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Expression of Two X-Linked Genes in Human Hair Follicles of Double Heterozygotes

(Lesch–Nyhan syndrome/gene inactivation/Lyon hypothesis)

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ABSTRACT **Expression of the two X-linked loci glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and hypoxanthine:guanine phosphoribosyltransferase (HGPRT; EC 2.4.2.8) was studied in single hair follicles of two females who were heterozygous for both of these genes (double heterozygotes). The coupling phase for these two loci was known to be *g6pd A* and *hgprt*$^-$ on the maternal X chromosome and *g6pd B* and *hgprt*$^+$ on the paternal X. Three phenotypic classes of hair follicles were observed in both double heterozygotes: G6pd A follicles with deficient HGPRT activity, G6pd B follicles with normal HGPRT activity, and G6pd AB follicles with intermediate HGPRT activity. These data directly demonstrate one of the predictions of the Lyon hypothesis that for two X-linked loci, those genes in *cis* position are turned on or off in a cell and its clone, while in *trans* only one gene or the other is expressed.**

The X-chromosome inactivation hypothesis (Lyon hypothesis) predicts that the mammalian female is a mosaic with respect to whether her paternal or maternal X chromosome is active in a somatic cell (1). Furthermore, this chromosomal differentiation is heritable somatically; that is, a cell with a paternal-X active and a maternal-X inactive should give rise to somatic progeny all with the same pattern of X-chromosome activity. At the level of single loci, these predictions have been directly demonstrated in cloning experiments of fibroblast cell cultures from human females heterozygous for one of four different X-linked genes: glucose-6-phosphate dehydrogenase (*g6pd*) (2), hypoxanthine : guanine phosphoribosyltransferase (*hgprt*) (3), Hunter's syndrome (4), and Fabry's disease (5). Similar, but less direct, evidence has been available in the mouse for individual loci from observations of variegation effects in animals heterozygous for X-linked genes that affect coat color and form (1).

For two X-linked loci, the Lyon hypothesis predicts that genes in *cis* configuration (i.e., both genes on the same X) should both be turned off or on in a cell and its clone, while when in *trans* configuration (i.e., the two genes are on different X chromosomes), only one gene or the other should be expressed. In man, only limited and incomplete evidence is available on this point. Nyhan *et al.* recently reported two sisters, both obligate heterozygotes at the *g6pd* and *hgprt* loci (double heterozygotes), who exhibited only a single G6pd type and single Hgprt type in their hematopoietic cells (6). As was determined from family analysis, the expressed G6pd and Hgprt types were in *cis* configuration, the single erythrocyte phenotype apparently being due to selective overgrowth of the hematopoietic precursor cells expressing the *hgprt*$^+$ allele. This observation is compatible with the X-inactivation hypothesis in that the expressed genes were in *cis* configuration, although only one of the alternative types was observed (*g6pd B*, *hgprt*$^+$; but not *g6pd A*, *hgprt*$^-$). In the mouse, several pairs of genes affecting coat color and texture have been studied; in some cases the above predictions of a single X-chromosomal inactivation have been observed (e.g., Tabby and Striated) and in other cases the predictions have not been substantiated (e.g., Tabby and Dappled) (7). Since these gene pairs studied in the mouse may often be acting in different cells or even structures, the interpretation of these data in terms of the X-inactivation hypothesis is not clear. The ideal system, therefore, for examining X-inactivation involves the study of pairs of autonomous genes that are normally expressed in the same cell.

Recent studies from this laboratory have documented the value of human hair follicles in the study of X-chromosome inactivation (8, 9). The hair follicle develops from a small number of precursor cells; since the cells of the scalp are not distributed at random, but exhibit a certain degree of clonal growth, the probability of obtaining a single follicle with only one of the two X-linked alleles being expressed in all cells approaches 50% (8). A female heterozygous for a single X-linked gene will show three phenotypic classes of hair follicles: those consisting of cells expressing only the wild-type allele, those with cells expressing only the mutant allele, and a third class consisting of both kinds of cells (8, 9). It thus follows that by studying the hair follicles of a female heterozygous for two X-linked genes for which the coupling phase is known it should be possible to determine if X-inactivation involves alleles on only one or on both X chromosomes. In this report, we present the results of such a study utilizing the two X-linked genes *g6pd* and *hgprt* in double heterozygotes.

