and 6TG (6 $\mu\text{g}/\text{ml}$) was used to select a second set of variants, set II. The spontaneous frequency at which clones resistant to both analogs arose was less than 10^{-8} . After mutagenesis this frequency increased to 10^{-6} . As shown in Fig. 1 (*lower half*), all clones isolated by the second procedure (set II) contained less than 0.3% of the parental HPRT activity. These measurements have been done both *in vivo* and *in vitro* with similar results.

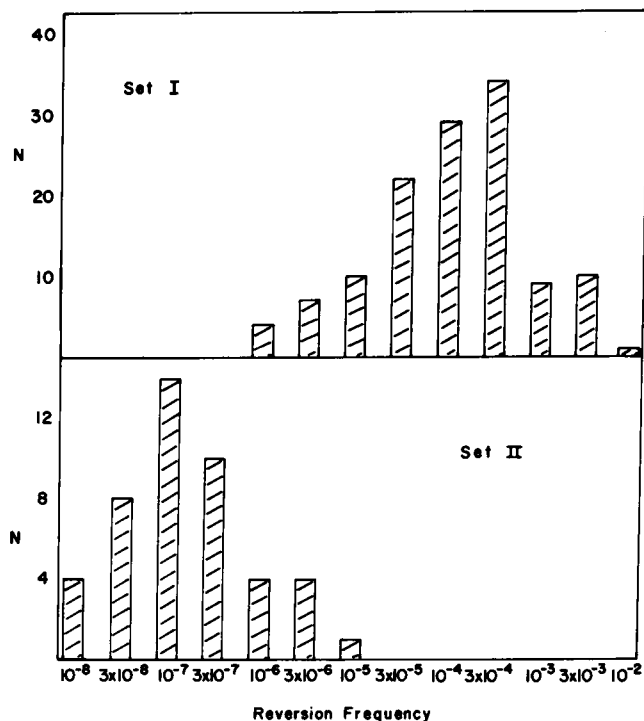


FIG. 2. The distributions of MNNG-induced reversion frequencies for the two sets (set I and set II) of mouse L cell variants resistant to 8AG and 8AG + 6TG respectively. N represents the number of variant cell lines observed within each reversion frequency bracket. 3×10^6 Cells per 100-mm plate (Falcon) of each variant cell were incubated at 37° for 2.5 hr with 3 $\mu\text{g}/\text{ml}$ of MNNG dissolved in regular medium (see Fig. 1) minus serum. Cell saturation on 100-mm plates is attained at about 1.5×10^7 cells per plate. The MNNG medium was then removed and fresh medium plus serum added. The cells were incubated at 37° in a 5% CO_2 atmosphere for 2 days to allow recovery from the mutagenic treatment. After mutagenesis there is a lag period (24–48 hr) before normal cell division resumes. The cells were then trypsinized and put down at 1/10 the cell density on new 100-mm plates with fresh medium. After 4 additional days of incubation at 37° to permit segregation, the selective medium, HAT medium (21), was added. Fresh HAT medium was added every 3 days. Clones capable of growing in HAT medium appeared in 10–14 days. At this time some of the clones were picked and further characterized, and the remaining clones were fixed with methanol and stained with Giemsa. The reversion frequency was calculated as the number of clones which grew up in the HAT medium divided by the number of cells present at the time of mutagenesis.

The MNNG-induced reversion frequencies for the two sets of variants (as measured by the regained ability to grow in HAT medium) differ markedly. Fig. 2 shows the distribution of reversion frequencies for the two sets of variants. It is observed that most of the variant cell lines in set I have MNNG-induced reversion frequencies in the range of 3×10^{-5} to 10^{-3} . The spontaneous reversion frequency of many of the high-reverting variants is not increased by treatment with MNNG. One variant in this set reverts spontaneously

