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### **Research Article**

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## Molecular Basis of Hypoxanthine-Guanine Phosphoribosyltransferase Deficiency in Ten Subjects Determined by Direct Sequencing of Amplified Transcripts

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### Abstract

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency is an inborn error of purine metabolism. Mutant HPRT gene sequences from patients deficient in enzyme activity have previously been characterized by cDNA cloning or amino acid sequencing techniques. The presence of HPRT-specific mRNA in nearly all deficient subjects, as well as the small size of the HPRT mRNA (1,400 bp), make the polymerase chain reaction (PCR) an alternative for the identification of mutations at this locus. In this report we use the PCR to identify previously undetermined mutations in HPRT mRNA from B lymphoblasts derived from 10 deficient individuals. Six of these variants contain single point mutations, three contain deletions, and one contains a single nucleotide insertion. Several of these mutations map near previously identified HPRT variants, and are located in evolutionarily conserved regions of the molecule.

### Introduction

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency is a disorder of purine metabolism responsible for two distinct clinical syndromes. Complete deficiency of HPRT causes the Lesch-Nyhan syndrome, which is characterized by hyperuricemia, hyperuricaciduria, and severe neurologic dysfunction consisting of hyperreflexia, choreoathetosis, mental retardation, and self-mutilation (1, 2). Patients with partial deficiency have severe gout and uric acid nephrolithiasis, but are usually spared neurologic dysfunction (3).

Early reports of HPRT deficiency demonstrated heterogeneity of kinetic and electrophoretic properties of the protein

(4, 5). The original hypothesis proposed to explain these observations was that different, independent mutations occurred in the structural gene (6). Subsequent molecular studies confirmed this observation (7, 8). More recently, amino acid sequencing and cDNA cloning have been used to identify the molecular basis of HPRT deficiency in 11 subjects (9–19).

Study of these naturally occurring mutants has revealed the structural and catalytic importance of certain regions of the molecule. However, the techniques used to identify these mutations are laborious, and have been dependent on sufficient residual levels of HPRT protein for amino acid sequence analysis or HPRT-specific mRNA for cDNA cloning.

In this report, we have used the polymerase chain reaction (PCR) to amplify HPRT-specific sequences from total RNA isolated from B lymphoblasts derived from 10 patients (20). Three of these subjects have partial deficiency and seven have Lesch-Nyhan syndrome. Direct sequencing of the amplified products allowed rapid identification of mutant gene sequences. This technique is applicable to the identification of mutations and carrier status for any inborn error of metabolism for which normal DNA sequence information is available. From these data, important structure-function relationships can be inferred.

### Methods

**Cell lines.** The methods used to establish and maintain B lymphoblastoid cell lines have been described (21). Cell lines J.M., M.S., and W.B. were derived from patients with partial HPRT deficiency. W.B. is the maternal uncle of M.S. Cell lines E.C., B.S., D.G., D.M., H.D., D.A., and K.M. were derived from unrelated patients with Lesch-Nyhan syndrome. Cell line GM558 is derived from a normal male (Human Mutant Cell Repository, Camden, NJ).

**PCR primers.** The two PCR primers that consistently gave us a high level of amplification were HT5' and HT3'. HT5' hybridizes to nucleotides –118 to –101 of the antisense strand of the HPRT transcript, while HT3' anneals to nucleotides 727–759 of the sense strand. The sequences of HT5' and HT3' are GCGAACCTCTCGGCTTTC and AAGCTCTACTAAGCAGATGGCCACAGAACTAGA, respectively. Primer HT3' is the reverse complement of the sense strand of HPRT, with nucleotide 759 listed first and nucleotide 727 given last.

**RNA isolation and in vitro amplification.** RNA was isolated from the above cell lines using guanidium isothiocyanate (22). Total cellular RNA (1 µg) was primed with oligo d(T)<sub>12–17</sub> (23; Pharmacia Fine Chemicals, Piscataway, NJ) and reverse transcribed in a total reaction volume of 50 µl. A portion of this reaction was diluted 1:5 in a buffer containing 25 mM KCl, 0.2% gelatin, 40 µM deoxynucleoside triphosphate (dNTP), 1 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 1 µg PCR primers (Figs. 1 and 2). The reaction mixture was overlaid with mineral oil, and DNA amplification was performed in a Thermocycler (Perkin-Elmer Cetus). 30 cycles of am-

