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Mutation at Autosomal Loci of Chinese Hamster Ovary Cells: Involvement of a High-Frequency Event Silencing Two Linked Alleles

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Two classes of cell lines heterozygous at the galactokinase (*glk*) locus have been isolated from Chinese hamster ovary cells. Class I, selected by plating nonmutagenized wild-type cells at low density in medium containing 2-deoxygalactose at a partially selective concentration, underwent subsequent mutation to the $glk^{-/-}$ genotype at a low frequency (approximately 10^{-6} per cell), which was increased by mutagenesis. Class II heterozygotes, isolated by sib selection from mutagenized wild-type cells, had a higher spontaneous frequency of mutation to the homozygous state ($\sim 10^{-4}$ per cell), which was not affected by mutagenesis. About half of the $glk^{-/-}$ mutants derived from a class II heterozygote, but not the heterozygote itself, were functionally hemizygous at the syntenic thymidine kinase (*tk*) locus. Similarly, a $tk^{+/-}$ heterozygote with characteristics analogous to the class II $glk^{+/-}$ cell lines underwent high-frequency mutation to $tk^{-/-}$, and most of these mutants, but not the $tk^{+/-}$ heterozygote, were functionally hemizygous at the *glk* locus. A model is proposed, similar to that for the mutational events at the adenine phosphoribosyl transferase locus (W. E. C. Bradley and D. Letovanec, Somatic Cell Genet. 8:51–66, 1982), of two different events, high and low frequency, being responsible for mutation at either of the linked loci *tk* and *glk*. The low-frequency event may be a point mutation, but the high-frequency event, in many instances, involves coordinated inactivation of a portion of a chromosome carrying the two linked alleles. Class II heterozygotes would be generated as a result of a low-frequency event at one allele, and class I heterozygotes would be generated by a high-frequency event. Supporting this model was the demonstration that all class I $glk^{+/-}$ lines examined were functionally hemizygous at *tk*.

The isolation and characterization of drug-resistant mutants of cultured somatic cells has proceeded rapidly in the last decade (for a review, see reference 22). The bulk of this work has been done with genetic systems in which the resistance is acquired in a single step, such as mutation to a dominant phenotype or mutation at a locus comprising only one functional allele (18). Recessive mutation at diploid loci is more difficult to study, but the process can be analyzed by isolating heterozygous mutants in partially selective medium, taking advantage of the fact that in these cells the affected gene product is frequently expressed at about 50% of the wild-type (W.T.) level. The second step of mutation to full drug resistance can then be easily studied.

With this approach, mutation at the adenine phosphoribosyl transferase locus (*aprt*) in the Chinese hamster ovary (CHO) cell line has been shown to occur in two steps (11), the second step occurring at a spontaneous rate of about 3×10^{-7} per cell per generation and increasing ~100-fold after mutagenesis. In recent work from this laboratory, a second type of *aprt* heterozygote has been isolated, capable of mutation to full drug resistance at high frequency (HF), independent of mutagenesis (5). These results led to the proposal that W.T. CHO (+/+) may become mutant at the *aprt* locus ($aprt^{-/-}$) in two steps, one a low-frequency (LF) event whose rate is greatly increased by mutagenesis and the other an HF event, possibly gene inactivation, with the interesting property of occurring nonrandomly among members of the population placed at risk. Either the HF or LF event could occur first, yielding the two types of heterozygotes, which have been called class I and class II, respectively. Mutants have not been seen to arise from two successive HF events. This model has recently been substantiated by biochemical studies (19) which showed that the product of a gene mutated by the LF

event was frequently a physically altered protein but that the HF event never resulted in detectable gene products being made.

Isolation of class II *aprt*$^{+/-}$ cells involved mutagenesis of the parental population to enrich for cells made heterozygous by the LF event, followed by sib selection (16), a procedure which permits isolation of mutants without exposure to the selective medium. The rationale for the latter was to ensure that the heterozygotes had not undergone any form of adaptation which might have affected their subsequent behavior in higher drug concentrations. We utilized a similar protocol to isolate analogous cell lines heterozygous at two linked loci, the galactokinase (GLK) locus (*glk*) and the thymidine kinase (TK) locus (*tk*). Comparison of these cell lines with class I heterozygotes has demonstrated that the general model proposed for mutation at the *aprt* locus seems to hold for both *tk* and *glk*. Furthermore, it was shown that the HF event usually involves a coordinated inactivation of a *tk* and a *glk* allele. This inactivation phenomenon, named allelic silence, was first reported in *ts*201, a *tk*$^{+/-}$ cell line derived from CHO (3), and has also been reported at the pair of syntenic loci specifying emetine resistance (*emt*^{r}) and chromate resistance (*chr*^{r}) in CHO (6).