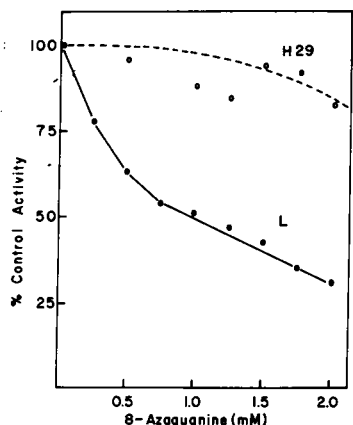


FIG. 3. The *in vitro* inhibition by 8AG of the HPRT-dependent conversion of [3 H]hypoxanthine to [3 H]IMP observed in extracts of the parental L cell line and the variant cell line H29. Cells were grown as described in Fig. 1. Confluent 100-mm plates were harvested at 4° as follows. The plates were washed twice with 5 ml of phosphate-buffered saline and drained. 0.5 ml of extraction buffer [20 mM Tris·HCl (pH 7.4)–10 mM MgCl₂–30 mM KCl–1 mM dithiothreitol–0.5% Triton X-100] was added and the plate was shaken over a 15-min period. The extract was removed with a Pasteur pipette and centrifuged for 5 min at 1000 × *g* to remove nuclei and cellular debris. 2 M KCl was then added to bring the final KCl concentration to 0.1 M for increased stability of HPRT. This extraction procedure yielded more protein (3–6 mg/ml) and higher specific HPRT activity than the freeze-thaw method. HPRT assays were performed in 50- μ l reaction mixtures containing 100 mM Tris·HCl (pH 7.9), 7 mM MgCl₂, 0.1 mM EDTA, 1 mM PRibPP, 0.21 μ M [3 H]hypoxanthine (Schwarz-Mann, 12 Ci/mmol), 1 mg/ml bovine-serum albumin, and the indicated concentration of 8AG. Because of the limited solubility of 8AG, it was always diluted in 0.1 N NaOH, neutralized with 0.1 N HCl, and the remaining components added. The reaction was started by the addition of 5 μ l of diluted cell extract, incubated at 39° for 11 min, and stopped by placing on ice after addition of 10 μ l of 7% trichloroacetic acid. To determine the amount of Hyp converted to IMP, 5 μ l of the reaction mixture was spotted on a 1 × 7-cm strip of polyethyleneimine cellulose (Brinkmann) previously spotted with 1 μ l of 10 mM hypoxanthine plus 10 mM IMP in 0.1 N HCl. The hypoxanthine and IMP were separated by ascending chromatography in *n*-butanol; acetic acid: H₂O (60:15:25). The chromatograms were then dried and the Hyp and IMP (*R_F* 0.5 and 0, respectively) were located under an UV light, cut out, and put into scintillation vials containing 0.3 ml of 0.9% NaCl. After a wait of 5 min to elute the IMP, 5 ml of Patterson-Greene scintillation fluid (2 liters of toluene, 1 liter of Triton X-100, 200 mg of POPOP, 8 g of PPO) was added and mixed well, and the vials were counted in a scintillation counter. The reaction mixture with no added enzyme gave 0.1% of the radioactivity in the IMP spot. With no 8AG present the diluted wild-type and H29 extracts gave 31% and 5.8% of the radioactivity in the IMP spot. ●—● relative L HPRT activity; ○—○ relative H29 HPRT activity.

with the staggeringly high frequency of 10^{-2} . On the other hand, the distribution of induced reversion frequencies for set II centers around 10^{-7} .

From these observations we conclude that 8AG is not the agent of choice for selecting HPRT⁻ mutants. Not only do a considerable proportion of the 8AG-resistant variants (set I) contain high levels of HPRT activity, but also their extremely high spontaneous reversion frequencies bring into question the genetic origin of such variants. On the other hand, the variants isolated in the presence of both 8AG and 6TG uniformly contain extremely low levels of HPRT activity, and both the forward and back mutation frequencies are much lower. These frequencies are in the range observed for mutagenic events in procaryotic organisms.

An operational definition (i.e., the ability of the revertants to grow in HAT medium) was used to determine the reversion frequencies of the variants in both set I and set II. As expected, set II revertants have high levels of HPRT (15–60% of the parental L cell level as measured *in vitro*). The

