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Isolation of Mammalian Cell Mutants Deficient in Glucose-6-phosphate Dehydrogenase Activity: Linkage to Hypoxanthine Phosphoribosyl Transferase

(somatic cell genetics/cell hybrids/synteny/Chinese hamster cells)

MAURICE ROSENSTRAUS AND LAWRENCE A. CHASIN

Department of Biological Sciences, Columbia University, New York, N.Y. 10027

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ABSTRACT **Mutants of Chinese hamster ovary cells deficient in glucose-6-phosphate dehydrogenase (D-glucose 6-phosphate:NADP 1-oxidoreductase, EC 1.1.1.49) activity were isolated after mutagenesis with ethyl methane sulfonate. The mutants were induced at frequencies of about 10^{-4} and do not differ in growth properties from wild-type cells. They were isolated by means of a sib selection technique coupled with a histochemical stain of colonies for enzyme activity. The lack of enzyme activity is not due to a dissociable inhibitor, and is recessive in hybrid cells. Multiple mutants that lack hypoxanthine phosphoribosyltransferase activity (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) and adenine phosphoribosyltransferase activity (AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) were isolated by further mutagenesis. By following segregation of wild-type phenotypes from heterozygous multiply marked hybrid cells, it was shown that the genes responsible for glucose-6-phosphate dehydrogenase activity and hypoxanthine phosphoribosyltransferase activity are linked in Chinese hamster cells, in agreement with the location of both on the X chromosome in humans. No linkage to adenosine phosphoribosyltransferase was found. The isolation of mutant cells carrying linked markers should prove useful for studying chromosomal events such as segregation, breakage, recombination, and X-chromosome reactivation.**

The induction and isolation of mutant mammalian cells carrying two mutations in the same chromosome would be useful in several ways for somatic cell genetics. By following the simultaneous behavior of two syntenic* markers, one can distinguish chromosome-wide events, such as segregation or X-chromosome inactivation, from locus-specific events such as gene mutation. Such mutants would also be useful in attempting to establish methods for the ordering of genes and mutations within a chromosome, taking advantage of chromosome breakage, translocations, or possibly mitotic crossing-over.

The X chromosome is a logical target for the isolation of mutations in syntenic genes. Since only one X chromosome is functional in mammalian somatic cells (1), single recessive mutations should be readily expressed. The genes for glucose-6-phosphate dehydrogenase (G6PD, D-glucose 6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49) and hypoxanthine phosphoribosyltransferase (HPRT, IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) are both X-linked in humans (2) and there is a strong evolutionary conservation of X-linkages among mammals (3). These two genes also appear to be linked in Chinese hamster cells, on the basis of gene dosage studies by Westerveld *et al.* (4), which we have confirmed (unpublished results). The facility with which mutations to HPRT deficiency are induced in Chinese hamster cells (5, 6) supports the idea that only one gene need be mutated. Furthermore, in some cases structural gene mutations have been implicated in the HPRT system (refs. 7 and 8; Chasin and Urlaub, in preparation). Mutants lacking G6PD activity have not been isolated in culture, one problem being the lack of a selective system. Human cell strains lacking G6PD activity have been derived from persons having a genetic deficiency for this enzyme (9), and a line of rat hepatoma cells has been reported that is similarly lacking enzyme activity (10). These cells generally grow as well as wild-type cells, indicating that G6PD is not necessary for cell viability under ordinary culture conditions. The $G6PD^+$ and $G6PD^-$ phenotypes can be distinguished at the cellular level by a histochemical staining technique for enzyme activity. If $G6PD^-$ cells can be induced at about the same frequency as HPRT-deficient cells (2×10^{-4}), then it is only necessary to scan about 10,000 colonies surviving mutagenesis to isolate a $G6PD^-$ clone that is recognizable histochemically. Since the staining procedure kills the cells, a sib selection technique (11) must be used in order to recover the mutants.

A Chinese hamster ovary cell line (CHO) was used for the present studies because of the high plating efficiency, rapid growth, and genetic stability of these cells in culture; and because they have been successfully used for the isolation of a wide variety of mutant phenotypes (see ref. 12 for review). Despite extensive chromosomal rearrangement, this line has retained close to a diploid amount of DNA (13).