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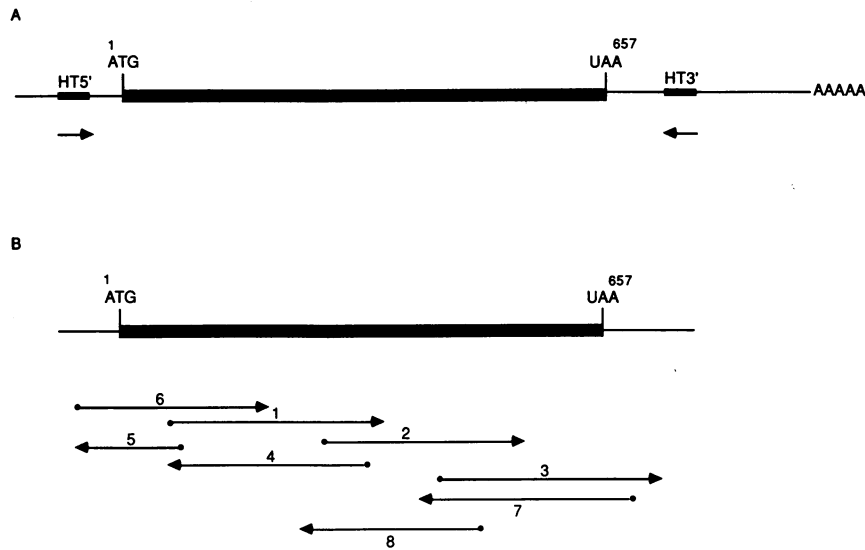
1. **Abbreviations used in this paper:** dNTP, deoxynucleoside triphosphate; HPRT, hypoxanthine-guanine phosphoribosyltransferase; PCR, polymerase chain reaction; PRPP, 5-phosphoribosyl-1-pyrophosphate.

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**Figure 1.** Strategy for PCR amplification and sequencing of amplified products of HPRT variants. (A) The PCR primers HT5' and HT3' were used to amplify HPRT sequences from oligo d(T) primed DNA:RNA hybrids synthesized using avian myeloblastosis virus reverse transcriptase. The relative priming sites (stippled boxes) and direction of extension for HT5' and HT3' (arrows) are indicated. The base number for the A of the ATG start codon and the terminal A of the UAA stop codon are indicated. (B) For direct sequencing of amplified products, phosphorylated primers specific for HPRT (1–8) were annealed and extended as described in Methods. The direction of extension is indicated by arrows. The extent of the overlapping sequence is represented approximately by the overlap of the arrows.

plification were done with each cycle consisting of 94°C, 1 min, 55°C, 1 min, and 72°C, 3 min. Each cell line was reverse transcribed and amplified a minimum of three times from independent RNA isolates to insure the authenticity of mutations found upon sequencing of amplified transcripts.

**Sequencing.** Sequencing of the amplified HPRT transcripts was done directly without cloning using dideoxynucleotide chain termination (24). All of the coding sequence of both strands was sequenced using eight HPRT-specific oligodeoxynucleotides that hybridize to HPRT coding sequence internal to the PCR primers. The primers were phosphorylated with [ $\gamma$ -<sup>32</sup>P]dATP (6,000 Ci/mmol) and T4 polynucleotide kinase and used in eight separate sets of sequencing reactions (13, 25). The regions to which these primers anneal, and the extent of