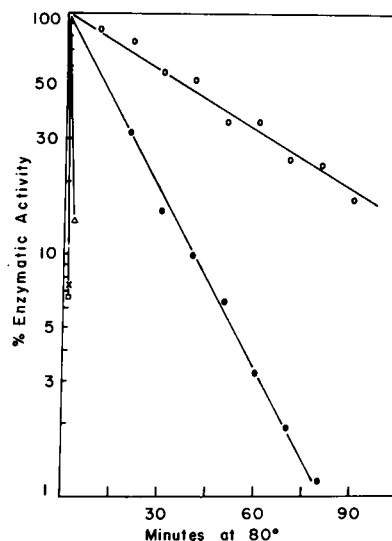


FIG. 4. Heat inactivation at 80° of partially purified HPRT from the parental L cell line and revertants of H355. Extracts were prepared as described in the legend to Fig. 3 and centrifuged at 220,000 × *g* for 60 min. The supernatants (0.5 ml) were mixed with blue dextran 2000 (Pharmacia) and applied to a column (0.8 × 23 cm) of Sephadex G-200 (10-ml bed volume) topped with 1.5 ml of Sephadex G-25. Elution volumes were 4.0, 6.5, and 7.0 ml for the blue dextran, HPRT activity, and bovine hemoglobin (measured by *A*₄₁₀), respectively. The recovery of HPRT activity was virtually quantitative and the specific activity increased about 5-fold. For routine processing of the tissue culture cell extracts, the blue dextran was used as a visual calibration to determine the HPRT-containing fractions. These Sephadex fractions, supplemented with 1 mg/ml of bovine-serum albumin, were distributed into tightly sealed plastic tubes for each time point and heated. After heating, centrifugation at 1500 × *g* for 3 min was sufficient to pellet the precipitated protein. The supernatants were assayed as described in the legend to Fig. 3, except that 50 mM Tris·HCl (pH 7.4), and 0.62 μ M [3 H]hypoxanthine (26.3 Ci/mmol) + 5 μ M unlabelled hypoxanthine were present. Incubation periods were chosen to preserve strict linearity with time. Unheated samples (zero time) had 20–50% of the [3 H]hypoxanthine converted to IMP. All points are HPRT activities relative to zero time. ○, L; ●, H355a; ×, H355b; △, H355c; and □, H355f.

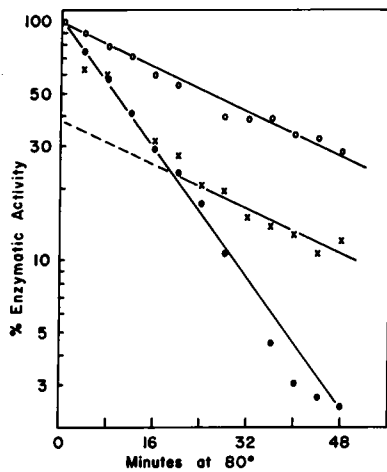


FIG. 5. Heat inactivation at 80° of partially purified HPRT from L, H355a, and the mixture of L + H355a. Procedures are described in the legend to Fig. 4. The mixture contained 39% L and 61% H355a HPRT activity. The broken line shows the curve expected for 39% L activity alone. O, L; ●, H355a; ×, mixture.

majority of set I variants cannot grow in HAT medium even though some contain considerable amounts of HPRT. A few of these variants (such as H29 to be discussed later) grow well in either 8AG or HAT medium and therefore were not included in these studies.

HPRT with altered kinetics

In the process of isolating revertants of clones resistant to 8AG (set I), we discovered several lines which grew well in either HAT medium or medium containing 3 $\mu\text{g}/\text{ml}$ of 8AG. These variant cell lines were found to contain high levels of HPRT activity, as measured both *in vivo* and *in vitro*. One explanation of these properties would be that these variants contain altered HPRT such that the enzyme can distinguish between Hyp and 8AG, and no longer uses the analog as a substrate. To test this possibility, an extract of one such variant, H29, was assayed for HPRT in the presence of increasing amounts of 8AG. The results shown in Fig. 3 indicate a marked reduction in sensitivity of the H29 enzyme to 8AG [measuring inhibition of IMP formation from [^3H]Hyp and phosphoribosyl pyrophosphate (PRibPP)], compared

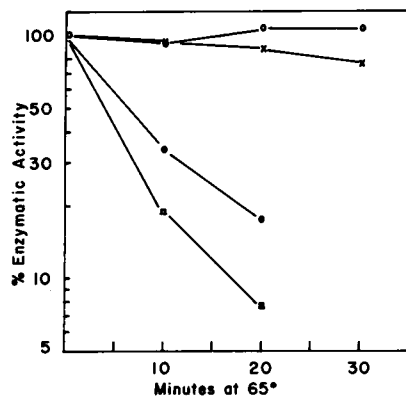


FIG. 6. Heat inactivation at 65° of partially purified HPRT from L and revertants of H355. Procedures are as described in the legend to Fig. 4. O, L; ×, H355a; □, H355b; ●, H355c; △, H355f. The symbols □ and △ are superimposed in the figure.

with enzyme from the parental L cells. The same extracts showed little difference in sensitivity to 6TG, which was not used in the selection of H29. H29 is sensitive to 6TG *in vivo*. This control ruled out possible differences in endogenous Hyp concentrations in the extracts.

As a further test for the existence of an altered purine-binding site in HPRT extracted from H29, the Michaelis constant for hypoxanthine was measured in the presence of excess PRibPP. The K_m values we calculated, using the method of Cleland (13) to evaluate the standard error, were $1.13 \pm 0.15 \mu\text{M}$ and $3.8 \pm 0.4 \mu\text{M}$ for wild-type and H29, respectively. Similar results have been obtained in several independent extracts and Michaelis-Menten kinetics were observed down to $0.02 \mu\text{M}$ [^3H]Hyp, indicating an endogenous Hyp concentration of less than $0.02 \mu\text{M}$.

These two altered properties of HPRT from H29 point to an altered amino-acid sequence, which in turn indicates a mutation in the HPRT structural gene.