MATERIALS AND METHODS

Cell lines. The cell lines used were derived from the Toronto strain of W.T. CHO, obtained from C. Stanners and M. Meuth. B211 and D41621 are cell lines W.T. for *tk* and *glk* but containing an altered ribonucleotide reductase, rendering the cells partially resistant to 5-bromodeoxyuridine (BrdU). Cell line 21BU1 is a derivative of B211 that is heterozygous at the *tk* locus, which was isolated by sib selection. D41621 is descended from D416, which is heterozygous at the *aprt* locus (5). The isolation of each of these lines has been described previously (4). All cell lines were grown at 37°C in plastic tissue culture dishes in α medium without DNA and RNA precursors (Flow Laboratories), supplemented with 5% fetal calf serum (Flow) and antibiotics. Periodic tests by autoradiography after [^{3}H]thymidine incorporation indicated that all cell lines were mycoplasma-free.

Mutant isolation. Cells were mutagenized by growing in α medium containing 150 μg of ethyl methane sulfonate (EMS) per ml for 24 h. This treatment results in about 50% killing and induces mutations at the hypoxanthine phosphoribosyl transferase locus (*hprt*) at a frequency of 10^{-4} (5). The cells were then cultured in α medium for 4 days to permit establishment of the phenotype before exposure to the selective medium. Cell lines partially deficient in GLK (putative *glk*$^{+/-}$ heterozygotes) were isolated in 12 mM 2-deoxygalactose (2-dgal), whereas cells with complete deficiency of GLK were isolated in 48 or 72 mM 2-dgal. At 48 mM, small surviving colonies were usually seen at frequencies 10- to 100-fold greater than the frequency of large, healthy colonies. Only the latter were picked and characterized. Selection for partially and fully TK-deficient mutants was performed in 0.10 and 0.60 mM BrdU, respectively. For isolation of partially resistant mutants it was found to be necessary to seed cells at densities of $\leq 2 \times 10^3$ per cm^2. In other selections, densities up to 6×10^3 cells per cm^2 were used. EMS, 2-dgal, and BrdU were all obtained from Sigma Chemical Co.

The isolation of mutants without exposure of the cells to selective medium was accomplished by sib selection (16). The rationale and procedures are explained in detail elsewhere (4).

Enzymology. For the TK and GLK enzyme assays, cell extracts were prepared as described previously (3). The enzymes were also assayed as described previously (3) except that separation of the phosphorylated product from unreacted substrate was accomplished by using DE81 paper (4). Adenine phosphoribosyl transferase was assayed on cell extracts as described by Jones and Sargent (11).

Karyotype. Metaphase spreads of cells arrested in mitosis by colchicine were prepared as previously described (3).

RESULTS

Isolation of class I and class II *glk* heterozygotes. The purpose of these experiments was to determine whether two different mutational events (HF and LF) might be involved in acquisition of the fully deficient phenotype at the *glk* and *tk* loci. The approach taken was to isolate heterozygous cell lines by the two methods outlined above; those isolated directly from nonmutagenized W.T. populations are referred to here as class I, and those isolated from mutagenized populations by sib selection are referred to as class II. When nonmutagenized W.T. populations were seeded in 12 mM 2-dgal, colonies appeared at a frequency of about 10^{-5}. When the plating density was sufficiently low ($\leq 2 \times 10^3$ per cm^2), these colonies, when allowed to develop for 2 weeks, formed healthy, tightly packed centers in a nearly confluent background of dying cells. Several of these colonies were picked and characterized (Table 1). The GLK specific activity was between 40 and 60% of W.T., and the killing curve in 2-dgal was that expected for a heterozygote (data for one cell line, 5SDG, is shown in Fig. 1), namely, increased resistance relative to W.T. but nevertheless with a steep drop in viability at 2-dgal concentrations higher than the D_{10}. These cell lines gave rise to colonies in 72 mM 2-dgal at about 10^{-6} per cell, a frequency increased to 0.3×10^{-4} to 1.0×10^{-4} per cell after mutagenesis. The fully resistant colonies were stable and deficient in GLK. The colonies of intermediate resistance were considered to be *glk*$^{+/-}$ heterozygotes, analogous to the *aprt*$^{+/-}$ cells isolated by Jones and Sargent (11) and Thompson et al. (20) and the class I heterozygotes of Bradley and Letovanec (5).

TABLE 1. Properties of 2-dgal-resistant mutants

Cell line[a]	Isolation condition[b]		GLK sp act[c]	Plating efficiency in 2-dgal (12 mM)	Mutation frequency[d] to GLK$^-$	
	Mutagenesis of parental line	Direct or sib selection			Spontaneous	EMS induced
CHO			1.00	1×10^{-3}	5×10^{-7}	5×10^{-7}
Class I						
5SDG	None	Direct	0.43	0.63	8×10^{-7}	3×10^{-5}
G754	None	Direct	0.59	ND[e]	ND	1.1×10^{-4}
G758	None	Direct	0.51	ND	ND	5×10^{-5}
G759	None	Direct	0.54	ND	ND	3×10^{-5}
Class II						
D2171	EMS	Sib	0.50	0.9	2×10^{-4}	2×10^{-4}
705	EMS	Sib	0.50	0.8	1.5×10^{-4}	ND

[a] G754, G758, G759, and 705 were derived from W. T. CHO cells; 5SDG and D2171 were derived from D41621.

