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Linkage Relationships of X-linked Enzymes Glucose-6-Phosphate Dehydrogenase and Hypoxanthine Guanine Phosphoribosyltransferase: Recombination in Female Offspring of Compound Heterozygotes

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In agreement with Lyon's hypothesis of X-chromosome inactivation [1, 2], somatic cells of mammalian females heterozygous for X-linked genes express only one or the other allele [3–7]. Inactivation of either the paternal or maternal X chromosome takes place early in embryonic development and remains stable in clonally derived somatic cells. In clones from individuals heterozygous for two X-linked genes, the alleles on the active X chromosome are consistently expressed together whether or not they are closely linked [8, 9]. While unselected tissues reveal mosaicism for X-linked genes, it is possible to determine the coupling phase for each X chromosome in compound heterozygotes by in vitro cell culture studies [9, 10].

Segregation of the X-linked genes for complete deficiency of the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT) which manifests clinically as the Lesch-Nyhan syndrome [11, 12] and for glucose-6-phosphate dehydrogenase (G6PD) variants A and B has been observed in two independent families through 3 generations. Preliminary studies of erythrocyte G6PD types in members of one family had disclosed two recombinants among four affected males and suggested hemizygous expression in the erythrocytes of two heterozygous females [13]. We now report the results of cell culture studies which have determined the genotypes of the females in both families. The data provide further evidence for selective events in the development of the hematopoetic system and increase to 13 the number of cases informative for recombination.

MATERIALS AND METHODS

Selection and Cloning of Skin Fibroblasts

Fibroblast cultures were established from skin biopsies and were grown in Eagle's minimum essential medium (MEM), in Ham's F10, or in Coon's modified F12* [14]

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* Reagents used in this study were purchased from the following companies: Coon's modified

containing 10% fetal calf serum, penicillin, and streptomycin in humidified air with 5% CO_2. For selection experiments, cells from early passages were plated at low density ($1–2 \times 10^2$ cells/60 mm dish). Cells containing HGPRT were selected for by adding 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine (HAT) [15] to F12 medium which contains glycine. For selection of HGPRT-deficient cells, either 6×10^{-5} M 6-thioguanine (6-TG; 2-amino-6-mercaptopurine) [16] or 2×10^{-5} M 8-azaguanine (8-AG) [17] was added immediately before use to the F10 medium from which hypoxanthine had been omitted. In each selective system, actively growing clones were isolated after 2–3 weeks and carried in selective medium continuously until analyzed biochemically. For this purpose a confluent monolayer of cells in a large plastic flask (Falcon) was rinsed with warm phosphate-buffered saline and exposed to 0.05% trypsin in sodium EDTA buffer for 3 min. The floating cells were suspended in medium containing fetal calf serum and counted in a Coulter counter. After centrifugation the cells were washed once each in serum-free medium and in EX-buffer (0.01 M Tris-HCL, pH 7.4, containing 0.0025 M $MgCl_2$ and 0.14 M KCl). Cell pellets were stored dry at 4° C overnight or at −70° C for several days and then assayed by gel electrophoresis. Replicate fibroblast cultures from each individual were maintained in MEM or F10 without selective agents and used for enzyme analysis as unselected cell controls.

Electrophoretic Analyses of HGPRT, APRT, and G6PD

The dry cell pellets were resuspended in EX-buffer at a concentration of 10^6 cells/100 μl and lysed by three cycles of freezing and thawing. After centrifugation at 24,000 *g* for 20 min at 4°C, aliquots of the supernatant fluid were analyzed for HGPRT and adenine phosphoribosyltransferase (APRT) activities and for G6PD isozymes by polyacrylamide gel disc electrophoresis [18–20].

Electrophoresis of hair root lysates from female members of family SB was performed in order to determine heterozygosity for HGPRT deficiency [21].

Attempt to Induce Mitotic Recombination

Fibroblasts from a 6-TG–resistant clone of subject II-2 of kindred HFJ (compound heterozygote) which were completely lacking in HGPRT activity and contained only G6PD A were exposed to agents capable of inducing mitotic recombination in yeast [22]. Ethylmethane sulfonate was added to F10 medium in concentrations of 5×10^{-3} M and 10^{-2} M for various times up to 4 hr; N-methyl-N′-nitro-N-nitrosoguanidine was used in concentrations of 5×10^{-6} M and 10^{-5} M for up to 3 hr. Fibroblasts from a patient with complete HGPRT deficiency were treated the same way as controls. Forty-eight hours after treatment with mutagenic agents, HAT was added to the culture medium in an attempt to recover clones in which HGPRT activity might have been restored.