HPRT with altered heat stability

Heat inactivation studies were done on HPRT from some of the revertants of set II mutants. The enzymes from one set of six such revertants, derived from a common 8AG-6TG resistant parent, H355, fell into three classes: three were extremely heat sensitive, one was intermediate in response, and two were similar to wild-type enzyme. In Fig. 4 the heat stabilities at 80° of the enzymes from four of the revertants (H355a, b, c, and f) are compared with that of the parental enzyme. HPRT from H355b, c, and f is inactivated extremely rapidly. HPRT from H355a is inactivated about 2.7 times as rapidly as enzyme from wild type.

A mixing experiment demonstrated that the increased sensitivity of H355a was not due to some property of the partially purified enzyme preparation used for the heat inactivation experiment, but was due to a difference in the enzyme itself (see Fig. 5). Before heating, the mixture contained 39% wild-type and 61% H355a HPRT activity. As shown in Fig. 5, the mixed extract gave the expected biphasic curve that extrapolated back to 39%.

In order to examine the enzymes from the three extremely heat-sensitive revertants more closely, we heated enzymes at 65° and compared them with HPRT from wild type and H355a (Fig. 6). Wild-type enzyme was repeatedly observed to be slightly activated by heating at this temperature. The inactivation of HPRT from H355b, H355c, and H355f proceeded at least 15 times as rapidly as that of H355a.

Thus, four of the H355 revertants have HPRT with altered heat stability. Again the simplest hypothesis is that the alteration in heat stability results from amino-acid sequence changes in the revertants.

Implications for somatic cell genetics

Evidence is rapidly accumulating to support the view that at least some of the phenotypic variants isolated from mammalian tissue culture cell lines arise from structural gene mutations. The strongest support for this hypothesis is the evidence that these variants contain altered enzymes. Chinese hamster cell variants that contain altered HPRT (i.e., having immunological crossreactivity but lacking enzymatic activity) (14) and altered RNA polymerase II (i.e., RNA polymerase which is α -amanitin-resistant) (15) have been reported. In this communication we report variants of mouse L cells con-

taining HPRT with altered kinetic constants and heat sensitivities.

We have stressed the importance of the choice of the selective methods for determining the class of variants which one isolates. Thus, 8AG proves to be a poor selective agent if the intent is to isolate HPRT⁻ mutants. Using a tighter selective procedure, 8AG in combination with 6TG, one uniformly obtains HPRT⁻ variants. These latter variants are excellent candidates for structural gene mutants, for not only are the forward and back mutation frequencies much lower, but revertants from this set exhibit altered temperature-sensitivity profiles.

Because 8AG is a less stringent selective agent, there may exist many mechanisms (mutational or nonmutational) whereby the cell can "leak" past the killing effect of 8AG. Some of the variants in set I which contain high levels of HPRT do have altered enzyme (e.g., H29). However, the H29 phenotype is rare. Only 1% of the variants in set I exhibit this phenotype.

One must still consider the problem posed by the ability to isolate recessive phenotypes from mammalian cells, since they are not haploid. A reasonable hypothesis is that in aneuploid lines, and even in "pseudodiploid" lines such as the Chinese hamster cell lines, the number of copies of any given chromosome, as a result of nondisjunction, is fluctuating. Thus, at any given time, a certain population of cells would be going through a haploid state for a given chromosome. This would permit segregation of a recessive genotype. Such a hypothesis would account for the requirement of the long segregation period following mutagenesis in order to isolate variants at a reasonable frequency (8).

Since HPRT may be X-linked in the mouse, we could be dealing with the special case of only a single chromosome being expressed in the cell (16). HPRT is X-linked in man (17) and perhaps in hamster (18). However, one can demonstrate the expression of more than one X chromosome in mammalian cell hybrids (19, 20).

In the introduction we raised the problem posed by Harris' observation that diploid, tetraploid, and octaploid hamster cell lines become resistant to 8AG with similar frequencies (10). Since the time required to select 8AG resistant variants is long, relative to the generation time, it is important to determine the karyotype of the resistant clones. We have already pointed out the difficulty of correlating 8AG resistance with the HPRT⁻ phenotype. Some of the 8AG resistant variants may indeed arise as a result of nonmutational events. It would be interesting to repeat such experiments using a

tighter selective procedure. Finally, we should point out the need to establish the number of copies of the chromosome of interest.

The same type of analysis should be considered in the case of the haploid amphibian cell line which gives rise to BrdU-resistant variants at a frequency similar to that seen in the "pseudodiploid" line, rather than at the square root of that frequency (9). The presence of two copies of the chromosome carrying the thymidine kinase gene as well as the consistent correlation of BrdU resistance with the thymidine kinase-negative phenotype should be established.

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