# SEPARATION OF LINKED MARKERS IN CHINESE HAMSTER CELL HYBRIDS: MITOTIC RECOMBINATION IS NOT INVOLVED

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ABSTRACT

A search for mitotic recombination was carried out using mutant subclones of cultured Chinese hamster ovary cells. Recombination events were sought between the linked loci specifying the enzymes hypoxanthine phosphoribosyl tranferase and glucose-6-phosphate dehydrogenase. It was shown by fluctuation analysis that markers at these two loci co-segregate from doubly heterozygous pseudotetraploid hybrid cells more than 90% of the time. The minority class of segregants, which had lost one marker without losing the other, were genetically analyzed to distinguish between the possibilities of mitotic recombination and deletion of chromosomal material. Nine clones in which a linkage disruption had occurred were studied, using further cell hybridization and segregation. In three cases, a recessive lethal loss of genetic information was indicated, suggesting the deletion mechanism. In six cases, it was demonstrated that no new linkage relationships had been established concomitant with linkage disruption. Thus, in all nine clones, the evidence indicated that mitotic recombination was not involved in the events that disrupted linkage between these two loci. If mitotic recombination takes place at all in this system, the rate must be less than about $10^{-6}$ per cell per generation.

SPONTANEOUS crossing over between homologous chromosomes is known to occur in many types of mitotically dividing eukaryotic cells. This phenomenon, called mitotic recombination to distinguish it from meiotic events, was first described by STERN (1936) to explain twin-spot formation in Drosophila somatic tissues. Since that time, mitotic recombination has been demonstrated in several other types of eukaryotic cells, including fungi (PONTECORVO and KAFER 1958; MANNEY and MORTIMER 1964), slime molds (KATZ and KAO 1974) and soybeans (VIG and PADDOCK 1968).

The demonstration of mitotic recombination in mammalian cells would be important for two reasons: (1) recombination between the centromere and a heterozygous locus in a diploid cell can lead to homozygosis for a mutant allele. A previously masked recessive mutant phenotype could then be expressed. Thus, mitotic recombination should be considered along with new mutations as a genetic mechanism for cellular variation; and (2) mitotic recombination could

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provide a method for genetic mapping in cultured mammalian cells. At present, segregation from interspecific hybrids can be used to assign genes to particular chromosomes or chromosomal regions (see RUDDLE and CREAGAN 1975). More recently, it has been possible to map the position of genes relative to one another by a combination of X-ray-induced chromosome breakage and cell hybridization (GOSS and HARRIS 1975, 1977a,b). However, there is no method for fine-structure genetic mapping of mutations in cultured mammalian cells. In lower eukaryotes, however, mitotic recombination has been used for fine structure mapping. For example, in yeast frequencies of intracistronic recombination as high as $10^{-3}$ have been achieved (YOST, CHALEFF and FINERTY 1967). Frequencies several orders of magnitude lower than this would suffice for fine-structure mapping.

There is considerable cytogenic evidence suggesting that mitotic recombination takes place in cultured mammalian cells. Exchange between sister chromatids is a widespread and frequent occurrence (TAYLOR 1958; LATT 1974), indicating that enzymes capable of carrying out exchanges between DNA molecules are present in mammalian cells. Recent biochemical evidence also supports the formation of recombinant DNA molecules in this process, based on the transfer of density labels (ROMMELAERE and MILLER-FAURÈS 1975; MOORE and HOLLIDAY 1976). Exchanges between homologous (or nonhomologous) chromosomes have also been observed cytologically, albeit at frequencies much lower than sister-chromatid exchange (LATT *et al.*, 1975). Interchromosomal exchanges have been demonstrated by autoradiography following cell fusion (ROMMELAERE, SUSSKIND and ERRERA 1973).

The most dramatic evidence for the occurrence of recombination between non-sister chromatids is the formation of "quadriradial" chromosome structures (GERMAN 1964). Recently, these structures have been shown to involve exchanges of chromosome material (CHAGANTI, SCHONBERG and GERMAN 1974). Moreover, while the frequency of quadriradials is normally low, it is increased by mitomycin C treatment, a known recombinogen (SHAW and COHEN 1965; HUTTNER and RUDDLE 1976), and in cells from individuals with Bloom's syndrome (CHAGANTI, SCHONBERG and GERMAN 1974; THERMAN and KUHN 1976).

Genetic evidence for mitotic recombination in mammals has been anecdotal rather than systematic (GRUNEBERG 1966; BATEMAN 1967; BOSTIAN *et al.*, 1969; KNIGHT, MALEK and HANLY 1974). Genetic tests in mammals have been limited by the unavailability of appropriate selective markers and the apparently low frequencies involved.

We have approached the question of mitotic recombination in mammalian cells by using established cell culture lines. Selective markers in Chinese hamster ovary (CHO) cells have been developed that allows the screening of relatively large numbers of cells for possible rare mitotic recombinants. Specifically, the loci specifying the enzymes hypoxanthine phosphoribosyl transferase (HPRT, EC 2.4.2.8) and glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) were focused on. These loci were chosen because they are known to be *X*-linked in humans (RUDDLE and CREAGAN 1975) and other mammals (COOK 1975; EPSTEIN 1969; HASHMI and MILLER 1976) and the *X*-linkage group appears to have been

conserved in the evolution of mammals (OHNO 1969). By studying *X*-linked markers, starting with pseudodiploid cells, the analysis is simplified in that only one functional copy of each gene need be considered, since the second *X* chromosome is inactivated during development in female mammalian cells (LYON 1972). In addition, the HPRT system is most valuable, since powerful methods are available to select for both forward and back mutations.

The basic approach has been to construct intraspecific hybrid cells carrying two different alleles at the *hprt* and *g6pd* loci. Both copies of these *X*-linked markers are expressed in such hybrids (CHASIN and URLAUB 1976). We have previously shown that the *hprt* and *g6pd* loci are linked in CHO cells, since coupled alleles segregate together from heterozygous hybrid cells (ROSENSTRAUS and CHASIN 1975). A small minority class of segregants was found in which the segregation of one marker had occurred without the segregation of the coupled allele at the linked locus. This disruption in linkage could be due to mitotic recombination occurring between the loci specifying HPRT and G6PD. The result of such an event could be homozygosis of the distal marker. We report here the isolation of segregants in which the linkage between *hprt* and *g6pd* has been disrupted. These clones have been subjected to further segregation analysis designed to indicate or rule out a recombinational origin. Studies of nine such segregant clones lead to the conclusion that mitotic recombination is not involved.

## MATERIALS AND METHODS

*Cells and culture conditions*: All cell lines used are derivatives of the K1 line (KAO and PUCK 1968) of Chinese hamster ovary cells. They are listed in Table 1, along with their relevant phenotypes.

Cells were grown as monolayers or in suspension culture in F12 medium (HAM 1965) supplemented with 10% heat-inactivated (30 min at 56°) fetal calf serum. Cultures were incubated at 37° in an atmosphere of 5% $CO_2$. All selective media were made with modified F12 (see below) containing 10% dialyzed fetal calf serum (CHASIN and URLAUB 1976).

F12 medium lacking glycine was used to distinguish between wild-type cells and glycine auxotrophs. Folinic acid was used to assign glycine auxotrophs to the *gly A* or *gly B* complementation group (KAO, CHASIN and PUCK 1969).

Cells deficient in HPRT activity were characterized by their ability to grow in the presence of 6-thioguanine (0.01 mM) and their inability to grow in medium containing azaserine (0.02 mM) to inhibit *de novo* purine biosynthesis with hypoxanthine (0.03 mM) as the sole source of purines (CHASIN 1973).

In some experiments higher 6-thioguanine concentrations were used, as indicated.

Cells deficient in adenine phosphoribosyl transferase (APRT, EC 2.4.2.7) were characterized in an analogous way by their resistance to 0.1 mM 2,6-diaminopurine and their inability to utilize adenine (0.05 mM) as a purine source (CHASIN 1974).

G6PD+ cells were selected by their resistance to treatment with diamide (0.05 mM) as described previously (ROSENSTRAUS and CHASIN 1977).