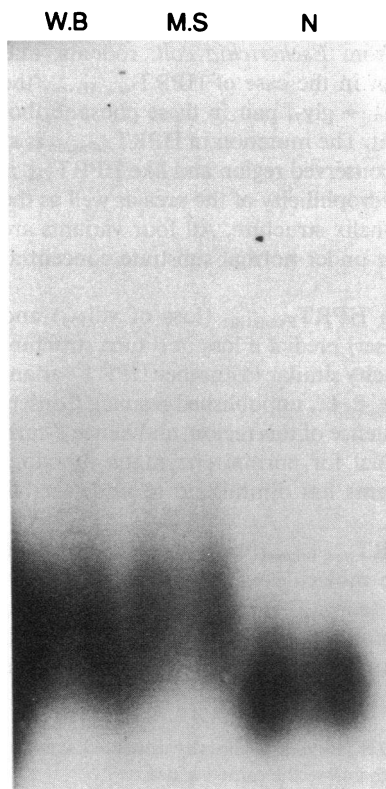
overlap of the sequence generated, are depicted in Fig. 2. Direct sequencing was done using a modification of the procedures described by Engelke et al. (26). 2  $\mu$ l amplified DNA was combined with 4  $\mu$ l of ddNTP mixture (600  $\mu$ M each dNTP and 40  $\mu$ M of the appropriate dideoxynucleotide), 4  $\mu$ l of 10 $\times$  buffer (1 $\times$  buffer is 20 mM Hepes, pH 7.5, 50 mM NaCl, and 10 mM MgCl<sub>2</sub>), and 100,000 cpm of one of the <sup>32</sup>P-labeled HPRT-specific primers. The samples, in a total reaction vol of 40  $\mu$ l, were denatured for 3 min at 95°C, then placed at 42°C for 5 min to allow the primer to anneal. This was followed by the addition of 1 U of Sequenase (U.S. Biochemical Corp., Cleveland, OH) and a 5-min polymerization step at 42°C. The denature/anneal/polymerize cycle was repeated twice. A solution of proteinase K (1 mg/ml), SDS (2%), and EDTA (100 mM, pH 8.0) was added, and the reaction mixture was incubated at 50°C for 30 min. The samples were precipitated on dry ice after the addition of EDTA, NaCl, glycogen (final concentrations 7 mM, 100 mM, and 0.02 mg/ml, respectively), and 2 vol of ethanol, and resuspended in 8  $\mu$ l of dideoxy sequencing dye (98% formamide, 10 mM EDTA, and 0.1% xylene cyanol FF and bromophenol blue). An aliquot of each reaction (4  $\mu$ l) was electrophoresed at constant wattage (60 W) for either 1.5 or 3 h through 4% polyacrylamide gels containing 8 M urea. The gels were fixed in 5% methanol/5% acetic acid for 10 min, transferred to 3MM paper (Whatman Laboratory Products Inc., Clifton, NJ), dried under vacuum at 80°C, and autoradiographed.

**Enzyme analyses.** HPRT enzyme activity was measured in membrane-free lysates as previously described (27). The final concentrations of hypoxanthine, 5-phosphoribosyl-1-pyrophosphate (PRPP), MgCl<sub>2</sub>, and Tris-HCl are 50  $\mu$ M, 200  $\mu$ M, 4.2 mM, and 50 mM, respectively. Nondenaturing PAGE was performed in 6% polyacrylamide gels using 500  $\mu$ g of protein from subjects W.B., M.S., K.M., and D.G. 5  $\mu$ g of total protein from GM558 cell lysate was used as a normal control. After electrophoresis the gels were overlaid with a reaction mixture containing 1 mM [8-<sup>14</sup>C]hypoxanthine (57 mCi/mmol), 15 mM PRPP, 30 mM MgCl<sub>2</sub>, and 100 mM Tris-HCl, pH 7.4. The gel was incubated at 37°C for 2 h and blotted onto polyethyleneimine cellulose (Sybron/Brinkman, Westbury, NY). The polyethyleneimine cellulose was then dried and autoradiographed (28).

**Secondary structure predictions.** The PEPTIDESTRUCTURE and PLOTSTRUCTURE programs for prediction of secondary structure were from the Genetics Computer Group, Madison, WI, and have been previously described (14, 29).

## Results

All seven cell lines derived from patients with the Lesch-Nyhan syndrome (D.A., D.G., H.D., K.M., D.M., E.C., and



**Figure 2.** Activity stain of HPRT from W.B. and M.S. (HPRT<sub>Arlington</sub>). Approximately 500  $\mu$ g of protein from membrane-free lymphoblast extracts isolated from cell lines W.B. and M.S., and 5  $\mu$ g of protein from a normal control (GM558) were electrophoresed through a 6% nondenaturing polyacrylamide gel and stained for HPRT activity (see Methods). The migration of HPRT<sub>Arlington</sub> from subjects W.B. and M.S. is less anodal than normal, consistent with the aspartic acid to valine substitution at amino acid 80.

B.S.) and two subjects with partial deficiency (W.B. and M.S.) had levels of HPRT activity < 0.1 nmol/min per mg (< 0.7% of control). HPRT activity in J.M. was 0.2 nmol/min per mg (1.4% of control).

Direct dideoxynucleotide sequencing of the PCR-amplified HPRT transcripts from these 10 cell lines revealed nine different mutations. RNA isolation, reverse transcription, amplification, and sequencing were repeated at least three times for each cell line. For each variant the same mutation was identified in independent amplifications, and was the sole departure from normal HPRT cDNA sequence. Thus, we conclude that these mutations are authentic and do not represent silent polymorphisms. These nine variants are designated HPRT<sub>Evansville</sub> (B.S.), HPRT<sub>Michigan</sub> (D.A.), HPRT<sub>New Haven</sub> (D.G.), HPRT<sub>Chicago</sub> (D.M.), HPRT<sub>New Briton</sub> (E.C.), HPRT<sub>Connersville</sub> (H.D.), HPRT<sub>Milwaukee</sub> (J.M.), HPRT<sub>Detroit</sub> (K.M.), and HPRT<sub>Arlington</sub> (W.B. and M.S.). The mutations in all variants except HPRT<sub>New Haven</sub> and HPRT<sub>Arlington</sub> have been confirmed by RNase mapping (Palella, T. D., unpublished observations) and are summarized in Table I.