RESULTS

Case Report and Pedigree Data

Kindred SB. The propositus (III-1) was born to a healthy 18-year-old Negro woman. The pregnancy had been complicated by hemorrhage in the third trimester, associated decrease in fetal movements, and excessive weight gain. The boy was delivered by cesarean section 2 weeks prior to term and weighed 6 lb 4 oz. He was

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F12—Grand Island Biological Co., Santa Clara, Calif.; fetal calf serum—Grand Island Biological Co., Santa Clara, Calif., and Flow Laboratories, Inglewood, Calif.; 6-thioguanine (2-amino-6-mercaptopurine)—Schwarz/Mann, Orangeburg, N.Y.; 8-azaguanine—Nutritional Biochemicals Corp., Cleveland, Ohio; ethylmethane sulfonate—Eastman Organic Chemicals, Rochester, N.Y.; N-methyl-N′-nitro-N-nitrosoguanidine—Aldrich Chemical Co., Milwaukee, Wis.

slow in developing motor control. At 7 months he was diagnosed as having athetoid cerebral palsy. At 2 years of age after an episode of herpes stomatitis, he compulsively began to bite his lower lip; 6 months later he started to mutilate his fingers. On examination at $2\frac{8}{12}$ years of age, he was below the third percentile for height, weight, and head circumference. The lower lip was partly missing and the index fingers were marked by scars and signs of infection. Generalized spasticity was most pronounced in the lower extremities. The deep tendon reflexes were hyperactive and there were positive Babinski responses bilaterally. He had pronounced extensor thrust and choreoathetoid movements and was unable to sit without support for longer than 30 sec. His head control was poor. His developmental quotient had been estimated at between 55 and 71 on different scales and was highest in social functions. Analysis of his erythrocytes revealed complete deficiency of HGPRT and the G6PD variant A.

Pedigree data and the results of in vitro studies are illustrated in figure 1. The patient's father could not be tested. His mother (II-2) had four siblings: II-3 died at the age of 9 months from an unknown cause; II-4 and II-5 were healthy 17-

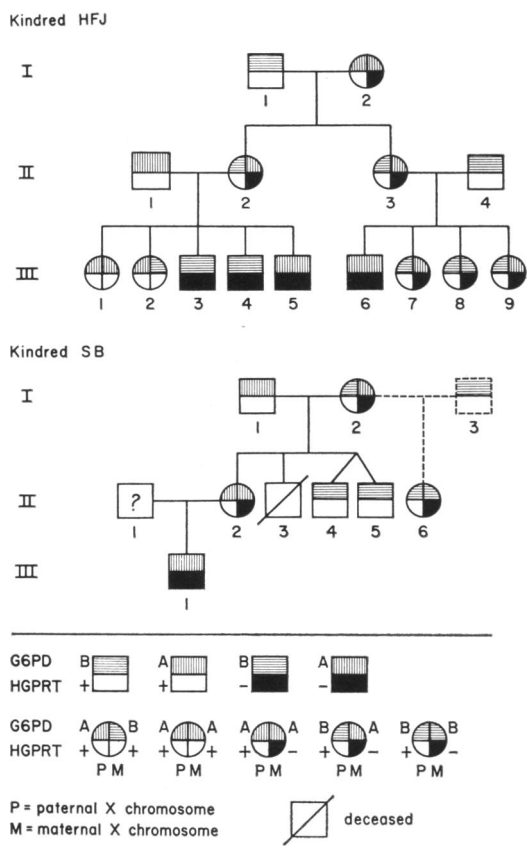

FIG. 1.—Pedigrees of kindreds HFJ and SB indicating the HGPRT and G6PD genotypes

year-old twins proven to be dizygotic; and II-6 appeared phenotypically different from the rest of the family, her G6PD type suggesting nonpaternity. As the family denied further cooperation, other genetic markers including Xg^a phenotypes could not be studied.

Kindred HFJ. A partial pedigree of this family has previously been published [13]. In addition to the females depicted there, three sisters of patient III-6 were included in the present study (III-7, 8, and 9). Analysis of hair roots [8] and skin fibroblasts [10, 13] of subjects II-2 and II-3 provided confirmation that in these two sisters the gene for G6PD A is on same X chromosome as the gene for HGPRT deficiency, while G6PD B is coupled with the normal HGPRT allele. The Xg^a phenotypes were not informative for linkage and failed to contribute information regarding inactivation of the Xg locus. There was no history of consanguinity between these two kindreds.