[b] Isolation conditions are described in the text. The selective medium was 12 mM 2-dgal. Isolation was by sib selection (see footnote *a*, Table 2) or by conventional direct picking of colonies which grew up in the selective medium.

[c] Expressed relative to W.T. W.T. specific activity is 1.57 nmol/min per mg of protein.

[d] Determined by plating cells, mutagenized or not in 72 mM 2-dgal.

[e] ND, Not determined.

Following the approach taken previously for the *aprt* locus, putative class II *glk* heterozygotes were isolated from mutagenized W.T. populations by sib selection (Table 2). Two cell lines, 705 and D2171, were isolated which had GLK specific activities (Table 1) and 2-dgal resistance (Fig. 1) characteristic of heterozygotes. D2171 was chosen for further study.

Reconstruction experiments. To determine whether the sib selection protocol had been necessary to avoid adaptation of the class II heterozygote to the partially selective medium, the conditions of a conventional selection procedure were reproduced. D2171 was seeded in medium containing 12 or 30 mM 2-dgal at about 200 cells per 20-cm^2 dish. In some experiments, 3×10^4 W.T. CHO cells were seeded with the D2171 cells, but it was subsequently determined that their presence had no effect on the ultimate phenotype of the cells being tested. The colonies

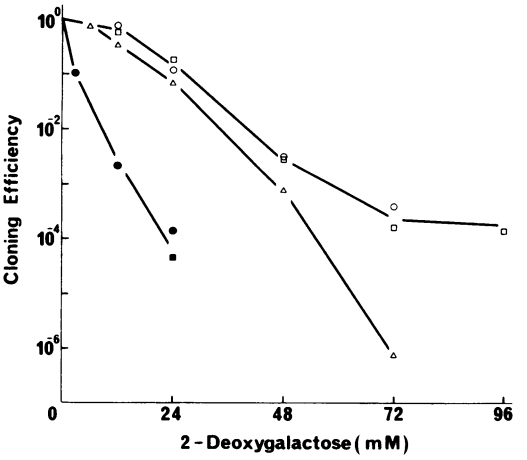

FIG. 1. Cloning efficiencies of W.T. and *glk* heterozygous cell lines in different concentrations of 2-dgal. Symbols: ●, D41621; ■, CHO; ○, D2171; □, 705; △, 5SDG.

TABLE 2. Sib selection of the *glk*$^{+/-}$ cell line D2171[a]

Enrichment round	No. of cells seeded	No. of populations	No. of populations with 2-dgal^r colonies
1	1.2×10^5	24	2
2	2.5×10^4	48	3
3	1,000	24	2
4	88	88	3

[a] A culture of D41621 cells was mutagenized, and the indicated number of viable cells was distributed into 24 2-ml-capacity Linbro wells and grown in α medium for 5 to 8 generations. Each population was trypsinized; 10% of each was placed in one 10-cm petri dish with 12 mM 2-dgal and most of the rest was kept in nonselective medium. Seven days later, 2-dgal-resistant colonies were visible in several dishes, and the population yielding the most vigorously growing colonies was chosen for the next round of enrichment. The cells of that population which had been maintained in α medium were trypsinized and counted, and the indicated number were distributed into 48 2-ml-capacity Linbro wells in α medium. The enrichment process was repeated until the fourth round when 88 colonies were picked from a dish of cells seeded very sparsely in α medium. These colonies were tested individually for 2-dgal resistance, and one of the resistant colonies was recloned in α medium and named D2171.

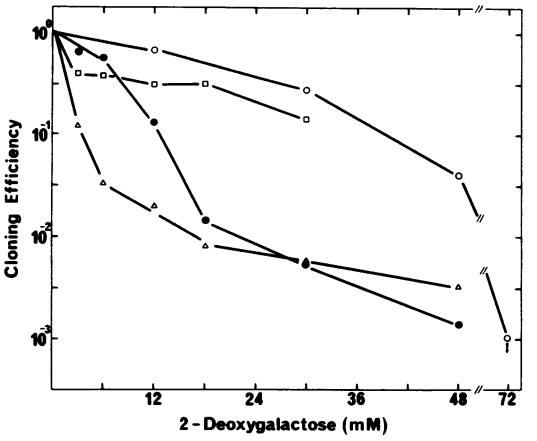

FIG. 2. Cloning efficiencies in 2-dgal of D2171 and three colonies picked from dishes containing either 12 or 30 mM 2-dgal. Symbols: ●, D2171; ○, W2c1; □, S5c1; △, W5c2. In all experiments such as this, the colonies scored as resistant were usually present in a background of many smaller colonies of sick cells. There was, therefore, some subjectivity in assessing cloning efficiencies, and this explains the variation in killing curve of D2171 from one experiment to another (compare Fig. 1).