This paper describes the isolation of Chinese hamster cell mutant lacking G6PD activity by the use of such a sib selection technique. Moreover, we demonstrate that the wild-type alleles for G6PD and HPRT are indeed linked (and presumably syntenic) in CHO cells.

MATERIALS AND METHODS

Cell Cultures. The cell lines used were all derived from the K1 clone (14) of Chinese hamster ovary cells (CHO). They are listed in Table 1, along with their relevant phenotypes.

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; CHO, Chinese hamster ovary cell line.

* Synteny refers to the presence of genes on a common chromosome. Linkage means that certain pairs of markers do not segregate at random.

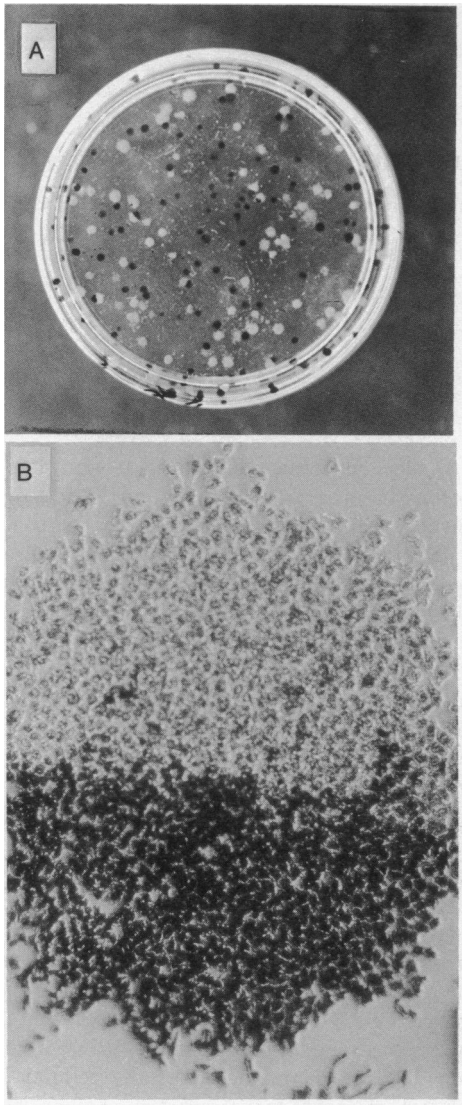

FIG. 1. A mixture of wild-type (G6PD$^+$) and mutant Y113 (G6PD$^-$) colonies stained for enzyme activity. G6PD$^+$ cells are stained blue by the formazan precipitate, while G6PD$^-$ cells stain yellow. (A) 35-mm dish, indirect light. (B) Phase contrast photomicrograph of two adjacent colonies (about 2-mm diameter).

Cells were grown as monolayers or in suspension culture in F12 medium (16) supplemented with 10% v/v fetal calf serum (Gibco). Dialyzed serum was used whenever selective growth conditions were employed. Thioguanine-resistant and diaminopurine-resistant mutants were induced by ethyl methane sulfonate and isolated as previously described (6, 17).

Hybrid cell clones were isolated by taking advantage of complementation between the *glyA* and *glyB* loci (21). The chromosome number of hybrids was close to the sum of the two parental strains.

Histochemical Stain for G6PD. The staining procedure of Wajntal and Demars (18) was modified during the isolation of the G6PD$^-$ mutants. After the isolation of these strains, reconstruction experiments (Fig. 1) showed that the following method optimally distinguished G6PD$^-$ from wild-type colonies in this cell line. The staining mixture contained: 0.33 mg/ml of nitroblue tetrazolium, 0.17 mg/ml of phenazine methosulfate, 0.17 mg/ml of NADP, 2.5 mg/ml of glucose 6-phosphate, 0.14 M NaCl, and 0.01% Triton X-100. The stock glucose 6-phosphate solution was adjusted to pH 6.5 with NaOH. Stock solutions of the individual components were stored at −20° and the staining mixture was prepared just before use.

TABLE 1. *Characteristics of cell lines*

Strain no.	Parent	GlyA	GlyB	HPRT	APRT	G6PD
51-11	Ref. 14	−	+	+	+	+
43-64	Ref. 14	+	−	+	+	+
DR31	Ref. 21	+	−	−	−	+
Y113	51-11	−	+	+	+	−
YH21	Y113	−	+	−	+	−
YHD13	YH21	−	+	−	−	−
YT22	DR31	+	−	−	−	−

GlyA and GlyB represent glycine requirements that are due to distinct complementing biosynthetic deficiencies (15).