*Isolation of HPRT− mutants*: The selection procedure used was essentially the same as previously described (CHASIN 1973). Log-phase cells were mutagenized using one of the following treatments: (1) ethyl methanesulfonate (EMS, 0.2 mg/ml, 18 hr); (2) 4,5′,8 trimethylpsoralen (0.005 μg/ml) for two hours, followed by exposure to black light (30 min, two General Electric F15T8BLB bulbs, 6.5 cm); or (3) exposure to adenovirus type 12 (500 PFU per cell). The mutagen-containing medium was removed, replaced with nonselective medium, and the cells were grown for seven days to allow expression of induced mutations. During this time, cells

TABLE 1

*Characteristics of cell lines*

| Cell line | Parent(s)* | G6PD | HPRT‡ | APRT§ | *gly*‖ | Modal chromosome number | Reference¶ |
|---|---|---|---|---|---|---|---|
| 5111 | K1 | + | +(3.4) | +(12) | *A* | 21 | Kao and Puck (1968) |
| 4364 | K1 | + | +(6.1) | +(4.6) | *B* | 20 | Kao and Puck (1968) |
| DR31 | 4B21 | + | *B*(5.3) | —(<0.01) | *B* | 20–21 | Chasin (1973, 1974) |
| 5A9 | 5111 | + | *A*(0.45) | + | *A* | N.D.†† | Chasin and Urlaub (1976) |
| AA41 | DR31 | + | *B* | +(3.2)** | *B* | N.D. | Chasin (1974) |
| Y113 | 5111 | — | + | + | *A* | 21 | Rosenstraus and Chasin (1975) |
| YHD13 | YH21 | — | — | —(0.023) | *A* | 20 | Rosenstraus and Chasin (1975) |
| YT22 | DR31 | T† | *B* | — | *B* | 19–20 | Rosenstraus and Chasin (1975) |
| AYT13 | YT22 | T† | +**(1.6) | — | *B* | 20 | |
| HTS11 | 5111 | + | —(0.45) | + | *A* | N.D. | |
| G5A12 | 5A9 | — | *A* | + | *A* | 21 | |
| YD12 | Y113 | +** | + | + | *A* | 20 | Rosenstraus and Chasin (1977) |
| 43YHD31 | 4364+YHD13 | + | + | + | + | 38–40 | |
| YY11 | YD12+YT22 | + | + | + | + | N.D. | |
| AAY11 | AA41+AYT13 | + | + | + | *B* | 39–40 | |
| AHT21 | HTS11+AYT13 | + | + | + | + | 36–39 | |
| 51YT211 | 5111+YT22 | + | + | + | + | 40 | |
| 9Y11 | 5A9+YT22 | + | + | + | + | 39–40 | Chasin and Urlaub (1976) |

* When two parents are given, the cell line is the product of somatic cell hybridization.
† Indicates a G6PD⁻ mutant containing residual G6PD activity that is more thermolabile than wild type.
‡ *A* and *B* represent two mutant alleles that complement intragenically. The numbers in parentheses are HPRT specific activities expressed as mIU per mg protein. The HPRT activities in *A* and *B* have a high $K_m$ for PRPP resulting in an HPRT-deficient growth phenotype.
§ The numbers in parentheses are APRT specific activities expressed as mIU per mg protein.
‖ *A* and *B* represent distinct, complementing mutations in the glycine biosynthetic pathway (Kao, Chasin and Puck 1969).
¶ Where no reference is given, the cell line was isolated during the course of this work.
** These lines are partial revertants at these loci.
†† N.D. Not determined.

were passaged when cultures became confluent. At least twice the number of mutagenized cells was subcultured. At the end of the expression period, cells were challenged in thioguanine-containing medium at a density of $2 \times 10^3$ cells/cm$^2$ (CHASIN 1973). Surviving clones were recloned in selective medium. Thereafter, cells were grown in nonselective medium.

*Isolation of G6PD- cells*: The isolation of G6PD- cells was achieved by a sib-selection procedure, using a histochemical stain for G6PD activity to indicate enzyme-negative colonies (ROSENSTRAUS and CHASIN 1975, 1977).

One new G6PD- mutant not previously described, cell line G5A12 (Table 1), was isolated by this method after mutagenesis with EMS (0.2 mg/ml, 18 hr). This mutant contains less than 1% of wild-type G6PD activity. It behaves as a recessive when hybridized to wild-type cells and does not complement the G6PD- mutant, YT22, as indicated by histochemical staining.

*Cell hybridization*: Cells were fused by incubating equal numbers of parental cells at confluent density ($1.2 \times 10^5$ cells/cm$^2$) in monolayer for 16 to 24 hr in the presence of $\beta$-propriolactone-inactivated Sendai virus (Connaught Laboratories) (VELASQUEZ, PAYNE and KROOTH 1971). Cells were harvested and seeded at the appropriate density in the appropriate selective medium. After five to seven days, colonies were isolated and recloned.

*Biochemical procedures*: Late log-phase cultures were harvested by either centrifugation of suspension cultures or trypsinization and subsequent centrifugation of monolayer cultures. The cell pellets were washed twice with isotonic saline. The cells were broken by (1) resuspending in 0.03 M Tris-HCl, pH 7.4 and sonicating with five sec bursts until greater than 90% breakage was achieved, as determined by phase-contrast microscopy; or (2) resuspending in 0.03 M Tris-HCl, pH 7.4 + 0.1% Triton X-100 (Union Carbide). To prepare extracts for the assay of G6PD, 1 mM NADP was added to cell suspensions before sonication. Nuclei (in the case of Triton X-100 treatment) and debris were removed by centrifugation at $12,000 \times g$ for 20 min at 4°. The $12,000 \times g$ supernatant was used for isoelectric focusing and HPRT assays. When extracts were to be used for G6PD assays the $12,000 \times g$ supernatant was centrifuged at $42,000 \times g$ for one hour at 4°. The extracts were then made 0.01 mM in NADP by filtration on Sephadex G-25 (ROSENSTRAUS and CHASIN 1977).

HPRT and G6PD activities were assayed as previously described (CHASIN and URLAUB 1976; ROSENSTRAUS and CHASIN 1977). Extract protein was measured by the method of LOWRY *et al.* (1951), using bovine serum albumin as a standard.

Isoelectric focusing was performed in polyacrylamide slab gels and HPRT activity was detected as previously described (CHASIN and URLAUB 1976).

*Fluctuation tests*: Independent cultures were initiated by seeding approximately 15 cells into the wells of a 24-well dish (Linbro). The cultures were expanded until a sufficient number of cells had accumulated, and then a sample of each culture was challenged in the appropriate selective medium. Surviving colonies were stained for G6PD activity or with crystal violet, and the number of colonies in each culture was recorded. The segregation rates were calculated from this data using the median method of LEA and COULSON (1949) or equation (8) of LURIA and DELBRUCK (1943). To solve equation (8), the tables of CAPIZZI and JAMESON (1973) were employed; alternatively, Figure 1 of LURIA and DELBRUCK (1943) was used for an approximate solution, and an exact solution was found using an iterative procedure with an electronic calculator. Since only a portion of each culture was challenged, calculations of variance and segregation rates were corrected as described in footnote 5 of LURIA and DELBRUCK (1943).

*Detection of sister-chromatid exchange*: Sister-chromatid exchange was detected by using the fluorescence plus Giemsa method of WOLFF and PERRY (1974). Cells were incubated for two generations (approximately 34 hr) in F12 medium lacking thymidine and supplemented with 0.01 mM 5-bromodeoxyuridine, 0.4 $\mu$M 5-fluorodeoxyuridine, 6 $\mu$M uridine and 10% dialyzed fetal calf serum. Mitotic cells were collected during the last three hours of incubation by adding 0.04 $\mu$g/ml of Colcemid to the cells. Chromosome spreads were prepared and stained as described by WOLFF and PERRY (1974). In our hands, the best differential staining of chromatid arms was obtained by leaving Hoescht-stained, cover-slip-sealed slides exposed to daylight on a window sill for at least one day.

*Materials*: Fetal calf serum was purchased from either Gibco or Microbiological Associates. Chemicals were generally purchased from Sigma or Fisher. Psoralen was generously supplied by R. S. COLE and 33258 Hoescht was generously supplied by S. LATT.

## RESULTS

*Search for recombination within the* hprt *locus*: In an initial series of experiments, evidence was sought for the reconstitution of a wild-type *hprt* gene by recombination between two independent mutants at this locus. Seven independent mutants were used to construct seven hybrid clones, employing unrelated markers to isolate the hybrids. Clones carrying one of the markers (EMS-1, Table 2) produce an ineffective HPRT enzyme with altered electrophoretic and kinetic properties that probably represents the result of a structural gene mutation (mutant line DR-31 of Table 1). The other six mutants produce less than 0.5% of wild-type HPRT levels. All seven hybrids exhibited an $HPRT^-$ phenotype (no complementation). As can be seen in Table 2, no evidence for intragenic recombination was found, either spontaneously, or after exposure to mitomycin C, EMS or psoralen plus black light. These treatments are known to induce recombination in other systems (HOLLIDAY 1964; YOST, CHALEFF and FINERTY 1967; ZIMMERMAN 1973; COLE 1973). The one small colony that appeared following mitomycin treatment was found only after fixation and staining. In any event, this frequency is not different from that expected from induced reversion (CHASIN and URLAUB 1976). A total of $7 \times 10^7$ cells was screened in these experiments, indicating that if intragenic mitotic recombination does take place in hybrid cells under these conditions, the frequency must be very low. Similar negative results have recently been reported by TARRANT and HOLLIDAY (1977), using three combinations of HPRT-deficient CHO cell mutants.

