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The CHO/HGPRT Mutagenicity Assay. II. Genetic Basis of 6-Thioguanine Resistance

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Running head: Genetic Basis of TG^r

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INTRODUCTION

An essential aspect of any attempt to study mutation induction in mammalian cells is the demonstration that genetic alterations are the bases for the altered phenotypes. Evidence consistent with a genetic basis for several altered phenotypes has been reported in many studies of mammalian cell mutagenesis. However, quantitative mutagenesis determinations present a different level of problems in this area. It is not sufficient that some of the variants have properties consistent with a mutation basis; it must also be demonstrated that all cells which give rise to colonies under selective conditions fulfill some accepted set of criteria for a mutation event.

We have defined reproducible conditions for the selection of 6-thioguanine-resistant (TG^r) variants of Chinese hamster ovary (CHO) cells. It is known that many such variants contain altered forms of the enzyme hypoxanthine (Hx)–guanine phosphoribosyl transferase (HGPRT). Our question was whether every variant colony which develops under our conditions has properties consistent with a genetic alteration at the HGPRT locus. We attempted to consider all other possible mechanisms for the TG resistance such as:

a) colony formation by wild-type cells which have escaped the toxic effects of TG as a result of the death of cells and release of purine bases and metabolites into the medium and/or as the result of depletion of medium TG due to inherent decay and cellular metabolism leading to a loss of selection stringency;

b) phenotypic resistance due to some transient, adaptive response in cellular physiology, such as increased rates of purine biosynthesis;

c) stable resistance as a result of mutations at other loci, such as alterations in TG uptake or alterations which affect intracellular levels of purine nucleotides or PRPP.

EXPERIMENTAL PROCEDURES

The cells, culture conditions, and mutant selection procedures were described earlier in this workshop.

The characterization of mutation clones has been described previously (O'Neill et al. 1977). Experimental details of the fluctuation analyses will be published elsewhere (J. P. O'Neill et al., submitted for publication).

RESULTS AND DISCUSSION

Table 1 summarizes the evidence for a genetic basis for the TG^r phenotype isolated under our selective conditions. (T-1) The spontaneous frequency has ranged between 0 and 10 in approximately 95% of 500 determinations over the past 3 years. Detailed fluctuation analyses have demonstrated the random nature of the appearance of the TG^r phenotype and yielded an estimation of the mutation rate. These studies will be described in more detail below. The mutation frequency is increased by various physical and chemical agents in these quasi-diploid cells, but there is no induction in quasi-tetraploid cells (Chasin 1973; Hsie et al. 1977). More than 200 mutant clones induced by different agents have been isolated, and the TG^r phenotype is stable after growth in non-selective medium for more than 100 generations. The spontaneous reversion measured by growth in aminopterin (AP) is less than 10^{-7} reversions/cell.

Isolated TG^r mutant clones have been characterized by four different methods. The first is based on the AP sensitivity expected of HGPRT-deficient cells. Most isolated TG^r clones are AP sensitive and retain this phenotype also after growth in non-selective medium. The disadvantage of this method is that it does not differentiate between $HGPRT^-$ mutants and Hx/TG uptake mutants. Furthermore, it might be expected that some $HGPRT^-$ mutants would also be AP positive, if the mutant enzyme activities had certain types of kinetic alterations. The second and third methods of characterization involve two kinds of studies utilizing Hx uptake and incorporation into cellular macromolecules as a test for HGPRT activity (O'Neill et al. 1977): (a) an autoradiographic procedure which also has the disadvantage of not easily differentiating uptake mutants from $HGPRT^-$ mutants; (b) a method utilizing mass cultures of isolated mutant clones which allows measurement of both soluble and incorporated Hx. This has proven to be a sensitive test for $HGPRT^-$ mutants since it is an assay utilizing growing cells. The fourth characterization method is the measurement of HGPRT activity in cell-free extracts. We measure the conversion of [^{3}H] Hx to inosine monophosphate, which is measured by a DEAE filter method. As summarized in Table 2, 97–99% of the mutants isolated by our selection method have characteristics which are consistent with mutation at the HGPRT locus, as defined by these four analyses. These mutants were isolated after treatment with a variety of mutagenic agents, including alkylating agents, ICR compounds, and UV.