Five to 7 day colonies on plastic tissue culture dishes were rinsed three times with isotonic saline containing 0.01% Triton X-100. The staining mixture was then added without allowing the dishes to dry and the dishes were incubated at 37° for 1–2 hr. Cells were then fixed with 10% formalin and counterstained with metanil yellow (19).

Isolation of G6PD$^-$ Mutants by Sib Selection. Cells were mutagenized (ethyl methane sulfonate, 0.2 mg/ml, 18 hr) and then distributed among 10 to 20 60-mm dishes at 2000 cells per dish. This corresponds to about 600 viable cells per dish, since only 30% of the cells routinely survive the mutagenesis. Nonselective F12 medium was used throughout the procedure. After 2 days (about 3 generations), each culture was trypsinized and split to give two sister cultures, again in 60-mm dishes. After incubation for 5–7 days, the cells had grown to individual colonies; one set of 20 dishes was then stained for G6PD activity, leaving the sister set as a source of viable cells. The sister cultures of those dishes containing the highest numbers of G6PD$^-$ colonies were then used to initiate a new set of 10 to 24 cultures. For this second cycle, only 200 to 600 cells were inoculated on each of ten 35-mm dishes or in each well of a 24 well dish (Linbro). The cultures were again incubated for 2 days, split, and grown to colonies; one set was stained, and the cultures whose stained sister dishes contained the highest frequency of G6PD$^-$ colonies were used to start a third round. This cyclical process was repeated, with the total number of cells per culture decreasing each time, until finally clonal cultures were derived that consisted entirely of G6PD$^-$ cells. As the number of cells per culture was reduced, the dish size was accordingly decreased. Multiwell dishes proved convenient for the final rounds.

Two sib selections were carried out. In the second experiment, dishes were heated at 45° for 5 min before staining for G6PD. Wild-type cells are not affected by this treatment, which was included to increase the chances of isolating a mutant containing a thermolabile enzyme.

Enzyme Assays. Late log phase cells were harvested as previously described (19, 20). The cells were suspended in 2 ml of 0.03 M Tris·HCl, pH 7.4, and were sonicated (Biosonik III)

TABLE 2. *Progressive enrichment of G6PD⁻ mutants by sib selection*

Round	No. of cultures screened	Average no. of colonies/culture	G6PD⁻ colonies: Overall frequency	G6PD⁻ colonies: Best frequency
		Exp. no. 1: Parent strain 51-11		
1	10	1400*	0.00057	0.0021
2	10	644	0.0047	0.012
3	10	305	0.018	0.043
4	24	33	0.010	0.12
5	96	3	0.048	1
6	12	1	1	—
		Exp. no. 2: Parent strain DR31		
1	18	922	0.00074	0.0022
2	24	133	0.0013	0.023
3	36	46	0.0036	0.033
4	96	N.D.	N.D.	0.5, 1.0
5a	4	1	1	—
5b	1	1	1	—

N.D. is not determined.

* For this round only, the number of colonies was estimated on the basis of the number of mutagenized cells plated and an assumed plating efficiency of 50%. In all other cases, colony counts were made on representative stained cultures.

with 5-sec bursts until more than 90% breakage was achieved, as judged by phase microscopy. The supernatant solution after centrifugation at 15,000 × *g* for 20 min was used for measurements of HPRT or APRT activity. In the case of G6PD assay, the final centrifugation was 42,000 × *g* for 60 min, and the supernatant was dialyzed overnight against 2 liters of the same buffer. Cell extracts could be stored at −70° with no significant loss of activity for at least a month.

G6PD and 6-phosphogluconate dehydrogenase [6PGD, 6-phospho-D-gluconate:NADP 2-oxidoreductase (decarboxylating), EC 1.1.1.44] activities were assayed as the production of NADPH by following the increase in absorbance at 340 nm on a recording spectrophotometer at room temperature. The initial change in absorbance was monitored in a reaction mixture containing 25 μmol of Tris·HCl, pH 7.4, 2.5 μmol of $MgCl_2$, 0.2 μmol of NADP, and up to 0.1 ml of extract in a total volume of 0.45 ml. After about 10 min, 0.05 ml of 0.02 M 6-phosphogluconic acid was added and the increase in absorbance due to 6PGD activity was recorded. Glucose 6-phosphate (0.05 ml at 0.02 M) was then added and the combined activities of 6PGD and G6PD were recorded. G6PD activity was calculated by subtraction. This indirect procedure was used because the G6PD reaction can generate the substrate for 6PGD, with the consequent production of additional NADPH.