The significance of this negative result with respect to the question of mitotic recombination in mammalian cells is compromised by the low sensitivity of the system. All seven of the markers used are probably located within the same gene and may be clustered within very small genetic distances. In addition, many of

TABLE 2

*Lack of recombination between different* hprt *mutant alleles in hybrid clones*

| Hybrid No. | Parental $hprt^-$ clones* | Treatment: | $HPRT^+$ colonies/number of survivors screened $\times 10^{-5}$ | | | |
|---|---|---|---|---|---|---|
| | | | None | Mitomycin C | EMS | Psoralen |
| 1 | EMS-1 × EMS-2 | | 0/150 | 0/4 | 0/5.5 | 0/8 |
| 2 | EMS-1 × EMS-3 | | 0/70 | 0/4 | 0/5.5 | 0/8 |
| 3 | EMS-1 × PS-1 | | 0/70 | 0/4 | 0/5.5 | 0/8 |
| 4 | EMS-4 × AD-1 | | 0/70 | 0/4 | 0/5.5 | NT† |
| 5 | EMS-1 × EMS-5 | | 0/70 | 1/57 | 0/5.5 | 0/8 |
| 6 | EMS-1 × AD-1 | | 0/70 | 0/4 | 0/5.5 | NT |
| 7 | EMS-4 × EMS-5 | | 0/70 | 0/4 | 0/5.5 | 0/4 |

* EMS-1 to 4, induced by EMS in four separate experiments; PS-1, isolated after treatment with psoralen plus black light; AD-1, isolated after treatment with adenovirus type 12.

† Not tested.

these mutants could represent overlapping deletions, although this is not the case for hybrid 2 (Table 2), since it is known that both of these markers revert (CHASIN and URLAUB 1976). Recombination between mutations at two functionally separate loci on the same chromosome should occur at much higher frequencies than intragenic recombination. We therefore directed our efforts to a search for intergenic mitotic recombination.

*Linkage of the* hprt *and* g6pd *loci*: Our basic approach was to construct intraspecific hybrid cells between mutant subclones of CHO cells carrying different alleles at the linked *hprt* and *g6pd* loci. Segregants from these hybrids that have lost one, but not the other, of two alleles that were originally coupled can be isolated and analyzed to distinguish the mechanism responsible for the disruption in linkage. Such mechanisms include chromosome breakage, mitotic recombination, gene conversion, and gene inactivation. The first step was to define the linkage characteristics of this system, in which linkage is defined as the nonrandom co-segregation of two genetic markers. In an initial report, the coupled wild-type alleles of *hprt* and *g6pd* were shown to co-segregate from heterozygous hybrid cells about 95% of the time (ROSENSTRAUS and CHASIN 1975). Subsequently, evidence was presented that several of the mutations used in these studies represent lesions in the structural genes for HPRT (CHASIN and URLAUB 1976) and G6PD (ROSENSTRAUS and CHASIN 1977). The linkage between various alleles at these two loci is further documented and quantified below.

*Segregation analysis*: The degree of co-segregation of two markers from heterozygous hybrid cells can be estimated on the basis of the relative frequencies of different classes of segregants in the population. However, this method is subject to errors due to the accumulation of one or another segregant class because of the chance early occurrence of that segregation event in any particular culture. This problem can be circumvented by carrying out a fluctuation analysis of segregation, using the method developed by LURIA and DELBRUCK (1943) for the appearance of bacterial mutations. The hybrid that has been studied most completely in this way is 51YT211, whose relevant genetic constitution (see Table 1) can be represented as:

$$\frac{hprt^{+}\ g6pd^{+}\ glyA^{-}\ glyB^{+}}{hprt^{B}\ g6pd^{T}\ glyA^{+}\ glyB^{-}}$$

The top line represents the contribution from the parental line 5111, which is wild type at the *hprt* and *g6pd* loci, and the bottom line represents the mutant YT22. The latter probably carries structural gene mutations at both of these loci, since the $hprt^{B}$ product is an electrophoretically and kinetically altered enzyme (CHASIN and URLAUB 1976), and cells carrying $g6pd^{T}$ produce a small amount of thermolabile G6PD (ROSENSTRAUS and CHASIN 1977). Each parent carries a glycine auxotrophic marker of a different complementation class, so that the hybrid can be isolated on the basis of its glycine independence with no selective pressure exerted on the *hprt* and *g6pd* markers. The mutant alleles at these loci are generally recessive in the most commonly used phenotypic deter-

minations. Hybrids behave as though they are HPRT+ in that they are sensitive to purine analogs such as 6-thioguanine and are able to utilize hypoxanthine as a sole purine source when *de novo* purine nucleotide synthesis is blocked by azaserine (CHASIN 1973). They also stain histochemically positive for G6PD activity (ROSENSTRAUS and CHASIN 1975). Segregants of this hybrid that have lost the *hprt*+ allele can be readily selected on the basis of their resistance to 6-thioguanine. Segregants that have lost the *g6pd*+ allele cannot be selected for, but such colonies can be recognized by their inability to stain for G6PD.

Three fluctuation tests were performed in which samples from 20 to 24 independent cultures were challenged in 6-thioguanine-containing medium, and surviving colonies were stained for G6PD activity. In the second and third tests, samples were plated in hypoxanthine plus azaserine medium as well, to demand the retention of *hprt*+; the resultant colonies were stained for G6PD activity to determine the frequency of the HPRT+ G6PD- phenotype. The results are shown in Table 3. The last line indicates that the loss of the two wild-type alleles together takes place at a rate 10 to 100 times higher than segregation of either marker alone. That is, *hprt* and *g6pd* co-segregate 90 to 99% of the time. The results of these fluctuation tests, plus three additional tests using three different hybrids, are summarized in Table 4.

Several additional points can be made regarding the different hybrids analyzed in Table 4. The second hybrid, 43YHD31, carries two different mutant alleles at the *hprt* and *g6pd* loci, showing that the co-segregation behavior is not limited to the particular alleles present in 51YT211. The mutant alleles in this second hybrid produce little or no HPRT or G6PD activity (Table 1). In the case of the third hybrid, YY-11, the *g6pd*+ allele is represented by a second-site revertant of what was originally a G6PD- mutant (see Table 1). This revertant produces an altered G6PD that is more thermolabile than the wild-type enzyme (ROSENSTRAUS and CHASIN 1977). It thus provides additional evidence that the linkage of the structural genes is being measured here. The fourth hybrid, 9Y11, represents a special case, since both *hprt* alleles are mutant. Each of these mutant alleles, $hprt^A$ and $hprt^B$, produces altered HPRT activity. However, it has been shown that these two alleles complement when together in a hybrid cell, resulting in a growth phenotype close to that of the wild type (CHASIN and URLAUB 1976). This hybrid should be able to segregate 6-thioguanine-resistant subclones by the loss of either the *A* or the *B* allele, so that about half of the HPRT- segregants should be G6PD+ and half should be G6PD-, which is the result found (Table 4, last line). Segregation of *g6pd*+ with retention of $hprt^A$ (and $hprt^B$) occurs at only one-tenth the rate of their co-segregation.

The linkage between $hprt^A$ and *g6pd*+ in hybrid 9Y11 can be directly demonstrated at the level of the molecular phenotype, since the gene products of $hprt^A$ and $hprt^B$ can be distinguished from each other and from the wild-type gene product by isoelectric focusing (CHASIN and URLAUB 1976).

The separation of these three activities is shown in Figure 1. It can also be seen that an extract of the hybrid displays bands of activity intermediate in position between the *A* and *B* activities. This is the expected result from the

TABLE 3

*Cosegregation of G6PD and HPRT in hybrid cell line 51YT211*

Genetic constitution: $\frac{hprt^{+}\ g6pd^{+}}{hprt^{B}\ g6pd^{T}}$

| | Experiment 1 | | Experiment 2 | | | Experiment 3 | | |
|---|---|---|---|---|---|---|---|---|
| Segregant phenotype: | HPRT- G6PD- | HPRT- G6PD+ | HPRT- G6PD- | HPRT- G6PD+ | HPRT+ G6PD- | HPRT- G6PD- | HPRT- G6PD+ | HPRT+ G6PD- |
| No. of cultures* | 20 | | 23 | | 24 | 24 | | 24 |
| No. of cells/sample | $5\times10^{4}$ | | $1.47\times10^{4}$ | | $1.76\times10^{4}$ | $9.27\times10^{3}$ | | $9.27\times10^{3}$ |
| Median No. segregants/sample | 59 | — | 36 | — | — | 48 | 3 | — |
| Mean No. segregants/sample | 72.4 | 0.2 | 64 | 5.70 | 1.08 | 95.4 | 7.33 | 2 |
| Variance | 2617 | 0.128† | 3599 | 246 | 0.99 | 14300 | 88.1 | 38.3 |
| Overall frequency of segregants | $145\times10^{-5}$ | $0.4\times10^{-5}$ | $435\times10^{-5}$ | $38.8\times10^{-5}$ | $6.1\times10^{-5}$ | $103\times10^{-4}$ | $7.9\times10^{-4}$ | $2.2\times10^{-4}$ |
| Segregation rate by median method | $29.9\times10^{-5}$ | — | $69.1\times10^{-5}$ | — | — | $137\times10^{-5}$ | $18.4\times10^{-5}$ | — |
| Segregation rate by LURIA-DELBRUCK (1943), eq. (8) | $23.5\times10^{-5}$ | $0.25\times10^{-5}$ | $57.6\times10^{-5}$ | $7.1\times10^{-5}$ | $1.5\times10^{-5}$ | $129\times10^{-5}$ | $13.8\times10^{-5}$ | $4.6\times10^{-5}$ |

* For experiment 1, each culture contained $10^{5}$ cells. For experiment 2, each culture contained $1.47\times10^{5}$ cells. For experiment 3, each culture contained $9.27\times10^{4}$ cells.