formed after 10 days were of various sizes, with those in 30 mM 2-dgal being generally much smaller than those in 12 mM 2-dgal. Several colonies representing different size classes were picked and tested for colony-forming ability in 2-dgal and for GLK levels. As shown in Fig. 2 and Table 3, a wide range of 2-dgal resistance was exhibited, from S5C2, which was virtually indistinguishable from a $glk^{-/-}$ mutant, to W5C2, which appeared even less resistant to 2-dgal than the parent. The reason for the small decrease in resistance as measured by the killing curve is unknown, but the phenomenon was frequently observed and seems to have been seen by others who have isolated 2-dgal-resistant CHO cells (21). Table 3 shows that a general inverse correlation existed between 2-dgal resistance and relative GLK specific activity, which was also observed by Whitfield et al. (21). It therefore appears that growth in 2-dgal results in selection for cells better adapted to growth in the drug, having undergone some secondary event which reduces GLK specific activity below the 50% of W.T. characteristic of heterozygosity, in some cases to 0. This change is hereditary since when subclones of the cell line isolated in the reconstruction experiment were isolated in nonselective medium, they exhibited a phenotype similar to the parental clone (data not presented).

Mutation to full 2-dgal resistance. To determine whether mutation to the $glk^{-/-}$ genotype occurred at a high rate as was seen with the class II $aprt^{+/-}$ lines described earlier (5), fluctuation tests were performed on D2171 and, for comparison, on the class I line, 5SDG. In these experiments the selective medium was 72 mM 2-dgal to allow distinction between spontaneous *glk* mutants and the cells adapting to the intermediate drug concentrations of the above experiment. The results (Table 4) show a difference between the two lines of about 30-fold in the mutation rates to the $glk^{-/-}$ phenotype. A further difference between these two lines was the effect of mutagenesis on the incidence of GLK^- mutants in the heterozygous population (Table 1). No increase in the mutation frequency to 72 mM 2-dgal resistance was seen after treatment of D2171 with EMS, whereas the same treatment of 5SDG increased the mutant frequency 30-fold.

Simultaneous silencing of *glk* and *tk* alleles. Previous work in this laboratory had shown that in one cell line (*ts*201, reference 3) an HF event resulted in coordinated inactivation of syntenic *tk* and *glk* alleles. To determine whether the HF mutation events resulting in high-level 2-dgal resistance shared this property, 11 resistant colonies were picked from among eight separate replicate cultures of the fluctuation test. Each was tested for functional hemizygosity at the *tk* locus (the test locus) by determining the specific activity of TK and the frequency of EMS-induced mutation to resistance to a high concentration of BrdU. The results are shown in Table 5. D2171 has the same TK specific activity as its parent and a mutation frequency to 0.6 mM BrdU resistance of 5×10^{-7} after mutagenesis. Five of the 11 2-dgal-resistant mutants (group A,

TABLE 3. Resistance and GLK specific activities in subclones of D2171 picked from plates containing 2-dgal[a]

Subclone	2-dgal concn of reconstruction experiment (mM)	Plating efficiency in 2-dgal		Relative GLK sp act (relative to W.T.)
		12 mM	30 mM	
W.T.				1.0
D2171		0.13	0.005	0.5
W2c1	12	0.67	0.28	0.12
W5c1	30	0.026	$<10^{-3}$	0.51
W5c2	30	0.02	0.006	0.52
W2c2	12	ND[b]	0.31	0.02
S2c2	12	ND	0.91	0.18
S5c1	30	0.31	0.14	0.21
S5c2	30	ND	1.01	<0.01

[a] D2171 cells were seeded at low density in several dishes containing either 12 or 30 mM 2-dgal. After 10 days, several colonies were picked, grown in nonselective medium, and tested for plating efficiency in 2-dgal and GLK specific activity.

[b] ND, Not determined.

TABLE 4. Fluctuation analysis of mutation rate to resistance to 72 mM 2-dgal

Cell line	No. of replicate cultures	No. of cells per culture		No. of cultures with N colonies where N is:					Range	Mean	Mutation rate[a]	Ratio: variance/mean
		Initial	Final	0	1–2	3–4	5–8	9–16	16–32				
5SDG	20	100	7.5×10^4	19	1	0	0	0	0	0–2	0.1	4×10^{-7}	2.1
D2171	15	100	7.5×10^4	4	2	5	0	3	1	0–19	5.1	1.2×10^{-5}	7.2

[a] Calculated by the Po method (13).

Table 5) had TK specific activities close to that of the $tk^{+/+}$ parent, D2171, and for most of these the mutation frequency to 0.6 mM BrdU resistance was $<2 \times 10^{-6}$. The sole exception was G2.1, whose induced frequency was 9×10^{-6}. These group A mutants seem to have arisen by a mutation affecting *glk* but not *tk*. Group B (Table 5) had characteristics suggesting functional hemizygosity at the test locus. The TK specific activity was at a level of 40 to 50% of the W.T., and for most of the mutants the EMS-induced mutation frequency of the TK^- phenotype was about 10^{-4}, close to the observed mutation frequency at the hemizygous *hprt* locus and about 200-fold higher than the parental frequency.