Adenine phosphoribosyltransferase (APRT, AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7), HPRT, and protein were assayed as previously described (17).

RESULTS

Sib Selection of G6PD⁻ Mutants. Two experiments were performed, each starting with a different subclone of CHO cells. In both experiments cells were first mutagenized by treatment with ethyl methane sulfonate. As described in *Materials and Methods*, the sib selection procedure involves growing a series of sister cultures into colonies, staining one set of sister cultures for G6PD activity, and using the culture with the highest number of negative colonies to initiate another round of sister cultures. The stepwise enrichment of G6PD⁻ cells in these experiments is shown in Table 2. Despite some fluctuation in the number of mutant colonies, a progressive increase in the frequency of mutant cells was achieved. After 4 to 5 rounds, the frequency of G6PD⁻ colonies was high enough (0.03–0.12) to warrant the establishment of clones, which were then tested individually with the histochemical G6PD stain.

TABLE 3. *Enzyme activity in extracts from wild-type and mutant strains*

Strain	Parent	G6PD: Specific activity*	G6PD: Percent of parental	6PGD: Specific activity*	6PGD: Percent of parental
51-11	—	54.8	100	43.3	100
Y113	51-11	<0.4†	<1	46.8	108
DR31	—	36.0	100	28.7	100
YT22	DR31	6.6*	18	32.4	113

* mIU/mg of protein.

† In order to maximize the sensitivity of the G6PD measurement in the case of the deficient mutants, these assays were run without correcting for the possible subsequent oxidation of the reaction product, catalyzed by 6PGD in the extract. This could result in an *over*estimate of the apparent G6PD activity by a factor of up to two.

The mutant frequency induced by ethyl methane sulfonate is given by the overall frequency of G6PD⁻ colonies at the end of round 1 in each experiment. The values of 5 to 7 × 10^{-4} are comparable to the figure of 2.5 × 10^{-4} found in many experiments in which thioguanine-resistant (HPRT⁻) mutants are induced by the same mutagen (6), supporting the idea that the genes for both G6PD and HPRT are functionally haploid in these cells. Unmutagenized cells of one parental strain, 51-11, gave no mutants among 2.6 × 10^4 colonies screened. The other parental strain, DR-31, gave two colonies that contained a mixture of G6PD⁺-staining cells and cells that stained as if they were G6PD⁻ among 2.3 × 10^4 colonies screened. The nature of these colonies is unconfirmed, since a sib selection on unmutagenized cells was not performed.

Mutant Characteristics. One mutant was recloned for further study from each of the two isolations. Each had been derived from a different subclone of the CHO K1 line, and each retained the genetic markers characteristic of the parental clone (Table 1). Both mutants have about a modal chromosome number of 20–21, like the parental cells. Strain Y113 (Exp. 1) has a stable phenotype, yielding no G6PD⁺ colonies among 10^5 cells screened by the histochemical stain. Populations of strain YT22 (Exp. 2), on the other hand, contained five lightly stained colonies among 1.9 × 10^4 screened. The mutants grow at approximately the same rate as wild-type cells in standard F12 medium and also in medium lacking sodium pyruvate, in which glucose is the only major carbohydrate.

The results in Table 3 show that soluble extracts of both mutants are deficient in G6PD activity. Strain Y113 has no measurable enzyme activity; strain YT22 does contain residual activity of up to 18% of the parental level. G6PD catalyzes the first reaction of the hexose monophosphate shunt.