† This value is not corrected for sampling variance since the small number of colonies detected (4) does not allow distinction between sampling variance and variance due to chance distribution of mutations.

TABLE 4

*Summary of cosegregation of G6PD and HPRT in hybrid cell lines*

| Hybrid cell line | Genetic constitution | Segregation rates ($\times 10^5$)* HPRT⁻ G6PD⁻ | HPRT⁻ G6PD⁺ | HPRT⁺ G6PD⁻ | Percent cosegregation G6PD with HPRT† | HPRT with G6PD‡ |
|---|---|---|---|---|---|---|
| 51YT211 | $\frac{hprt^{+}\ g6pd^{+}}{hprt^{B}\ g6pd^{T}}$ | 23.5 | 0.25 | N.D.§ | 99 | |
| | | 57.6 | 7.07 | 1.53 | 89 | 97 |
| | | 129 | 13.8 | 4.65 | 90 | 97 |
| 43YHD31 | $\frac{hprt^{+}\ g6pd^{+}}{hprt^{-}\ g6pd^{-}}$ | 14.8 | 0.46 | N.D. | 97 | |
| YY11 | $\frac{hprt^{+}\ g6pd^{R}}{hprt^{B}\ g6pd^{T}}$ | 33.7 | 0.320 | N.D. | 99 | |
| 9Y11 | $\frac{hprt^{A}\ g6pd^{+}}{hprt^{B}\ g6pd^{T}}$ | 128 | 110 | 14.2 | | 90 |

* As determined by equation (8) of LURIA and DELBRUCK (1943).

† $\frac{\text{Rate HPRT}^-\text{G6PD}^-}{\text{Rate HPRT}^-\text{G6PD}^- + \text{Rate HPRT}^-\text{G6PD}^+}$

‡ $\frac{\text{Rate HPRT}^-\text{G6PD}^-}{\text{Rate HPRT}^-\text{G6PD}^- + \text{Rate HPRT}^+\text{G6PD}^-}$

§ N.D., Not determined.

formation of hybrid enzyme molecules, since the Chinese hamster HPRT appears to be a trimeric enzyme with three identical subunits (OLSEN and MILMAN 1974). Extracts were made from 6-thioguanine-resistant subclones derived from the hybrid 9Y11 and were analyzed for the presence of $HPRT^A$, $HPRT^B$, or hybrid enzyme, by isoelectric focusing. The G6PD phenotype of each subclone was also determined by histochemical staining. The results are shown in Table 5. There is a complete concordance between the retention of $hprt^A$ and $g6pd^+$. Moreover, about half of the segregants have lost $hprt^A$ and half have lost $hprt^B$, indicating that there is an approximately equal probability of segregating each allele, without bias toward one type of segregant in the selection method. This conclusion was also indicated less directly by the data in the last line of Table 4.

In all of the hybrids described so far, the wild-type alleles of *hprt* and *g6pd* were present in coupling. Two hybrids were also constructed with the wild-type

TABLE 5

*Cosegregation of G6PD and HPRT in hybrid cell line 9Y11*

| Genetic constitution: | $\frac{hprt^{A}\ g6pd^{+}}{hprt^{B}\ g6pd^{T}}$ | |
|---|---|---|
| Segregant phenotype: | G6PD⁺ | G6PD⁻ |
| $HPRT^A$ | 9 | 0 |
| $HPRT^B$ | 0 | 13 |

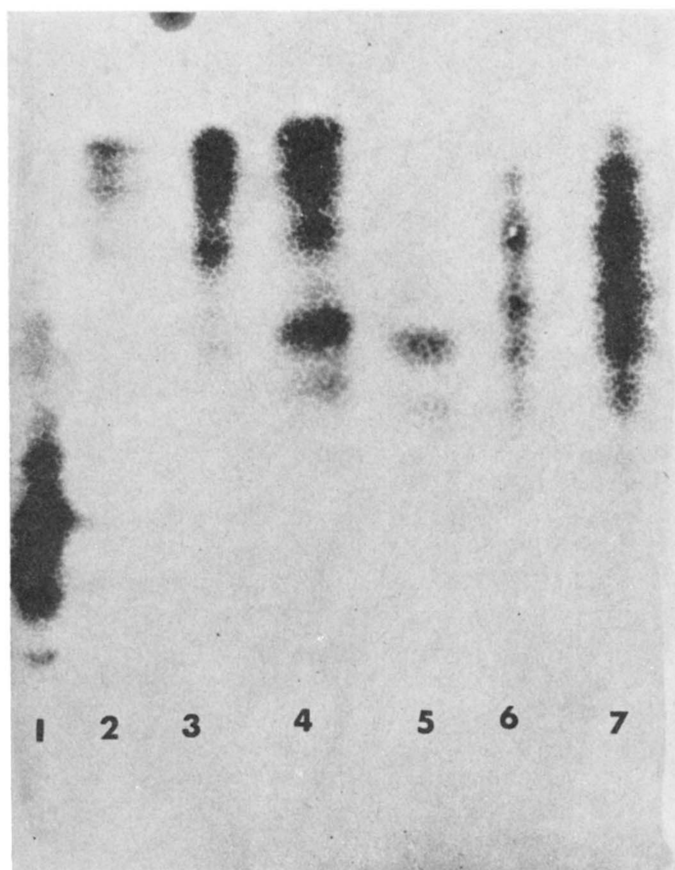


FIGURE 1.—Isoelectric focusing of HPRT activity in wild-type, mutant and hybrid cell extracts. Extracts were prepared and gels were run as described in MATERIALS AND METHODS. *Lane 1*, wild type (5111); *Lane 2*, $HPRT^B$ (YT22); *Lane 3*, extracts prepared from a 1:1 mixture of $HPRT^A$ (5A9) and $HPRT^B$ (YT22) cells; *Lane 4*, extract prepared from a 10:1 mixture of $HPRT^A$ (5A9) and $HPRT^B$ (YT22) cells; *Lane 5*, $HPRT^A$ (5A9); *Lane 6*, hybrid cell extract ($hprt^A/hprt^B$: cell line used was a $G6PD^-$ subclone of 9Y11); *Lane 7*, more concentrated hybrid cell extract (see Lane 6).

TABLE 6

*Frequencies of segregants when wild-type alleles are in repulsion*

| Hybrid cell line | Genetic constitution | No. of cells screened | Frequency of segregant phenotypes ($\times 10^5$) $HPRT^-$ $G6PD^-$ | $HPRT^-$ $G6PD^+$ | $HPRT^+$ $G6PD^-$ |
|---|---|---|---|---|---|
| AAY11 | $\frac{hprt^+ \quad g6pd^T}{hprt^B \quad g6pd^+}$ | $10^8$, $2\times10^7$ | 0.002, 0.005 | 20, 35.5 | N.D.* |
| | | $2\times10^7$ | 0.045 | 129 | N.D. |
| AHT21 | $\frac{hprt^+ \quad g6pd^T}{hprt^- \quad g6pd^+}$ | $1.5\times10^7$ | 0.140 | 568 | 939 |

* N.D., Not determined.

alleles in repulsion. These hybrids were used to test the possibility that the segregants being isolated resulted from a preferential association of epigenetically determined phenotypes. If this were the case, then double segregant phenotypes (*i.e.*, $HPRT^-$ $G6PD^-$) should still be the majority class when wild-type alleles are in repulsion. If genetic linkage were the basis of the segregation patterns, then double segregants should be very rare when the wild-type alleles are in repulsion, since at least two events (*e.g.*, mutation plus chromosome loss) would be necessary to lose both wild-type markers. The results in Table 6 indicate that the latter holds true.

*Analysis of nonparental segregants*: The data presented above show that the majority of segregants from doubly heterozygous hybrid cells lose the coupled markers simultaneously. The interesting question remains as to how the minority class, the class that displays a new nonparental phenotype with respect to HPRT and G6PD, is generated. While such phenotypes could, in principle, arise *via* new point mutations, the rate of appearance of these nonparental type segregants is generally too high ($2.5–142 \times 10^{-6}$ per cell per generation, Table 4) to be accounted for by this mechanism. The spontaneous mutation rate to $HPRT^-$ in parental pseudodiploid CHO cell lines is $2 \times 10^{-7}$ per cell per generation (CHASIN 1973; unpublished results). It is more probable that the nonparental phenotypes result from a disruption of linkage between the *hprt* and *g6pd* loci. Such a disruption in linkage could occur by two different mechanisms: (1) chromosomal deletion, including terminal deletion (equivalent to chromosome breakage) and interstitial deletion; and (2) mitotic recombination, including gene conversion. Experiments designed to distinguish between these mechanisms in two types of hybrid-segregant systems are described below.