Hair Root Studies

Hair roots from the females of kindred SB were analyzed for activities of HGPRT and APRT after electrophoretic separation of these enzymes [21]. When HGPRT activity is considered in relation to the activity of the autosomal enzyme APRT, individual hair roots from heterozygotes for complete HGPRT deficiency typically fall into one of three categories (fig. 2). The theoretical basis and practical application of this approach to carrier detection were established by Gartler and colleagues [23, 24, 8]. The results obtained on the female members of kindred SB leave little doubt that all three of them are heterozygous for the Lesch-Nyhan syndrome (table 1). This interpretation is in agreement with results obtained using tissue culture methods.

G6PD and HGPRT Expression in Erythrocytes, Fibroblasts, and Clones of Selected Fibroblasts

In tables 2 and 3, the results obtained in unselected fibroblasts and erythrocytes from each individual have been compared with the G6PD and HGPRT phenotypes

TABLE 1

RESULTS OF HAIR ROOT STUDIES IN FEMALES OF KINDRED SB

SUBJECT	NO. HAIR ROOTS WITH: Normal HGPRT (>30% APRT)	Intermediate HGPRT (<30% APRT)	Absent HGPRT	TOTAL
Possible heterozygotes:				
SB I-2	0	5	17	22
SB II-2	4	13	9	26
SB II-6	0	7	16	23
Controls:				
Normal individuals ($N = 12$)	48	0	0	48
Lesch-Nyhan patients ($N = 3$)	0	0	8	8

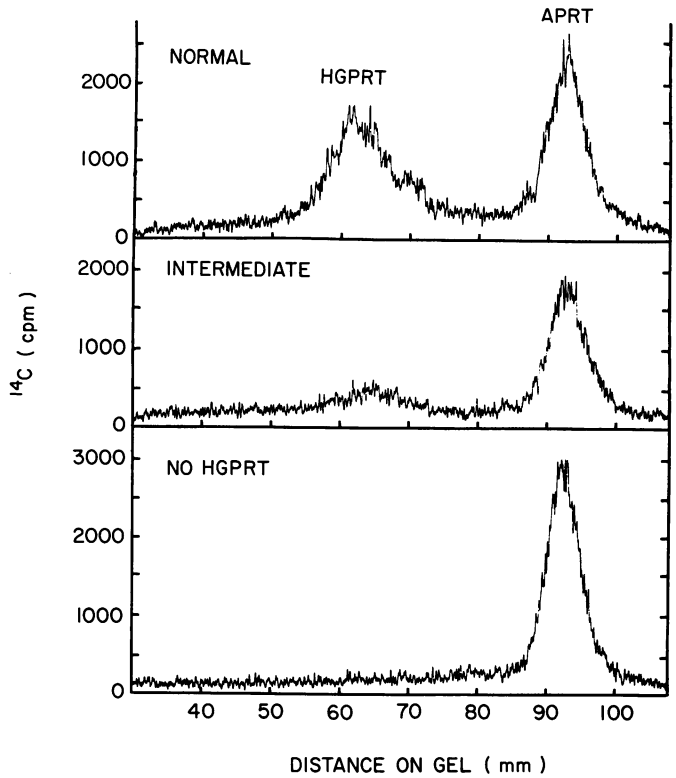

FIG. 2.—Radioelectropherograms of ^{14}C-IMP and ^{14}C-AMP reflecting activities of the enzymes HGPRT and APRT in three different single hair roots from subject II-2 of kindred SB.

in fibroblast clones isolated in selective media. All clones which lacked HGPRT activity contained normal amounts of APRT, indicating that the cells were metabolically active. The G6PD activity demonstrated the presence of a functional X chromosome. The expression of G6PD and HGPRT alleles in clones derived from compound heterozygotes provided information on the cis/trans arrangement of these alleles in each individual (fig. 1, table 2).

Although cells from subject I-2 of kindred HFJ were not cloned, assumptions about her genotype can be made. The A/+ phenotype observed in blood and unselected fibroblasts together with the transmission of an X chromosome carrying A/— to both daughters favors the interpretation that she was a Lesch-Nyhan carrier and homozygous for G6PD A. Because of the latter, recombination in subjects II-2 and II-3 of kindred HFJ cannot be determined. Seven female offspring of compound heterozygotes were informative for linkage. Three instances of recombination (SB II-6, HFJ III-1, and III-8) and four nonrecombinants (SB II-2, HFJ III-2, III-7, and III-9) were observed (table 2).