It therefore seems that the event responsible for mutating the *glk* allele in the cell lines of group B also rendered the cell functionally hemizygous for *tk*. The explanation we favor is that gene inactivation had occurred over a region of the chromosome which carried both the W.T *glk* allele and the linked *tk* allele. The somewhat lower than expected mutation frequency ob-

TABLE 5. Tests for functional hemizygosity at the *tk* locus of 2-dgal-resistant cell lines[a]

Cell genotype	Cell line	Enzyme sp act (relative to W.T.)		EMS-induced mutation frequency to TK^-
		GLK	TK	
$glk^{+/-}$ (class II)	D2171	0.50	1.00	5×10^{-7}
$glk^{-/-}$ Group A	G2-1	<0.03	0.91	9×10^{-6}
	G6-2	0.10	1.0	$<2 \times 10^{-6}$
	G12-2	<0.03	0.83	$<1 \times 10^{-6}$
	G14-1	<0.02	0.99	$<1 \times 10^{-6}$
	G16-2	<0.02	1.12	$<1 \times 10^{-6}$
Group B	G4-1	<0.02	0.51	1.6×10^{-4}
	G6-1	<0.02	0.50	7×10^{-5}
	G9-1	<0.02	0.40	1.0×10^{-4}
	G10-1	0.12	0.43	1.1×10^{-5}
	G12-1	0.05	0.41	1.1×10^{-4}
	G16-1	0.10	0.58	1.2×10^{-5}
$glk^{+/-}$ (class I)	5SDG	0.43	0.53	3×10^{-5}
	G754	0.59	0.31	7.5×10^{-5}
	G758	0.51	0.31	7×10^{-5}
	G759	0.54	0.43	1.0×10^{-5}

[a] From eight different replicate cultures of the fluctuation test of D2171 described in Table 4, a total of 11 $glk^{-/-}$ colonies were picked and grown up in 72 mM 2-dgal. Mutants G6-1 and G6-2 were from the same population, as were G12-1 and G12-2 and G16-1 and G16-2. Four independent class I heterozygotes were also tested; 5SDG was derived from D41621 and the rest from CHO pro^-.

served in two mutants in this group, G10-1 and G16-1, may reflect incomplete inactivation of the affected chromatin. Consistent with this is the relatively high level (~10% of W.T.) of GLK remaining in the mutants, suggesting that the inactivation may have been only 80% complete. If the TK expression by the linked allele was at a similar level, the cell line would not behave as truly hemizygous. In one of the two lines (G16-1) this appeared to be the case, since the TK activity was about 10% above the 50% expected of a functionally hemizygous cell. The other line, with 43% of W.T. TK, still may have had partial expression of the inactivated test allele, since the enzyme specific activity of heterozygous cell lines is known to be as low as 30% of W.T. (5, 11).

The class I heterozygotes were also tested for hemizogysity for the *tk* locus (data for four lines shown in Table 5). The TK specific activities were between 30 and 50% of the W.T., and the EMS-induced mutation frequencies to full BrdU resistance ranged from 1×10^{-5} to 7.5×10^{-5}, which, although slightly lower than expected for a hemizygous locus, were very close to the respective frequencies to 72 mM 2-dgal resistance (Table 1). On the other hand, the derivatives of these lines which were resistant to 72 mM 2-dgal all had TK levels unchanged from $glk^{+/-}$ parents and were still functionally hemizygous for *tk* (data not presented). The event leading to the $glk^{+/-}$ genotype of these mutants, therefore, seems to be the same as that leading to the high frequency events yielding the cell lines of group B, Table 5, namely, the simultaneous inactivation of both a *glk* allele and a *tk* allele. On the other hand, the second step resembles the LF event described earlier, with respect to both frequency and the lack of inactivation at the test locus.

If the interpretation offered above is correct, then it should be possible to detect a similar coordinated inactivation of *tk* and *glk* alleles when selecting for mutants at the *tk* locus. To test this, we used 21BU1, a $glk^{+/+}$ $tk^{+/-}$ cell line isolated by sib selection from a mutagenized population of cells W.T. at the *tk* locus but with an altered ribonucleotide reductase (4). This line was a class II heterozygote, since the mutation rate was 8×10^{-6} as compared to 8×10^{-7} for 5SDG (data not presented), which is considered a class I $tk^{+/-}$ in this context. Five independent derivatives, all resistant to 0.6 mM BrdU but with various growth rates in this medium, were picked and characterized. Two, B8-1 and B9-2 (Table 6), clearly appeared hemizygous at the test locus (*glk*), with GLK levels of 40 to 50% of W.T. and EMS-induced mutation frequencies to 48 mM 2-dgal resistance of about 10^{-4}. B1-2 and B3-4, two other mutants which grew somewhat slower in 0.6 mM BrdU, exhibited reduced levels of GLK and mutation frequencies at the test locus intermediate between the parental 21BU1 (6×10^{-7}, Table 6) and the frequency expected for a single event. These cell lines may be similar to G10-1 and G16-1, having experienced only partial inactivation since the TK and GLK specific activities are in both cases significantly above 0 and 50%, respectively. For one of the five mutants, B10-1, the two criteria are not completely in agreement, since the enzyme activity is only slightly reduced (89% of W.T.), but the mutation frequency is 13-fold higher than that of W.T. However, there is good overall agreement between enzyme levels and mutation frequencies, and perfect concordance cannot always be expected given the statistical nature of mutation.