(A) *Loss of* g6pd *with retention of* hprt

*Theory*: The hybrid 9Y11 has the genetic constitution $hprt^A$ $g6pd^+/hprt^B$ $g6pd^T$. This hybrid has an $HPRT^+$ phenotype due to the complementation between the *A* and *B* alleles. The loss of the $g6pd^+$ allele without co-segregation of the coupled $hprt^A$ allele results in an $HPRT^+$ $G6PD^-$ nonparental phenotype. As was shown in Table 4, segregants of this type arise at a rate of $1.4 \times 10^{-4}$ per cell per generation. The scheme in Figure 2 illustrates how such clones could result from mitotic recombination, followed by the appropriate distribution (50% probability) of sister chromatids at mitosis, or from chromosome breakage. The gene order shown is not necessarily correct, but is used for illustration because it represents the most general case in that only a single event is necessary to segregate either the *hprt* or the *g6pd* marker. A single recombination or breakage event would also suffice if the order were centromere—*hprt*—*g6pd*. In the case of the third possible gene order, centromere—*g6pd*—*hprt*, two events would be necessary to generate $HPRT^+$ $G6PD^-$ segregants. This last arrangement is the one observed in humans (RUDDLE and CREAGAN 1975). If this order also exists in CHO cells, then selection of this phenotype does not provide a sensitive test for mitotic recombination. On the other hand, this last order could yield $HPRT^-$

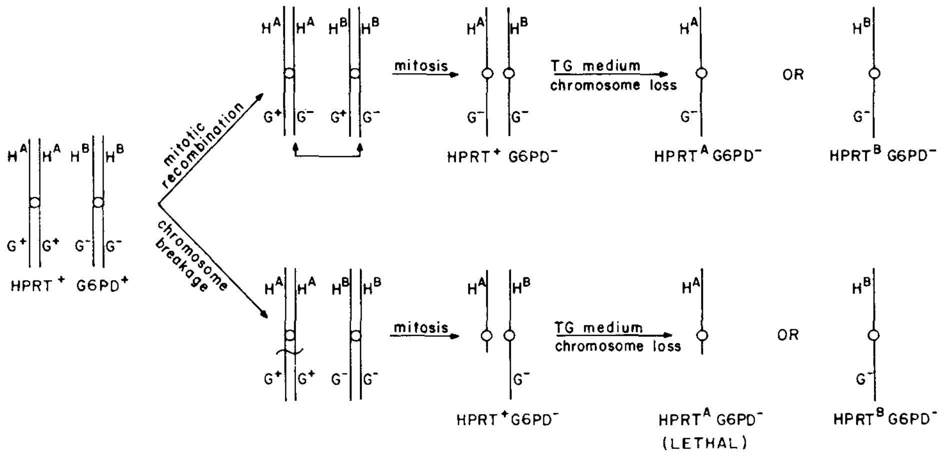


FIGURE 2.—Mechanisms for generating of nonparental HPRT+ G6PD- segregants from hybrid strain 9Y11. (H = *hprt*, G = *g6pd*, TG = 6-thioguanine.)

G6PD+ segregants by a single mitotic recombination event. The generation of this phenotype is dealt with in the next section.

The two mechanisms of mitotic recombination and chromosome breakage leading to HPRT+ G6PD- segregants from hybrid 9Y11 may be distinguished by subjecting the nonparental segregant clones to a second selection, a selection for the segregation of either the $hprt^A$ or the $hprt^B$ allele to generate 6-thioguanine-resistant (HPRT-) subclones. As shown in Figure 2, if mitotic recombination had occurred, then 6-thioguanine-resistant subclones possessing either HPRT$^A$ (lose $hprt^B$) or $HPRT^B$ (lose $hprt^A$) should be recovered at approximately equal frequencies, as was the case for the original hybrid 9Y11 (Table 5). However, if the HPRT+ G6PD- segregants result from chromosome breakage, then 6-thioguanine-resistant subclones possessing HPRT$^A$ may not be recovered, if it assumed that chromosome loss is responsible from most segregation in this system and that vital genes were located on the chromosomal region lost when the original $g6pd^+$ marker was lost. Since these assumptions are not proven, the described experiment cannot be used to prove mitotic recombination (both *A* and *B* alleles recovered), but can be used to rule out (only the *B* allele recoverable).

*Isolation of nonparental segregants*: HPRT+ G6PD- segregants of 9Y11 cannot be directly isolated since there is no selective method for the G6PD- phenotype. An indirect sib-selection procedure was therefore used, based on gradual enrichment for cultures containing (statistically) higher numbers of colonies that stain negatively for G6PD activity (ROSENSTRAUS and CHASIN 1975). The selection was carried out in hypoxanthine plus azaserine medium to force the retention of both *hprt* alleles. Because this is a rather arduous procedure, only three independently arising segregants of this type were isolated. These three segregants (X9Y11, Y9Y11 and Z9Y11) still carry both *hprt* alleles, as expected, since isoelectric focusing of extracts yields the characteristic hybrid HPRT pattern (Figure 1). They are glycine-independent and APRT+ (see Table 1), indicating

TABLE 7

*HPRT⁻ segregation rates in subclones of 9Y11*

| Clone: | X9Y11 | Y9Y11 | Z9Y11 |
|---|---|---|---|
| No. of cultures | 18 | 20 | 24 |
| No. of cells/sample | 3880 | $1.68 \times 10^{4}$ | 9725 |
| Median No. segregants/sample | 7.5 | 19.5 | 22 |
| Mean No. segregants/sample | 14.4 | 30.5 | 39.7 |
| Variance | 430 | 497 | 4136 |
| Overall frequency of segregants | $3.71 \times 10^{-3}$ | $1.82 \times 10^{-3}$ | $4.08 \times 10^{-3}$ |
| Segregation rate by median method | $8.12 \times 10^{-4}$ | $3.77 \times 10^{-4}$ | $7.11 \times 10^{-4}$ |
| Segregation rate by LURIA-DELBRUCK (1943), eq. (8) | $8.66 \times 10^{-4}$ | $3.35 \times 10^{-4}$ | $7.60 \times 10^{-4}$ |

The culture size was twice the sample size for Y9Y-11, and 1.2 times for the others.

that the parental alleles at these loci have not been lost. When grown at 40°, colonies stain negatively for G6PD activity, indicating the absence of the G6PD+ activity. However, when extracts are prepared from cells grown at 37°, reduced levels of G6PD activity (3.6, 4.3 and 7.8 mIU/mg as compared to 41.6 mIU/mg for 9Y11) are found, and this activity is thermolabile (less than 10% activity remaining after 20 minutes at 40°) compared to 9Y11 (84% activity remaining after the same treatment). These properties are characteristic of the *g6pd*$^{T}$ gene product (ROSENSTRAUS and CHASIN 1977) and show that this allele is still present in these three segregants.

*Characterization of 6-thioguanine resistant secondary segregants*: Independent thioguanine-resistant secondary segregants were isolated from each of these clones, as described in MATERIALS AND METHODS. The spontaneous rate of segregation to drug resistance was determined for each clone by fluctuation analysis (Table 7). These rates are similar to the rates in the original hybrid 9Y11 (Table 4). The HPRT phenotypes of 47 6-thioguanine-resistant subclones derived from the three nonparental segregants were determined by isoelectric focusing, and the results are shown in Table 8. No HPRT$^{A}$ subclones were found. All subclones,

TABLE 8

*Biochemical characterization of thioguanine-resistant subclones of nonparental type segregants derived from hybrid 9Y11*

| Cell line | No. of clones tested | HPRT phenotype of segregants | | |
|---|---|---|---|---|
| | | HPRT$^{A}$ | HPRT$^{B}$ | Other |
| X9Y-11 | 15* | 0 | 15 | 0 |
| Y9Y-11 | 17† | 0 | 14 | 3‡ |
| Z9Y-11 | 15† | 0 | 15 | 0 |

* Represents at least 13 independent segregation events.
† All of these clones are independent segregants.
‡ The HPRT type resembled that of the parental line 9Y11; these three segregants can give rise to colonies in 6-thioguanine medium, as well as in hypoxanthine plus azaserine medium.

with the exception of three from Y9Y11, had an $HPRT^B$ phenotype. The three exceptions gave rise to an isoelectric focusing pattern intermediate between a 1:1 hybrid (9Y11) and $HPRT^A$. Moreover, they exhibit a leaky growth phenotype in that they are both resistant to 0.01 mM 6-thioguanine, yet are able to utilize hypoxanthine as a purine source. It is possible that they resulted from a duplication of the chromosome bearing $hprt^A$ and that this gene dosage effect has compromised the complementation.

The lack of $HPRT^A$ segregants is not consistent with a mechanism of mitotic recombination. This result does support the idea that all three nonparental segregants arose as a result of a chromosome breakage event that deleted genes necessary for cell viability. This result also argues against point mutations at the *g6pd* locus as the source of the nonparental segregants, since such mutations would not be expected to yield a recessive lethal chromosome.