T-2

Let's now return to a point mentioned earlier — the determination of the spontaneous mutation rate. We initiated fluctuation analysis studies when we were reasonably certain that our TG^r clones fulfilled several criteria for a genetic basis for the phenotype. We wanted (a) to determine whether the TG^r colonies arise randomly in a population rather

than as a result of the selective pressure, and, if this appears to be the case, (b) to determine the rate of appearance. The problem of phenotypic expression was particularly important in this context. Although we have studied this phenomenon primarily with mutagenized cultures, we assumed that a spontaneous event would also require an expression period due to phenotypic delay. What this would mean is that any spontaneous event which occurs during the last 6–8 cell generations prior to selection of the culture would not be expressed, and the mutant cell could not give rise to a colony in TG medium. Keeping this in mind, we started with 50 replicate cultures of 50–200 cells and grew them to a density of 5–6 x 10^6 cells. Aliquots of 1–2 x 10^6 cells from each replicate culture were plated for selection, but essentially no TG^r colonies were found. For this reason, subsequent experiments were performed so that an expression time was allowed. The results are summarized in Table 3. In the first column are the results of experiments in which multiple samples from a single culture were plated for selection. The variance/mean ratio is close to 1, as expected if there were no significant error in the methodology other than sampling error. In the second column are the results obtained with multiple replicate cultures in which phenotypic expression was accomplished through subculture of 10^6 cells from each replicate for 7 days. In the third column are the results obtained with cultures in which expression was accomplished by maintaining the cells in an arrested state (O'Neill and Hsie 1979). In both cases, the variance/mean ratio is higher than would be predicted by simple sampling error. These results are consistent with the conclusion that the TG^r phenotype arises as a random event and are also consistent with a genetic basis for the phenotype. In addition, the data in Table 3 show that the experimental S.D./mean ratio is greater than that calculated from the mutation rate determinations; that is, the random

T-3

nature of the appearance of the TG^r phenotype is greater than might be expected from the mutation rate. This is usually ascribed to mutations which arose early during the growth of the cells.

Our conclusion from these studies is that the TG^r phenotype which is quantified by use of the CHO/HGPRT system does represent genetic alterations, and that we are performing quantitative mutagenesis determinations with this system.

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TABLE 1

Evidence for the Genetic Basis of TG Resistance in the CHO/HGPRT System

A) Spontaneous mutation frequency in the range of 0–10 mutants/10^6 clonable cells.

B) Spontaneous mutation rate in the range of 6–38 x 10^{-8} mutations/cell/generation.

C) Mutation frequency increased by mutagenic agents in a concentration-dependent manner as expected for a gene located on the functionally monosomic X chromosome.

D) No induction of mutants in near-tetraploid cells as expected for a recessive phenotype.

E) Isolated mutant clones show phenotype stability after growth under non-selective conditions (greater than 100 generations).

F) Spontaneous reversion frequencies of isolated mutants are less than 10^{-7} reversions/cell.

G) Altered HGPRT activity in greater than 97% of the TG^r mutants, as measured by four methods.

TABLE 2

Altered HGPRT Enzyme Activity in TG^r Mutants[a]

A)	AP sensitivity of mutant colonies (1170/1189 = 98.4%).
B)	Reduction of Hx incorporation into macromolecules in mutant colonies by autoradiography studies (121/122 = 99.2%).
C)	Reduction of Hx incorporation into macromolecules in isolated mutant clones (63/65 = 96.9%).
D)	Reduced or altered HGPRT enzyme activity in cell-free extracts of isolated mutant clones (96/98 = 97.9%).

[a]The data are summarized from Hsie et al. 1975; O'Neill et al. 1977; Riddle and Hsie 1978; and J. P. O'Neill et al., unpublished.

TABLE 3

Fluctuation Analysis of TG Resistance in CHO Cells

	Replicate sample (control)	Multiple cultures	
		Subculture expression	Arrest expression
No. of experiments	3	2	2
No. of cultures	1, 1, 1	50, 50	48, 44
Mean/10^6 clonable cells	0.53, 3.9, 2.6	2.9, 3.2	3.8, 6.3
Range	0-2, 1-7, 0-6	0-32, 0-33	0-35, 0-45
Variance/mean	1.0, 0.6, 0.9	10.1, 11.9	13.9, 15.6
Mutation rate (mutations/cell/ generation x 10^{-8})			
F_o calculation	—	10.1, 9.6	8.2, 6.6
Mean calculation	—	32.3, 33.7	27.6, 38.8
S.D./mean			
Experimental	—	1.8, 1.9	1.9, 1.6
Calculated	—	1.6, 1.6	1.5, 1.2