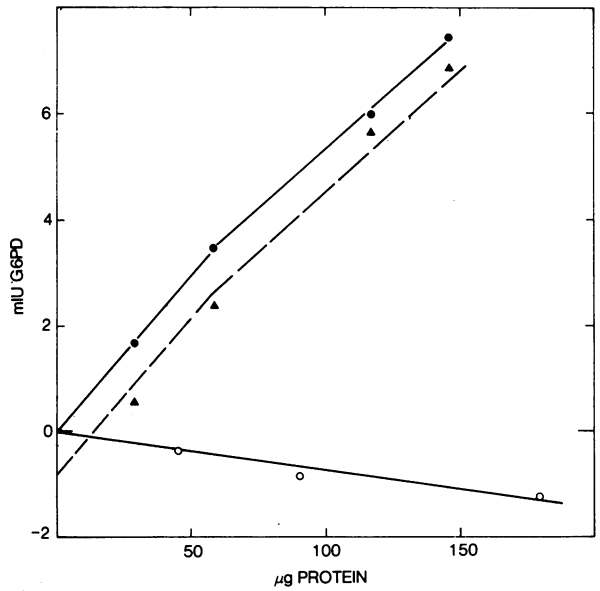

FIG. 2. The effect of an extract of the G6PD$^-$ mutant strain Y113 on wild-type (strain 51-11) G6PD activity. G6PD activity was calculated by subtracting 6PGD activity from the total 6PGD plus G6PD activity for both the wild-type and mutant extracts. The slight negative activity associated with the mutant is probably due to inhibition of 6PGD. (●) 51-11; (○) Y113; (▲) 51-11 in the presence of Y113 extract (89.5 μg of protein). The broken line represents the expected result from the algebraic sum of the two activities.

Levels of 6-phosphogluconate dehydrogenase, the second enzyme in the pathway, are normal in both of the mutant strains (Table 3). A mixing experiment demonstrated that the lack of G6PD activity in strain Y113 was not due to a dissociable inhibitor in the extract: enzyme activity of a wild-type strain is fully expressed in the presence of the mutant extract (Fig. 2).

Linkage Analysis. The rationale for the isolation of the G6PD$^-$ mutants was based on the idea that both the G6PD and HPRT genes were located on the same functionally haploid chromosome. Linkage between the positive alleles of these two genes can readily be tested by their cosegregation from heterozygous hybrid cells. For this purpose, a thioguanine-resistant subclone of G6PD$^-$ mutant strain Y113 was selected after mutagenesis with ethyl methane sulfonate. This new mutant, YH21, contains less than 0.05% of wild-type HPRT specific activity. Strain YH21 was fused with strain 43–64 which is wild-type at all relevant loci except for a complementing glycine requirement (see Table 1). A hybrid clone was isolated in glycine-free medium. Its genotype can be represented:

$$\frac{HPRT^+\ G6PD^+\ glyA^+\ glyB^-}{HPRT^-\ G6PD^-\ glyA^-\ glyB^+}.$$

As expected, the hybrid has almost the sum of the parental chromosome numbers, and it is glycine-independent and thioguanine-sensitive, as expected from the known recessive nature of the *gly* and *HPRT* mutations. The hybrid also stains positive for G6PD, indicating that the G6PD$^-$ character of the mutant is recessive to G6PD$^+$.

Although the hybrid is sensitive to thioguanine, resistant subclones can be isolated. We have previously shown that the segregation of thioguanine sensitivity from such a hybrid occurs spontaneously at a rate several orders of magnitude greater than that expected for spontaneous mutation, and is presumably due to chromosome loss (21). Thioguanine-resistant segregants lose HPRT activity (unpublished results).

Co-segregation of the G6PD$^+$ and HPRT$^+$ phenotypes from this hybrid clone was monitored by staining for G6PD activity those colonies that had grown up in the presence of thioguanine. Co-segregation of these two markers would yield a G6PD$^-$ HPRT$^-$ phenotype. Lack of co-segregation would result in most colonies being G6PD$^+$ HPRT$^-$. In order to score independent segregation events, a series of cultures was grown in nonselective medium, each starting with a small inoculum (about 10 cells) so that it was improbable that any segregants were present initially. When a sufficient number of cells had accumulated, each culture was challenged in thioguanine-containing medium (6). Drug-resistant colonies appeared at a frequency of about 10^{-3}. Out of 24 cultures, only one contained any thioguanine-resistant, G6PD$^+$ colonies; even in this case these colonies were only a small minority (3/61). Since a *minimum* of 25 segregation events must have occurred, HPRT$^+$ and G6PD$^+$ have co-segregated *at least* 96% of the time.