(B) *Loss of* hprt *with retention of* g6pd

*Theory*: The hybrid 51YT211 carries the wild-type alleles for HPRT and G6PD in coupling: $hprt^+ \ g6pd^+/hprt^B \ g6pd^T$. The loss of the $hprt^+$ allele without co-segregation of the coupled $g6pd^+$ allele results in an $HPRT^-$ $G6PD^+$ nonparental phenotype. About 1–10% of all $HPRT^-$ segregants from this type of hybrid retain the $G6PD^+$ phenotype (Table 4). Figure 3A shows that $HPRT^-$ $G6PD^+$ clones could result from mitotic recombination or from chromosome breakage. As noted before, the gene order presented is the simplest one in that only a single event is necessary to segregate either marker. If the order were that found in humans, centromere—*g6pd*—*hprt*, then likewise one event would suffice to generate $HPRT^-$ $G6PD^+$ segregants from this hybrid, but if the order were centromere—*hprt*—*g6pd* two breakage or recombination events would be required.

A critical difference between the two mechanisms is that mitotic recombination will produce a new linkage between $hprt^B$ and $g6pd^+$, but chromosome breakage will not. This prediction can be tested, as diagrammed in Figure 3B. $HPRT^-$ $G6PD^+$ segregants are fused with G5A12 cells ($hprt^A \ g6pd^-$) and pseudohexaploid triple hybrid clones are selected in hypoxanthine plus azaserine medium on the basis of the complementation between $hprt^A$ and $hprt^B$. Then independent 6-thioguanine-resistant segregants are isolated from the triple hybrids, and their G6PD and HPRT (*A* or *B*) phenotypes are determined. In the case of mitotic recombination, (Figure 3B-1), the great majority of 6-thioguanine-resistant segregants would have the $HPRT^B$ phenotype (*i.e.*, lose $hprt^A$) since there would be two copies of the $hprt^B$ in the triple hybrid. Those segregants that do have only $HPRT^A$ (*i.e.*, lose both $hprt^B$ alleles) would also be $G6PD^-$, since the lone $g6pd^+$ allele would co-segregate with the linked $hprt^B$ allele (see Figure 3B-1). In the case of chromosome breakage, $HPRT^A$ and $HPRT^B$ segregants should be recovered with equal probability. More importantly, $HPRT^A$ segregants would be $G6PD^+$, since $hprt^B$ and $g6pd^+$ would not be linked (see Figure 3B-2).

*Segregation in control pseudohexaploid clones*: It was anticipated that pseudohexaploid hybrid cells would be less stable than the usual pseudotetraploids, and

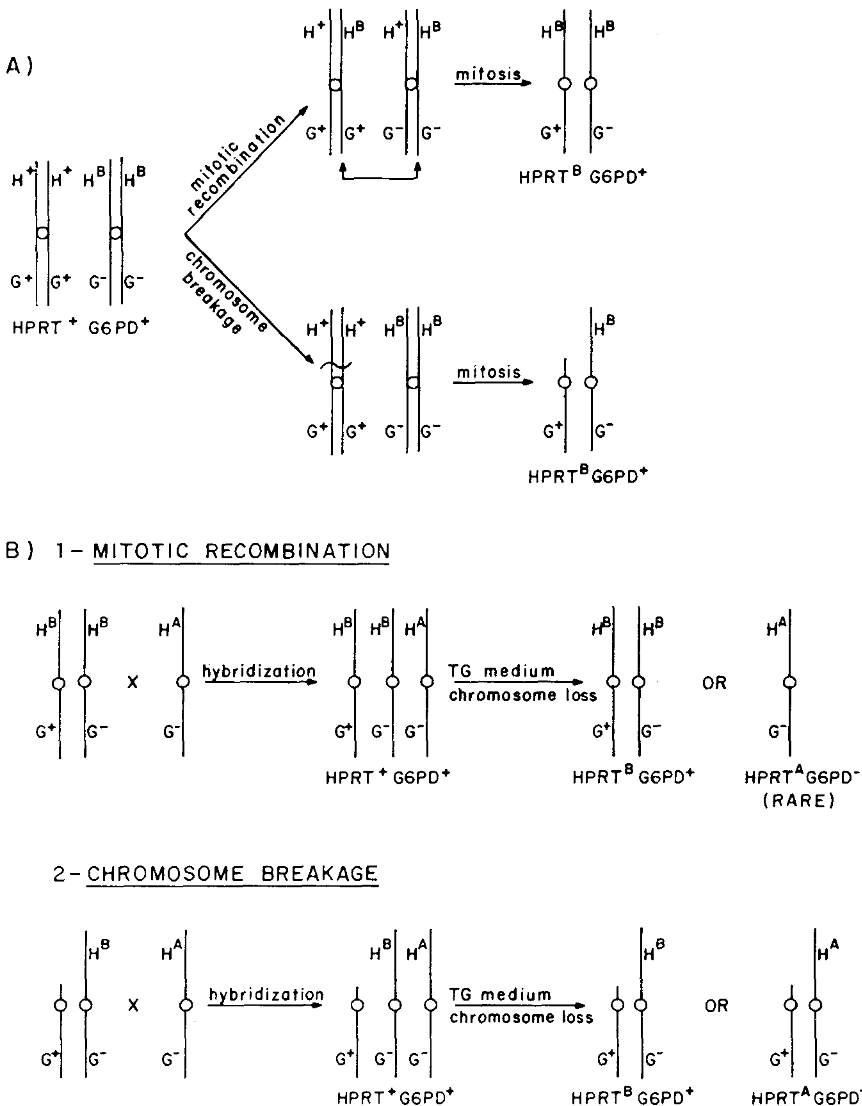


FIGURE 3.—(A) Mechanisms for generating nonparental HPRT- G6PD+ segregants from 51YT211. (B) Method for distinguishing between chromosome breakage and mitotic recombination. (H = *hprt*, G = *g6pd*, TG = 6-thioguanine.)

that the complementation between the *A* and *B* alleles of *hprt* might be altered as a consequence of gene dosage and/or larger cell size. For these reasons, two control clones were constructed to simulate the two types of cells predicted by the models described above. The first control clone has the *hprt* genotype expected under the mitotic recombination model. It was constructed by fusing a spontaneously arising pseudotetraploid (38 chromosomes) derivative of cell line YT22 with 5A9 cells and isolating hybrids in glycine-free medium. Its genetic consti-

tution at the two loci in question can be represented: *hprt*$^{B}$ *g6pd*$^{T}$/*hprt*$^{B}$ *g6pd*$^{T}$/ *hprt*$^{A}$ *g6pd*$^{+}$. This clone has a modal chromosome number of 55 (almost the sum of the parental chromosomes), stains positively for G6PD and has wild-type growth characteristics for all heterozygous markers (*glyA*, *glyB*, APRT, and HPRT). For the selection of 6-thioguanine-resistant subclones from this cell line, it was necessary to increase the drug concentration seven-fold to 0.07 mM, since at lower concentrations there was interference from background growth of the hybrid at moderate cell densities ($10^3$/cm$^2$). Segregation for 6-thioguanine resistance was analyzed by a fluctuation test, the results of which are presented in Table 9, columns 2 and 3. Segregant colonies were stained for G6PD activity to indicate whether they had lost the *hprt*$^{A}$ allele (were G6PD$^-$) or the presumed two *hprt*$^{B}$ alleles (were G6PD$^+$). The HPRT$^-$ G6PD$^-$ segregation rate was about 200 times higher than the HPRT$^-$ G6PD$^+$ segregation rate. Therefore, as predicted, the loss of the single *hprt*$^{A}$ allele was much more frequent than the loss of the two *hprt*$^{B}$ alleles.

For construction of the second control clone, an HPRT$^-$ G6PD$^-$ segregant of the heterozygous pseudotetraploid hybrid line 51YT211 was isolated. This segregant was presumed to have arisen by chromosome loss, resulting in the genotype: – –/*hprt*$^{B}$ *g6pd*$^{T}$. These cells were fused with 5A9 cells and triple hybrids selected in hypoxanthine plus azaserine medium on the basis of the HPRT *A* and *B* complementation. The control clone studied had 55 chromosomes and wild-type phenotypes at all heterozygous loci. If there really were just one copy of each of the two *hprt* alleles in this clone (– –/*hprt*$^{B}$ *g6pd*$^{T}$/*hprt*$^{A}$ *g6pd*$^{+}$), then the segregation rate of each should be about the same, resulting in approximately equal