There were three males in kindred SB. The fraternal twins II-4 and II-5 were nonrecombinants. The propositus III-1 was not informative for linkage since his

TABLE 2

G6PD AND HGPRT TYPES FOUND IN ERYTHROCYTES, UNSELECTED FIBROBLASTS, CLONES IN HAT, AND CLONES IN 8-AG OR 6-TG IN FEMALES OF KINDREDS SB AND HFJ

KINDRED AND INDIVIDUAL	ERYTHROCYTES		SKIN FIBROBLASTS						COUPLING PHASE		RECOMBINATION
			Unselected		Selected in HAT		Selected in 8-AG or 6-TG				
	G6PD	HGPRT	G6PD	HGPRT	G6PD	HGPRT	G6PD	HGPRT			
SB:											
I-2	B	+	AB	+	B (8)*	+	A (11)	−	B +	A −	...
II-2	A	+	A	+	A (4)	+	A (7)	−	A +	A −	NR
II-6	B	+	B	+	B (10)	+	B (4)	−	B +	B −	R
HFJ:											
I-2	A	+	A	+	Not done		Not done		A +	A −	...
II-2	B	+	AB	+	B (13)	+	A (9)	−	B +	A −	...
II-3	B	+	AB	+	B (10)	+	A (6)	−	B +	A −	...
III-1	A	+	A	+	A (3)	+	No growth		A +	A +	R
III-2	AB	+	AB	+	A (11) B (1)	+ +	No growth		A +	B +	NR
III-7	B	+	AB	+	B (3)	+	A (7)	−	B +	A −	NR
III-8	B	+	B	+	B (13)	+	B (7)	−	B +	B −	R
III-9	B	+	AB	+	B (2)	+	A (4)	−	B +	A −	NR

NOTE.—R = recombinant, NR = nonrecombinant.
* No. clones analyzed.

mother (II-2) was homozygous for G6PD A. In kindred HFJ, two of four informative males represented crossover products [13].

The clonal data provide evidence that subject II-6 of kindred SB is homozygous for G6PD B. Since her alleged father shows type A in his blood, nonpaternity was considered. It is conceivable that clones of type B/+ could have arisen by mitotic crossing over between the two loci if subject II-6 were truly A/+ and B/—. In order to test for this possibility in vitro, A/— cells from a compound heterozygote were placed in HAT medium with and without previous exposure to mutagenic agents. If clones developing in this selective medium were tested for G6PD type and HGPRT activity, two combinations would be possible. While clones of type

TABLE 3

G6PD AND HGPRT TYPES FOUND IN ERYTHROCYTES AND FIBROBLASTS FROM MALES OF KINDREDS SB AND HFJ

KINDRED AND INDIVIDUAL	ERYTHROCYTES		FIBROBLASTS		RECOMBINATION
	G6PD	HGPRT	G6PD	HGPRT	
SB:					
I-1	A	+	...	...	...
II-4	B	+	...	...	NR
II-5	B	+	...	...	NR
III-1	A	—	A	—	...
HFJ:					
I-1	B	+	...	...	...
II-1	A	+	...	...	...
II-4	B	+	...	...	...
III-3	B	—	B	—	R
III-4	B	—	B	—	R
III-5	A	—	A	—	NR
III-6	A	—	A	—	NR

NOTE.—R = recombinant, NR = nonrecombinant.

B/+ would indicate reactivation of the silent X chromosome or incomplete selection of the initial cell population, a clone of type A/+ could suggest a reverse mutation in the HGPRT locus on the active X chromosome or mitotic recombination with reactivation of part of the inactive X. In four separate experiments, none of the 10^5–10^6 cells surviving the drug treatment formed clones in HAT medium while they were still able to grow in regular F10. Therefore, mitotic recombination with reactivation of part of the silent X chromosome, if it occurs in cell culture, does not appear to be a frequent event and would not be expected to interfere with studies on the coupling phase in compound heterozygotes.

The fibroblast and clonal data reported so far on subject II-3 of kindred HFJ have all been based on cells from the initial skin biopsy. A second biopsy taken from the same site and handled in the same way grew out cells which appeared to contain only G6PD B in the unselected state. In repeated selection experiments exposing a total of 5×10^6 cells to 6-TG, we were unable to recover a single A/— clone. However, a small number of clones isolated and propagated in selective medium turned out to be B/+ on biochemical analysis.