MATERIALS AND METHODS

Patients C-1 and C-2 were previously reported by Nyhan *et al.* and correspond to II-2 and II-3, respectively, in the published pedigree (6). Single hair follicles were plucked from their scalps and only those follicles with visible bulbs and

Abbreviations: HGPRT, hypoxanthine:guanine phosphoribosyltransferase (EC 2.4.2.8); Hgprt, phenotype denoting ability or otherwise to synthesize this enzyme; *hgprt*, gene directing this synthesis; G6PD, G6pd, and *g6pd*, similarly for glucose-6-phosphate dehydrogenase (EC 1.1.1.49); APRT, adenine phosphoribosyltransferase (EC 2.4.2.7).

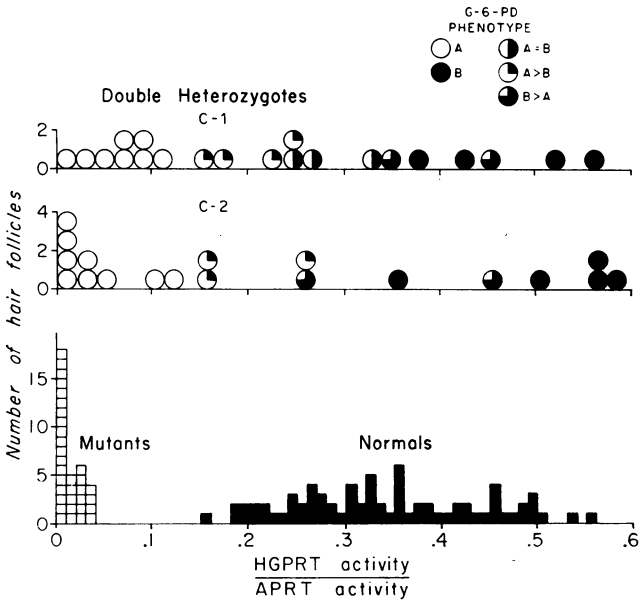

FIG. 1. *Lower half:* HGPRT activity in single hair follicles of mutants and normals. HGPRT activity was determined in single hair follicles (9) and is expressed as a ratio of HGPRT activity to APRT activity (HGPRT/APRT). Each box represents one hair follicle. Normals, ■; mutants, □. *Upper half:* Correlation of G6pd phenotype and HGPRT activity in single hair follicles of two female double-heterozygotes. Each *circle* represents one hair follicle on which both G6pd phenotype and HGPRT activity were determined. G6pd phenotype is indicated by the legend in the upper right. HGPRT activity, expressed as HGPRT/APRT, is indicated by the position of the circle on the horizontal axis.

sheaths were used. Each follicle was placed onto a small piece of filter paper (0.5 cm × 1.0 cm) that had been dampened in buffer A (25 mM Tris-chloride (pH 7.4)–25 mM $MgCl_2$–3 μg/ml of bovine serum albumin–1.6 mg/ml (each) of penicillin and streptomycin). The filter paper with the hair follicle attached to it was then put into a test tube and stored at −20°C until further use. The following assays were performed on each single hair follicle: electrophoresis of G6PD, determination of HGPRT activity, and determination of adenine:phosphoribosyltransferase (APRT; EC 2.4.2.7) activity.

Determination of G6PD isozymes by electrophoresis

Each hair follicle, attached to the filter paper, was frozen (dry ice) and thawed three times, inserted into a slot in a 12% starch gel, and was then electrophoresed for 19 hr at 2.5 V/cm at 4°C. After the initial 30 min of electrophoresis, sufficient G6PD activity had migrated into the gel that the hair follicle could be removed from the filter paper insert for assay of HGPRT and APRT (see below). The preparation, electrophoresis, and staining of the gels were as described (8). With this method, the mobilities of G6PD isozymes A and B are clearly different by visual inspection and the two isozymes can be separated in an artificial mixture or in material obtained from a G6pd heterozygote (8). When the two isozymes are present in an artificial mixture of unequal proportions, as prepared from a red-cell lysate, the G6PD isozyme of lesser concentration is visible when it represents as little as 10% of the total mixture. To obtain this degree of sensitivity, it is necessary to apply a minimum activity of G6PD of 8.2 nmol/min to the gel. It was not possible to assay the G6PD content of each hair follicle quantitatively prior to its electrophoresis, but it was apparent from the intensity of the gel staining that the amount of G6PD activity electrophoresed in some hair follicles was less than 8.2 nmol/min and, therefore, a minor component comprising more than 10% of the G6PD could have gone undetected. Two controls (erythrocyte lysate and an extract of cultured skin fibroblasts from a patient known to be G6pd type AB) were routinely coelectrophoresed with each group of eight hair follicles and they served as reference standards for the qualitative determination of G6pd phenotype.