TABLE 9

*HPRT$^-$ segregation rates in prototype pseudohexaploid cell lines*

| Genetic constitution: | *hprt*$^{A}$ *g6pd*$^{+}$ / *hprt*$^{B}$ *g6pd*$^{T}$ / *hprt*$^{B}$ *g6pd*$^{T}$ | | *hprt*$^{A}$ *g6pd*$^{+}$ / *hprt*$^{B}$ *g6pd*$^{T}$ / —— | |
|---|---|---|---|---|
| Segregant phenotype: | HPRT$^-$ G6PD$^-$ | HPRT$^-$ G6PD$^+$ | HPRT$^-$ G6PD$^-$ | HPRT$^-$ G6PD$^+$ |
| No. of cultures† | 24 | | 24 | |
| No. cell challenged/sample | $9.6\times10^{3}$ | | $1.6\times10^{4}$ | |
| Median No. segregants/sample | 89.5 | — | 27 | 20.5 |
| Mean No. segregants/sample | 123 | 0.08 | 42.1 | 44.7 |
| Variance | 9948 | 0.07‡ | 1587 | 4966 |
| Average frequency of segregants | $129\times10^{-4}$ | $0.09\times10^{-4}$ | $26.1\times10^{-4}$ | $27.8\times10^{-4}$ |
| Segregation rate by median method | $21.9\times10^{-4}$ | — | $5.0\times10^{-4}$ | $4.1\times10^{-4}$ |
| Segregation rate by LURIA-DELBRUCK (1943), eq. (8) | $20.3\times10^{-4}$ | $0.09\times10^{-4}$ | $4.8\times10^{-4}$ | $5.1\times10^{-4}$ |

* HPRT$^-$ segregants were selected in 0.07 mM 6-thioguanine.
† Each culture contained 1.2 times the number of cells per sample.
‡ This value is not corrected for sampling variance since the small number of colonies detected (2) does not allow distinction between sampling variance and variance due to chance distribution of mutations.

segregation rates for G6PD+ and G6PD- phenotypes among HPRT- segregants. This is, in fact, the result obtained, as shown by the fluctuation test in Table 9, columns 4 and 5. In this case, 0.07 mM 6-thioguanine was again used in the isolation of segregant clones.

*Isolation of nonparental segregants and the effect of mitomycin C*: Starting with the heterozygous hybrid 51YT211 (*hprt*+ *g6pd*+/*hprt*$^B$ *g6pd*$^T$), nonparental HPRT- G6PD+ segregants were selected by first subjecting the hybrid population to treatment with diamide to eliminate G6PD- cells (ROSENSTRAUS and CHASIN 1977) and then immediately switching to 6-thioguanine medium to select against HPRT+ cells. Thus, nonparental segregants of this type can be rather readily isolated. However, their subsequent analysis is laborious, and only six independent segregants of this kind have been studied. One of these clones arose spontaneously and five were isolated after treatment with mitomycin C. This drug was used in order to increase the chance of observing a recombination event, since mitomycin C is a potent recombinogen in microbial systems (HOLLIDAY 1964) and in Drosophila (SUZUKI 1965) and is known to increase cytologically detectable crossing over between chromatids and even chromosomes in cultured mammalian cells (SHAW and COHEN 1965; LATT 1974; HUTTNER and RUDDLE 1976). Separate cultures of 51YT211 cells were treated for two hr with 0.3 $\mu$g/ml of mitomycin C, resulting in about a 50% loss in plating efficiency. The survivors were subcultured every two days and periodically tested for the presence of 6-thioguanine-resistant segregants. As can be seen in Figure 4, mitomycin C treatment increased the frequency of nonparental HPRT- G6PD+ segregants about six-fold. It should also be noted that an expression period of two to five days (three to eight generations) was necessary in order to detect this promotion of segregation by mitomycin C. In control experiments, a mitomycin C treatment of this type resulted in a three-fold increase in sister-chromatid exchange, as detected by the fluorescence plus Giemsa method (data not shown), indicating that this mitomycin C treatment was about as effective as those reported in the literature for mammalian cells.

*Characterization of pseudohexaploid secondary segregants*: Each of the six nonparental type clones was fused with G5A12 cells (*hprt*$^A$ *g6pd*-) and hybrids were selected in hypoxanthine plus azaserine medium on the basis of HPRT A and B complementation. Six triple-hybrid clones were isolated and shown to have a pseudohexaploid chromosome number (52–65 overall range) and to contain HPRT activities with an isoelectric focusing pattern characteristic of the complementing A and B mutant HPRT enzyme activities. Secondary segregants resistant to 0.07 mM 6-thioguanine were selected as part of a fluctuation test, the results of which are shown in Table 10. The segregation rates are comparable to that found in a complementing pseudotetraploid hybrid (9Y11, see Table 4). From 11 to 16 6-thioguanine-resistant secondary segregant clones were isolated from each of the triple hybrids. Each was tested for its G6PD-staining phenotype and for the presence of HPRT A or B activity by isoelectric focusing. The results are shown in Table 11. As can be seen in column 4, *hprt*$^B$ is readily lost without the co-segregation of *g6pd*+ in each of the six triple hybrids tested. In one case, two HPRT$^A$

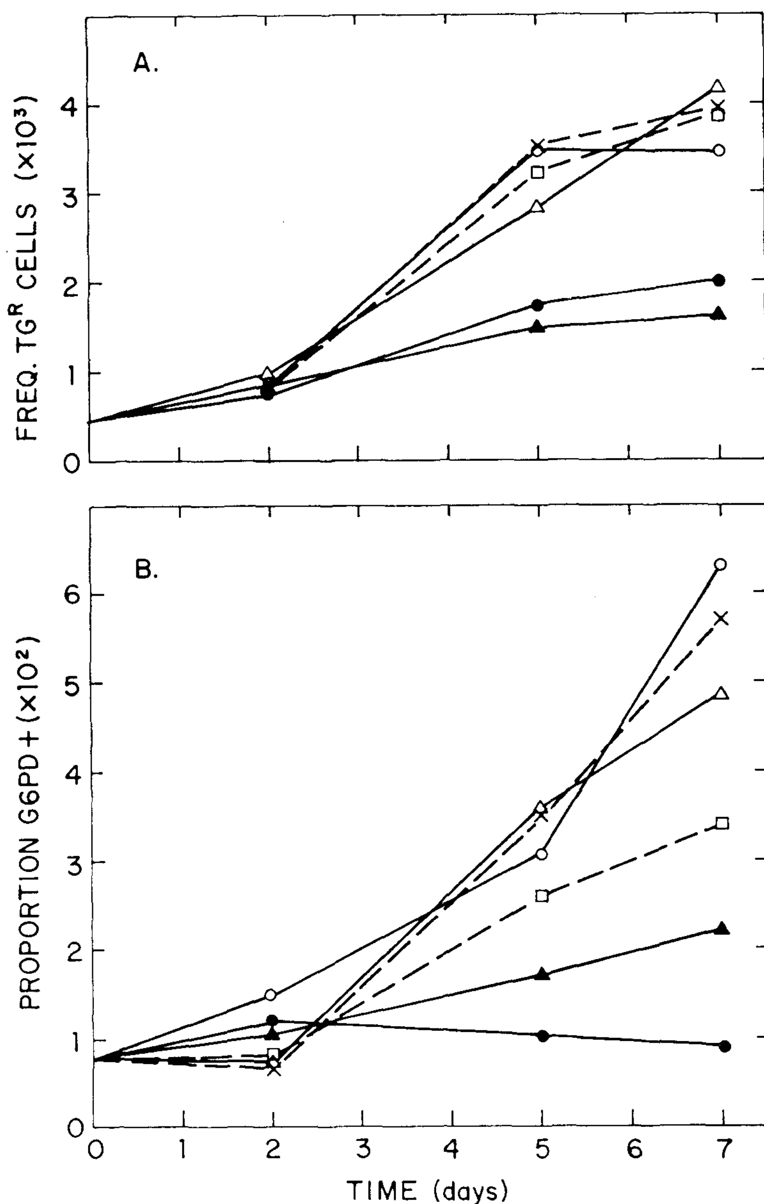


FIGURE 4.—Accumulation of HPRT⁻ segregants in a population of mitomycin C-treated cells. A population of 51YT211 cells was grown in suspension for two days in hypoxanthine plus azaserine medium. Separate monolayer cultures were then established in nonselective medium. The separate cultures served either as controls or were treated with 0.3 $\mu$g/ml mitomycin C for two hrs. The mitomycin C treatment reduced plating efficiency by 50%. Control cultures were initiated with $5 \times 10^5$ cells; mitomycin C-treated cultures consisted of $10^6$ cells. Every other day, cells were subcultured, carrying over $7 \times 10^5$ cells for each culture. At the times indicated, a sample of cells from each culture was challenged in thioguanine medium. After one week, surviving colonies were stained for G6PD activity. (A) Frequency of HPRT$^-$(TG$^R$) segregants. (B) Fraction of HPRT$^-$(TG$^R$) segregants that are G6PD+. To get the overall frequency of HPRT$^-$ G6PD+ segregants, multiply the ordinate value in (A) by the ordinate value in (B). (●, ▲) = control cultures. (□, X, △, ○) = mitomycin C-treated cultures.