To verify that enzyme specific activities did not vary randomly in these cell lines, two mutants from group B, Table 5 (G4-1 and G12-1) and mutants B8-1 and B9-2 (Table 6) were tested for adenine phosphoribosyl transferase specific activity. The enzyme levels were within 10% of the parental value (data not presented).

Partial coordinated inactivation of *tk* and *glk*. As mentioned above, the behavior of some mutants (G10-1 and G16-1, B1-2 and B3-4) suggested that the affected *tk* and *glk* alleles had been only partially inactivated. If incomplete inactivation is a frequent outcome of this mutational event, then the phenotypes of the D2171 subclones picked from dishes containing the partially selective media of the reconstruction experiment (Fig. 2, Table 2) could have resulted from such incomplete inactivation of the W.T.

TABLE 6. Tests for functional hemizygosity at the *glk* locus of BrdU-resistant cell lines[a]

Cell genotype	Cell line	Enzyme sp act (relative to W.T.)		EMS-induced mutation frequency to GLK^-
		TK	GLK	
$tk^{+/-}$ (class II)	21BU1	0.50	1.0	6×10^{-7}
$tk^{-/-}$ [b]	B1-2	0.18	0.61	3×10^{-5}
	B3-4	0.25	0.71	2×10^{-6}
	B8-1	<0.03	0.49	1.3×10^{-4}
	B9-2	<0.01	0.42	1.6×10^{-4}
	B10-1	0.09	0.89	8×10^{-6}

[a] Five independent spontaneous mutants picked from dishes containing 0.6 mM BrdU were grown up in 0.6 mM BrdU and tested in a manner analogous to that described in the footnote to Table 5.

[b] The genotype $tk^{-/-}$ is assigned to these cell lines to distinguish them from the heterozygous lines. It is possible that at least some are not $tk^{-/-}$ in the conventional sense.

glk allele, and if so, an equivalent inactivation would be detectable at the *tk* locus. Several of these subclones were therefore tested, and in all cases a reduction in the TK specific activity was observed which was close to the reduction in the GLK activity. The results are presented in Fig. 3A (open circles), showing that TK reduction plotted against GLK reduction gives a line with a slope of about 1. The results strongly suggest that in each case the adaptation to partially selective medium occurred by coordinated, but partial, inactivation of the two alleles. It is noteworthy that the plating efficiencies of D2171 in the media of the reconstruction experiment were 1 to 20%, so the events resulting in incomplete inactivation can apparently occur at a very high frequency. An accurate estimate of this frequency is difficult to make since the event may only occur at some time after seeding in the partially selective medium. Thus, only one cell in a slowly growing colony would suffer the inactivation and would subsequently overgrow that colony due to its competitive advantage.

Also shown in Fig. 3A (closed circles) are the points representing fully 2-dgal-resistant mutants of groups A and B (clustered in the lower right and upper right of the figure, respectively). In Fig. 3B are shown similar data for the derivatives of 21BU1 selected in 0.6 mM BrdU, again showing a distribution of points along a line with a slope of about 1. The sole exception in this case is the point representing B10-1, which may fall into the same category as the group A mutants of D2171.

Effect of azaC on reversion of TK$^-$ mutants. The cytidine analog 5-azacytidine (azaC) is known to induce hypomethylation of DNA in mammalian cells (12) and has been used to investigate the role of methylation of cytidine residues in inactivation of X chromosomes (14), the integrated herpes *tk* gene (7), and cellular *tk* genes in V79 cells (9). When two TK$^-$ cell lines described in this work were grown in the presence of various concentrations of the drug for 1 day, one reproducibly yielded HAT-resistant revertants at frequencies above background (G9-1B, Table 7), although the maximum frequency, at about 10 to 20 mM azaC, was less than 10^{-4}. The second cell line, B8-1, did not revert under the conditions used. These data suggest that methylation may be involved in inactivation, at least in G9-1B. As discussed below, the fact that the other cell line did not revert under the conditions used does not necessarily rule out methylation as the general mechanism.

DISCUSSION

A model has recently been proposed (5, 19) suggesting that two different mutational events occur at the autosomal diploid *aprt* locus to yield the fully drug-resistant phenotype. In this paper we show that a similar model can serve to describe events at two other autosomal genes in CHO, the synthetic *tk* and *glk* loci. The model is shown in Fig. 4. The first mutational event, yielding heterozygosity, can occur by either LF, mutagenesis-sensitive event or by an HF event.