DISCUSSION

Recombination between the X-linked loci for HGPRT and G6PD was found to have occurred in five of 13 offspring of compound heterozygous females. This result raises the question whether the observed recombination fraction $\theta = .38$ means that the two loci are within measurable distance on the X chromosome. Under the assumption of a true recombination fraction of $\theta = .50$, the probability of finding this result or a more extreme outcome would be $P = .29$ (for $N = 13, i = 5$) according to binomial tables [25]. Thus the observed recombination fraction is not signifi-

cantly different from $\theta = .50$, a value which would be expected for loci on different chromosomes. Taking into account the possibility of double crossovers which would result in reduction of the apparent recombination fraction, the map distance between both loci is calculated as 51 cM using a modification of Kozambi's formula [26].

This result excludes close linkage of HGPRT and G6PD on the X chromosome. It appears probable that there is no measurable distance between these loci. The total genetic length of the X chromosome has been estimated indirectly to be 150–160 cM, based on autosomal chiasma counts in male meiosis. There have been no direct observations of crossovers between the two X chromosomes in female meiosis. However, because of the existing evidence that crossovers are more frequent in females, it has been assumed that the genetic length of the human X chromosome might be in the range of 250 cM [27]. Correlation of our results with data on cytological mapping of the X chromosome would support this assumption. Mapping of genes on the X chromosome by interspecific cell hydridization using human X-autosome translocations has failed to demonstrate separation of the loci for HGPRT and G6PD. Both loci were initially assigned to the short arm/centromere region [28]. Using the same translocation, Ricciuti and Ruddle [29] have assigned both loci to a portion of the long arm. Evidence from a different translocation has supported this interpretation and further defined the localization of both genes in the distal half of the long arm [30]. After prolonged propagation in culture, Miller et al. [31] observed mitotic separation of HGPRT and G6PD in two of six clones of man-mouse somatic cell hybrids. They attributed this to chromosomal breakage between the two loci with selective retention of the HGPRT-bearing portion.

Our results provide further evidence for hemizygous expression of G6PD in red blood cells from heterozygotes for the Lesch-Nyhan syndrome [13, 32, 33]. The erythrocytes of five compound heterozygotes (HFJ II-2, II-3, III-7, III-9, and SB I-2) have been found to contain only G6PD B which is in coupling with $HGPRT^+$. Their unselected fibroblasts were G6PD AB, and all of the $HGPRT^-$ clones contained G6PD A. In contrast, subject III-2 of kindred HFJ who was heterozygous for G6PD but not for HGPRT deficiency had erythrocytes of both types A and B. This finding supports the idea that selection against the HGPRT-deficient erythropoetic cells is responsible for hemizygous expression. Alternative explanations, such as selection against red blood cells containing G6PD A or a strictly monoclonal origin of erythrocyte precursors, are rendered less likely.

The observation that fibroblasts of only one type grew out of a skin biopsy from a proven heterozygote has obvious implications for the use of cell culture methods in heterozygote detection. The finding that clones developing in medium containing 6-TG turned out to be B/+ indicated that resistance to purine analogues does not necessarily prove HGPRT deficiency. Similarly, DeMars and Held [34] have found rare spontaneous 8-AG–resistant mutants of diploid human fibroblasts most of which contained HGPRT activity. If only a small number of purine analogue–resistant clones are found, it might be advisable for heterozygote detection that

they be tested further by enzyme assay or autoradiographic studies before being accepted as HGPRT-deficient mutants.

SUMMARY

Segregation of the X-linked genes for HGPRT deficiency and for the G6PD variants A and B has been observed in two kindreds. In females, heterozygosity for HGPRT deficiency was determined by hair root analysis and by cloning and selection of skin fibroblasts. Cell clones selected for the presence or absence of a functioning HGPRT gene on the active X chromosome were analyzed for their G6PD type. The results allowed determination of the coupling phase of the alleles in these individuals and led to assignment of gene pairs to the paternally or maternally derived X chromosome. Among the seven female and six male children of three compound heterozygous females, five instances of crossovers were observed. The recombination fraction $\theta = .38$ excludes close linkage and suggests absence of linkage when analyzed statistically.

Hemizygous expression of G6PD was found in erythrocytes from five compound heterozygotes, while red cells of both types A and B were present in a G6PD heterozygote who did not carry the gene for HGPRT deficiency.

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