TABLE 10

*$HPRT^-$ segregation rates in triple hybrids*

| Clone: | GCA-32 | GMA-12 | GMB-12 | GMC-12 | GMD-21 | GRB-11 | GRB-11-1 |
|---|---|---|---|---|---|---|---|
| No. of cultures | 21 | 22 | 22 | 24 | 20 | 22 | 17 |
| No. of cells/sample | 3800 | 3925 | 6150 | 6400 | $2.37\times10^{4}$ | 6600 | $1.35\times10^{4}$ |
| Median No. segregants/sample | 24 | 45.5 | 78.5 | 89 | 56 | 104 | 38 |
| Mean No. segregants/sample | 49.1 | 63.6 | 84.2 | 122 | 97.8 | 113 | 52.5 |
| Variance | 2822 | 4388 | 2943 | 7726 | 10916 | 7925 | 1197 |
| Overall frequency of segregants | $12.9\times10^{-3}$ | $16.2\times10^{-3}$ | $13.6\times10^{-3}$ | $19.1\times10^{-3}$ | $4.13\times10^{-3}$ | $17.1\times10^{-3}$ | $3.89\times10^{-3}$ |
| Segregation rate by median method | $19.5\times10^{-4}$ | $31.0\times10^{-4}$ | $30.6\times10^{-4}$ | $57.8\times10^{-4}$ | $6.05\times10^{-4}$ | $35.8\times10^{-4}$ | $7.82\times10^{-4}$ |
| Segregation rate by LURIA-DELBRUCK (1943), eq. (8) | $23.8\times10^{-4}$ | $28.5\times10^{-4}$ | $23.1\times10^{-4}$ | $30.2\times10^{-4}$ | $6.90\times10^{-4}$ | $27.7\times10^{-4}$ | $7.31\times10^{-4}$ |

Each culture contained 1.2 times the number of cells per sample. GRB-11-1 is a subclone of GRB-11.

TABLE 11

*Characterization of thioguanine resistant segregants from triple hybrids*

| Cell line | Recombinant parent* | No. of clones tested | No. of segregants with phenotype HPRT$^A$ G6PD$^+$ | HPRT$^B$ G6PD$^+$ | HPRT$^A$ G6PD$^-$ | HPRT$^B$ G6PD$^-$ |
|---|---|---|---|---|---|---|
| GCA-32 | CA-11 | 11† | 10 | 1 | 0 | 0 |
| GMA-12 | MA-11 | 16‡ | 7 | 9 | 0 | 0 |
| GMB-12 | MB-11 | 15§ | 7 | 5 | 0 | 0 |
| GMC-12 | MC-21 | 15‖ | 10 | 5 | 0 | 0 |
| GMD-21 | MD-21 | 15¶ | 5 | 8 | 0 | 0 |
| GRB-11 | RB-21 | 16‖ | 14 | 1 | 1 | 0 |
| GRB-11–1 | RB-21 | 11‖ | 10 | 0 | 1 | 0 |

* CA-11 is a spontaneously occurring recombinant-type clone. All others were isolated after treatment with mitomycin C.
† Represents at least nine independent segregants.
‡ Represents at least 13 independent segregants.
§ Represents at least 14 independent segregants. Three clones were G6PD+ and possessed the hybrid enzyme characteristic of the parent cell line.
‖ All of these clones are independent segregants.
¶ Represents at least 12 independent segregants. Two G6PD+ clones yielded enzyme bands that were intermediate in character between hybrid and B type HPRT activity.

G6PD$^-$ segregants were isolated, indicating the loss of both *hprt*$^B$ and *g6pd*$^+$. However, this must be compared to 24 segregants of the same hybrid and its subclone (GRB-11 and GRB-11–1, Table 11) in which *hprt*$^B$ was lost, but *g6pd*$^+$ was retained. The lack of co-segregation of *hprt*$^B$ and *g6pd*$^+$ in these six hybrids is not consistent with the mitotic recombination model (Figure 3B).

In four of the six triple hybrids, the *hprt*$^A$ and *hprt*$^B$ alleles were lost at about the same frequency, as expected from the chromosome breakage model (Figure 3B). In two hybrids, GCA-32 and GRB-11, the segregation of *hprt*$^B$ is favored, a result not predicted by either model. In the case of GRB-11, this tendency was verified in a subclone. The duplication of the chromosome bearing the *hprt*$^A$ allele in the triple hybrid or in the parent (G5A12) cells could explain this result.

While these results are consistent with the chromosome breakage model, point mutations could also be involved (see DISCUSSION). The main conclusion from this experiment is that mitotic recombination was not involved in the original linkage disruption, the mitomycin C treatment notwithstanding.

## DISCUSSION

The average segregation rate for the *hprt* locus in this system is about $5 \times 10^{-4}$ per cell per generation. The segregation rate for one linked marker without the other is one-tenth to one-hundredth the rate for the co-segregation event, or of the order of $10^{-5}$ per cell per generation. In none of the nine minority clones analyzed could the separation of linked markers observed have arisen by mitotic recombination. Therefore, if mitotic recombination can take place between the *hprt* and *g6pd* loci, the maximum rate would be about $10^{-6}$ per cell per generation. Further-

more, treatment with mitomycin C did not yield any evidence of mitotic recombination despite the stimulation of sister-chromatid exchange and increasing the frequency of marker separation six-fold (Figure 4).

The genetic mechanisms that are responsible for the disruption of linkage in this system include deletion of chromosomal material and point mutation. Gene inactivation would be an alternative explanation, but is not distinguishable from the actual loss of genetic information in these studies. Linkage disruption is strongly suggested in the case of the three nonparental segregants derived from the complementing hybrid clone 9Y11, since recessive lethal chromosomes were apparently produced concomitantly with loss of the *g6pd*$^+$ allele. In the second hybrid, 51YT211, evidence is less direct, but the numbers argue in favor of deletions rather than point mutations. In this case, *hprt*$^+$ is lost, while the linked *g6pd*$^+$ is retained. The loss of HPRT activity by mutation in parental pseudodiploid cells takes place at a rate of about $2 \times 10^{-7}$ per cell per generation. The rate of appearance of spontaneous HPRT$^-$ G6PD$^+$ cells from the doubly heterozygous hybrid is about two orders of magnitude greater than this. Implicit in this argument is the assumption that deletions, as opposed to point mutations, would be lethal in a pseudodiploid cell, and therefore could not contribute to the mutation rate. In hybrid cells, however, they would be freely expressed because of the two-fold redundancy of genetic information. In the case of mitomycin C treatment, the situation is more equivocal, since exposure of pseudodiploid parental wild-type cells to this treatment was mutagenic, yielding 6-thioguanine-resistant colonies at a frequency of $2 \times 10^{-5}$. This is about 20 times higher than the usual spontaneous background frequencies. Mitomycin C induced the HPRT$^-$ G6PR$^+$ phenotype in heterozygous hybrid cells at a frequency of $1.6 \times 10^{-4}$ (Figure 4).

Lack of mitotic recombination has also been reported by PIOUS and his coworkers using a different system. They analyzed near-diploid human lymphoid cell lines that are heterozygous for major histocompatability antigens. Subclones that lost one antigenic specificity, but not a linked isozyme marker, were isolated (PIOUS, HAWLEY and FORREST 1973). Gene dosage for the remaining allele was determined by estimating the amount of antigen per cell (PIOUS, BODMER and BODMER 1974). A mechanism of mitotic recombination would have predicted a doubling of the remaining antigen, but this was not found. In these cells, mitotic recombination might be tantamount to deletion in that recessive lethals could be exposed. This restriction does not apply to the hybrid cells analyzed here.

In both of these systems, the negative conclusions regarding mitotic recombination cannot be generalized, since only a small region of the genome is being investigated. In fact, cytogenetic evidence points to a nonrandom distribution of crossover points in quadriradial structures (BOURGEOIS 1974; HUTTNER and RUDDLE 1976; SHAW and COHEN 1965; THERMAN and KUHN 1977), with exchanges more often seen near centromeric heterochromatin (GERMAN, CRIPPA and BLOOM 1974; HUTTNER and RUDDLE 1976; KUHN 1976). Thus, it is still possible that recombination takes place at relatively high levels in mitotically

dividing mammalian cells, but is restricted to "hot spots", none of which happen to lie between *hprt* and *g6pd* in CHO cells.

The chromosomal deletions responsible for the disruption in linkage between *hprt* and *g6pd* could be of two types: terminal or interstitial. If terminal deletions are the predominant type, then the gene order *hprt*-centromere-*g6pd* is suggested, since either marker can be lost without the other at roughly the same rate (Table 4, line 1). However, it could well be that interstitial deletions contribute significantly, in which case no conclusions about relative gene order can be drawn. In fact, in Drosophila interstitial deletions are much more common than terminal deletions (ROBERTS 1975). Cytogenetic studies of nonparental segregants may resolve this question (FARRELL and WORTON 1977).

If the *hprt* and *g6pd* loci are located on the same chromosome arm, then the possibility remains that mitotic recombination is frequently occurring between the centromere and the proximal marker. In this case parental-type segregants would result. This possibility could be tested by gene dosage measurements and/or twin-spot experiments analogous to those of ZIMMERMANN (1973).

The overall question of whether any mitotic recombination takes place in mammalian cells awaits the availability of additional linked genetic markers in a variety of linkage groups. While "hot spots" of recombination may indeed be found, the negative results reported here suggest that the general frequency of mitotic recombination is probably too low to allow fine-structure (intracistronic) genetic mapping.

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