A second experiment was designed to include a control showing a lack of co-segregation between G6PD$^+$ and another drug marker, sensitivity to diaminopurine. Resistance to this adenine analog is not related to thioguanine resistance, and the two drug sensitivity alleles are not linked in this cell line (21). For this experiment, strain YH21 was mutagenized with ethyl methane sulfonate and a diaminopurine-resistant mutant deficient in APRT was isolated. This strain, YHD13, was fused with strain 43–64. A hybrid clone was isolated in glycine-free medium. Its genotype can be represented:

$$\frac{HPRT^+\ G6PD^+\ APRT^+\ glyA^+\ glyB^-}{HPRT^-\ G6PD^-\ APRT^-\ glyA^-\ glyB^+}.$$

The hybrid has a wild-type phenotype for all characteristics. Segregants that have lost the APRT$^+$ allele become resistant to diaminopurine. For this experiment one diaminopurine-resistant clone was isolated from each of 10 independent cultures and tested for G6PD histochemical activity. All 10 clones that had lost their diaminopurine sensitivity retained the G6PD$^+$ phenotype. On the other hand, among 22 independent cultures selected for thioguanine resistance, only two had G6PD$^+$ colonies, and these were in the minority when they occurred (5/30, 6/49). At least 24 segregation events must be represented in these 22 cultures, so HPRT$^+$ and G6PD$^+$ co-segregated at least 92% of the time.

Analyzing the data as a fluctuation test and using the median method of Lea and Coulson (22), we calculated the average number of independent segregation events per culture to be approximately 10 in both experiments. On this basis, G6PD$^+$ co-segregates with HPRT$^+$ about 99% of the time.

DISCUSSION

The successful isolation of G6PD$^-$ mammalian cell mutants is in itself significant for two reasons. First, it demonstrates the feasibility of using a sib selection method for the isolation of cell variants in the absence of selective pressure. Although this procedure is limited to variant frequencies of the order of 10^{-5}, it could prove useful for the isolation of single-step

mutants, segregants from hybrid cells, or variants altered in the expression of a specialized function. Second, it supports the premises on which the isolation was based: that single gene mutations can be induced in mammalian cells at frequencies of about 10^{-4}, and that recessive mutations are readily expressed if they are located on X chromosomal material.

Another assumption that was made in attempting these experiments was that G6PD activity is not essential for cell viability. Other studies with both bacterial (23) and mammalian cells (9, 10) had indicated that this is so, as do the present results. Although the principal role of G6PD and the hexose monophosphate shunt is thought to be the production of ribose and of NADPH, these cells evidently possess alternative pathways for these compounds that are entirely sufficient for cell growth. We are presently trying to devise culture conditions that would select against G6PD$^-$ cells, with some preliminary success using chemicals that deplete the cell of reduced glutathione (24, 25), thus creating an increased need for reducing power in the form of NADPH (26).

The two mutants isolated are deficient in G6PD activity, and in at least one case the lack of activity cannot be attributed to a dissociable inhibitor in the extract. However, there is no direct proof that mutations in the structural gene for the enzyme are the cause. The linkage of the wild-type alleles for G6PD and HPRT production is consistent with this explanation, since the two structural genes for these enzymes are on the same chromosome in humans (2). The characterization of the physical properties of the residual enzyme in strain YT22 and in revertant strains may provide more direct evidence on this point. Revertants would also be interesting to study with regard to the regulation of G6PD synthesis, since Yoshida has shown that a single amino-acid substitution in the human enzyme can increase the rate of enzyme synthesis several-fold (27).

Although the viability of G6PD$^-$ mutants was assumed in the original sib selection, the method is not limited to nonessential genes. Since the indicating colonies do not survive the staining procedure, the dishes can be heated to temperatures that do not affect wild-type enzyme activity prior to staining. Mutants with temperature-sensitive enzymes that function normally at 37° can then be isolated.

Mutations in other X-linked genes such as phosphoglycerate kinase, alpha-galactosidase, and phosphorylase kinase may also be isolable by sib selection. The presence of three markers on one chromosome would facilitate studies attempting to demonstrate mitotic recombination in cultured somatic mammalian cells. The doubly marked mutants described here can be used to look for mitotic recombination; and, in addition, to study the role of chromosome loss in mutation, chromosome breakage in cell hybrids, and to test for the reactivation of the X chromosome in female mammalian cells.

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