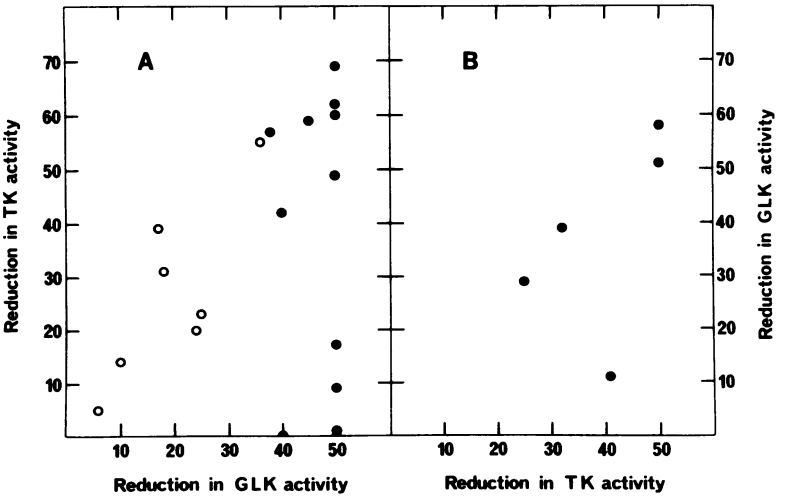

FIG. 3. Relationship of the reductions in specific activities of TK and GLK in various derivatives of D2171 ($glk^{+/-}$) and 21BUI ($tk^{+/-}$) isolated in selective media. Units were chosen such that for each enzyme 1 U = 1% of the specific activity of W.T. ($glk^{+/+}$ $tk^{+/+}$) cell lines. (A) D2171 seeded in 2-dgal either partially selective (12 or 30 mM, open circles) or fully selective (72 mM, closed circles). (B) 21BU1 seeded in 0.6 mM BrdU.

TABLE 7. AzaC-induced reversion frequencies of TK^- mutants[a]

AzaC concn (μM)	Reversion frequencies ($\times 10^{-5}$)			
	B8-1[b]	G9-1B[c]		
		Expt 1	Expt 2	Expt 3
0	<0.1	0.05	0.05	0.05
3	<0.2	1	<1	<0.3
10	<0.2	6	<1	0.3
15	<0.2		<1	0.3
20	<0.2		3	1.0
30	<0.2	<1	<1	0.3

[a] Cells were grown in α medium containing the indicated concentration of azaC for 24 h. After 3 further days of cultivation in α medium, the cells were plated in HAT. The concentration of 2-dgal used to select for GLK^- cells was 48 mM.

[b] TK^- line isolated from 21BU1 by spontaneous mutation to resistance to 0.6 mM BrdU.

[c] TK^- line isolated from G9-1 by mutagenesis and one-step selection to resistance to 0.6 mM BrdU. G9-1 is a GLK^- *tk* hemizygous line isolated from D2171 (Table 5).

In the former case, the heterozygotes (class II) can subsequently suffer mutation at the remaining W.T. allele by an HF event; in most cases this event, called HF2, involves inactivation not only of the gene whose product was selected against, but of one allele at the linked test locus, rendering the latter functionally hemizygous. In the remainder of cases, the HF event, HF1, was localized so that the test locus remained functionally dizygous. Class I heterozygotes arising after an HF event (Fig. 4) can only undergo mutation to full drug resistance by an LF event. In principle, class I cell lines could probably arise by either an HF1 or HF2 event, but out of six of this cell type characterized so far, all are functionally hemizygous at both *tk* and *glk* and so only the HF2 event is depicted in the figure.

In addition to the spreading effect, HF2 differs from HF1 in that the inactivation can be partial. This was concluded from the coincidence in the extent of reduction of TK and GLK activities in all colonies picked from either partially or fully selective medium in which the inactivation appeared to be partial (Fig. 3A and B). Genetic data are consistent with this in that those derivatives of class II heterozygotes in which partial inactivation had apparently occurred (G10-1 and G16-1, Table 5; B1-2 and B3-4, Table 6) underwent mutation at the test locus at frequencies 4- to 50-fold lower than that expected for a functionally hemizygous locus, but significantly higher than the 5×10^{-7} frequency characteristic of dizygosity.