Determination of HGPRT activity and APRT activity

Upon its removal from the filter paper insert (see above), each hair follicle was placed in 0.03 ml of buffer A for determination of both its HGPRT activity and APRT activity by a double-label assay (9). This assay uses the substrates [^{3}H]adenine and [^{14}C]hypoxanthine. APRT activity (Δ nmol [^{3}H]AMP formed per hr at 37°C per hair follicle) serves as a reference for comparison of the HGPRT activity (Δ nmol [^{14}C]IMP formed per hr at 37°C per hair follicle) of different hair follicles (9). Therefore, the HGPRT activity of each follicle is expressed as a ratio of HGPRT activity to APRT activity (HGPRT/APRT). Although some HGPRT activity and APRT activity of each follicle migrated into the gel during electrophoresis of G6PD, control studies indicated that most of the HGPRT and APRT remained in the hair follicle and that the ratio of HGPRT to APRT for each follicle was unaltered by the short period of electrophoresis.

Isotopes

[^{14}C]hypoxanthine (40 Ci/mol) and [^{3}H]adenine (1.6 Ci/mmol) were obtained from Schwartz BioResearch Co.

RESULTS

The HGPRT activity of single hair follicles from two males with HGPRT deficiency (proved by clinical manifestations of Lesch–Nyhan syndrome and by HGPRT assays of skin fibroblasts and red cells) and 12 normal subjects (consisting of six males and six females, ranging in age from 8 to 50) is shown in the lower half of Fig. 1. Although the normal hair follicles exhibit a wide range of HGPRT/APRT, there is no overlap with the values from the two affected hemizygotes. Moreover, normals and mutants can be clearly differentiated from Lesch–Nyhan heteroxygotes (9). Both C-1 and C-2, known to be heterozygotes for both the *hgprt* and *g6pd* genes, show the expected three classes of follicles with respect to HGPRT activity: those with only mutant activity, those with intermediate activity, and those with normal activity (upper half, Fig. 1). Similarly, both women showed three classes of hair follicles with respect to their G6PD isozymes: those with only type A, those with only type B, and those containing both types A and B.

A striking finding emerged when the HGPRT activity and G6PD isozyme type of each follicle in these two women were correlated. As presented in the upper half of Fig. 1, all hair follicles containing only the A isozyme of G6PD had the lowest activity of HGPRT. A number of these follicles were clearly in the mutant range for HGPRT and some had HGPRT activities higher than the mutant, but well below normal. In all hair follicles in which both G6PD A and B were clearly detected, the corresponding HGPRT activity fell in a

broad intermediate range and each follicle showed the expected correlation between G6PD B content and HGPRT activity (upper half, Fig. 1). Finally, all hair follicles that had only detectable the B isozyme of G6PD had normal HGPRT activity and, in fact, the highest HGPRT activities observed in these two women were found in the G6PD B follicles.

DISCUSSION

From analysis of family data previously reported by Nyhan *et al.* (6), it is known that the two double heterozygotes C-1 and C-2 carry the alleles for *g6pd A* and *hgprt*$^-$ on their mother's X chromosome and the alleles for *g6pd B* and *hgprt*$^+$ on their father's X chromosome. If X activation involves genes that are *cis* in position, then in these two women all *hgprt*$^-$ follicles should be *g6pd A*, all follicles with intermediate levels of *hgprt* activity should contain both *g6pd A* and *B*, and all *hgprt* normal follicles should be *g6pd B*. The actual data, as presented in Fig. 1, fit well with the above predictions. One exception, however, is the finding of G6pd A hair follicles that have HGPRT activity that is slightly above mutant levels. Since the assay for G6PD at best misses a minor component of 10% or less, it is possible that these follicles contained some G6PD B.

Our data, therefore, provide evidence at the biochemical level that genetic inactivation of these two loci has occurred on the same X chromosome. The importance of this finding is that it shows that two different genes, which are known to be widely separated on the X chromosome (6, 10), are not being inactivated independently. Rather, a single chromosomal effect, as predicted by the Lyon hypothesis, is demonstrated, since these two loci on the X are correlated in genetic expression. Furthermore, in contrast to the selection that is observed in hematopoietic cells (6), it can be concluded from these studies that there is no selection against cells of human hair follicles deficient in HGPRT.

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