The fact that partial inactivation can occur at

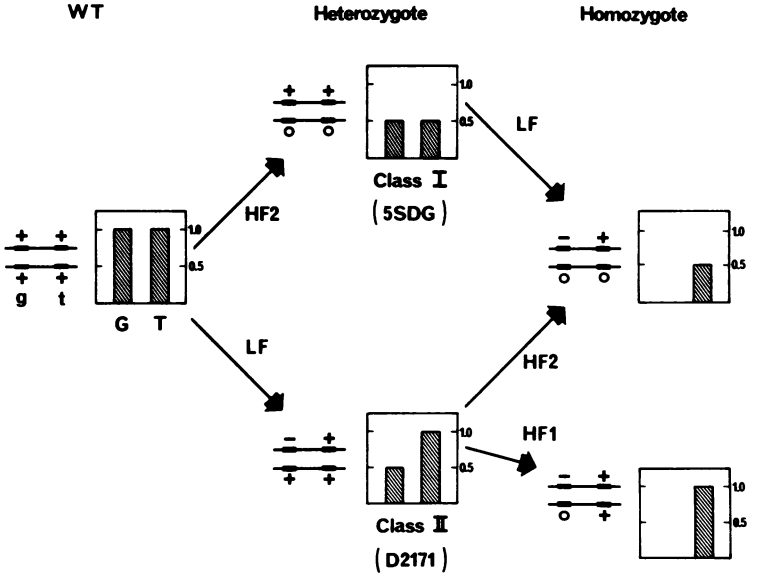

FIG. 4. Model of mutational events occurring at the *glk* locus. Horizontal lines represent the genotypes of W.T. heterozygous and homozygous negative cell lines at the two linked loci *glk* and *tk*, represented by g and t, respectively. (No particular order is implied.) Beside each schematic of the genotypes are bar graphs representing the level of expression of the enzymes, G and T representing GLK and TK, respectively. Symbols: +, W.T.; − mutant as a result of an LF event; 0, mutant, or inactivated, as a result of an HF event, either HF1 (no inactivation at the linked test locus) or HF2 (one allele at the test locus inactivated). Class I heterozygotes arise as a result of an HF event and class 2 as a result of an LF event. Prototype cell lines for the two classes are 5SDG and D2171, respectively.

frequencies higher than full inactivation raises the possibility that the phenotype of a cell and its descendants growing in partially selective medium can evolve through a series of such events, each resulting in more complete inactivation and each rendering the altered cell better adapted to its selective medium. This may be similar to the long-term development of BrdU-resistant cell lines described by Harris and Collier (10). Similarly, it is possible that the fully drug-resistant mutants described in this paper are in fact the product of several events acting in concert to achieve the observed phenotype. This would be consistent with the apparent higher frequency of partial inactivation events.

The coordinated inactivation of linked genes, which has been called allelic silence (3), has also been observed by Campbell and Worton (6) at the loci emt^r and chr^r in CHO cells. In addition, G. Adair (personal communication) has observed functional hemizygosity at the *glk* locus in fully BrdU-resistant derivatives of the $tk^{+/-}$ line AT3-2 of CHO (1). This phenomenon is therefore not an anomaly of a particular mutant cell line or even of a specific chromosome of CHO. It is possible that the event may be similar to that responsible for the extensive regions of functional hemizygosity in CHO (18). A further possible manifestation of HF events (either HF1 or HF2) may be the higher-than-expected recovery of certain recessive mutants such as some of the aminoacyl-tRNA synthetases (2). It remains to be seen whether the HF events can occur in other established cell lines or in cells of other species. Of particular interest, for example, is the possible implication of this type of mutation in the HF of emergence of altered phenotypes, including drug resistance, in human tumor populations.

What are the mechanisms of the HF events? Although the only difference between HF1 and HF2 definitely shown so far lies in whether or not a linked allele is inactivated simultaneously, it is anticipated that different molecular mechanisms are responsible for the two events. For example, HF1 may be analogous to the HF event which occurs at the *aprt* locus, which appears to involve a large deletion of DNA, including the entire *aprt* gene (18a). It is possible that a similar molecular event occurs at the *tk* and *glk* loci. On the other hand, HF2, or allelic silence, may be analogous to X inactivation in that long sequences of DNA are affected coordinately (*tk* and *glk*, at least in humans, are probably 1.2×10^6 base pairs apart (17). Another similarity is that inactivated X-linked genes can be reexpressed after azaC treatment (14) as can the inactivated emt^r gene (22).

As shown in Table 7, this drug also induced reversion of one TK^- cell line which had undergone an HF2 event. It was not shown clearly that it was the HF2 inactivation rather than the LF event which was reversed by azaC, but we have found no evidence for azaC-induced reversion of LF events in *aprt* mutants (data not shown). Furthermore, if LF events are indeed point mutations (as is proposed elsewhere) (19), it is unlikely that azaC would have an effect on their reversion. The results suggest, therefore, that the HF2 event involved methylation of cytidine residues. The frequency of reversion was about the same as is seen in some other systems where methylation is known to be responsible for inactivation (7), but much lower than the theoretical frequency of $>10^{-1}$, which has been reported in other systems (9; Worton et al. *in* M. L. Pearson, ed., *Proceedings of the Workshop on Gene Transfer, Frederick, Maryland*). Some unknown factors must influence the frequency of reactivation via hypomethylation, and it is possible that these factors may have had a particularly strong effect in the case of B8-1, rendering the reversion frequency undetectably low. Thus, the negative results with this cell line are not inconsistent with the HF event being associated with methylation of cytidine residues.

Other possible mechanisms for HF2 exist, but appear less likely. Chromosome loss and reduplication (8) would result in two identical chromosomes, but the data in Table 5 and 6 clearly indicate functional hemizygosity at the respective test loci. Similarly, mitotic recombination cannot explain the functional loss of one allele at the test locus. Simple chromosome loss would yield a cell line with an altered karyotype, which has not been detected (data not shown). One possibility we cannot exclude is that the HF2 event is similar to the correction phenomenon recently described (15). This is an HF event resulting in mutation of all copies of the *aprt* gene in a cell line into which this gene had been transfected and subsequently highly amplified. Such a mutational event may be a reflection of the amplification and may not inactivate long regions of presumably heterogeneous DNA sequences. In any event, molecular probes will be necessary to distinguish among the various possibilities.

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