HPRT<sub>Chicago</sub> has a single nucleotide (T) insertion at base 56, 57, or 58. The sequence CCTTGA becomes CCTTTGA resulting in a shift in the reading frame and the occurrence of a stop codon (UGA) at this position. The deletions in HPRT<sub>Evansville</sub>, HPRT<sub>Michigan</sub>, and HPRT<sub>Connersville</sub> predict shortened translation products as well. In HPRT<sub>Connersville</sub> all of exon 8 is deleted, resulting in a change in the reading frame and the occurrence of a stop codon 15 nucleotides downstream from the exon 7:exon 9 junction. HPRT<sub>Michigan</sub> is missing a codon, predicting a translation product shortened by a single amino acid. The deletion in HPRT<sub>Evansville</sub> predicts the replacement of those nucleotides coding for the carboxy-terminal 4 amino acids and stop codon (lys-tyr-lys-ala-stop) with a sequence that encodes an additional 28 amino acids before a stop codon is reached. The predicted translation product of HPRT<sub>Evansville</sub> is thus 24 amino acids longer than the normal protein.

Two of the five variants arising from point mutations predict a change in the electrophoretic properties of the enzyme, HPRT<sub>New Haven</sub> and HPRT<sub>Arlington</sub>. In a previous report HPRT<sub>New Haven</sub> was shown to be an acidic variant by Western blotting (7). This is consistent with the predicted glycine to glutamic acid substitution at amino acid 70. The predicted electrophoretic alteration in HPRT<sub>Arlington</sub> was confirmed using an HPRT activity assay on native protein in polyacrylamide gels (see Methods). The hypoxanthine, PRPP, and MgCl<sub>2</sub> were > 20, 75, and 7.2 times higher than the substrate concentrations used under normal assay conditions so that any kinetic incapacity would be overcome. As shown in Fig. 2, HPRT<sub>Arlington</sub> is a basic variant, consistent with the aspartic acid to valine substitution.

## Discussion

In the past, the basis of HPRT deficiency states has been characterized through the use of amino acid sequencing (9–12), cDNA cloning (13–19), and in one recent case, by denaturing gradient gel electrophoresis followed by amplification of the region of interest by the PCR (30). These methods are time consuming and labor intensive. More recently, the sequence of PCR-amplified transcripts from HPRT<sub>London</sub> and normal HPRT have been reported (17, 31). In both cases sequences

were determined by cloning into M13 bacteriophage and sequencing. However the misincorporation rate of Taq polymerase ( $2 \times 10^{-4}$ /nucleotide per cycle [20]) necessitates sequencing many independent clones to authenticate mutations. In this report we use direct sequencing of PCR-amplified products to circumvent both of these problems in the identification of nine previously undescribed mutations.

The molecular basis of HPRT deficiency in 20 of 24 deficient subjects from our original survey population have now been defined. This includes two recently acquired variants, HPRT<sub>Arlington</sub> and HPRT<sub>Detroit</sub>. Of the remaining four HPRT variants from this population, four have undetectable levels of HPRT mRNA on Northern blot analysis, but have no gross genetic rearrangements on Southern blots. The remaining variant is currently under study. Thus, the population of 20 variants<sup>2</sup> consists of 13 point mutations (65%), 5 deletions (25%), 1 single nucleotide insertion (5%), and 1 duplication (5%).

Although the deletions in HPRT<sub>Connersville</sub> and HPRT<sub>Evansville</sub> and the insertion in HPRT<sub>Chicago</sub> predict grossly altered protein structures, single amino acid substitutions or small deletions may affect protein stability, kinetic capacity, or subunit interactions as well (9–18). To investigate whether those variants with point mutations or a single codon deletion may cause structural perturbations, we used the PEPTIDESTRUCTURE and PLOTSTRUCTURE programs of the Genetics Computer Group to predict structural changes (29). As summarized in Table II, changes in secondary structure are predicted in four of the six variants, while hydrophobic changes are predicted in all cases. All six variants map near previously defined mutations.

The increase in hydrophilicity predicted for HPRT<sub>New Haven</sub> (gly<sub>70</sub> → glu) is similar to two previously defined mutants, HPRT<sub>Flint</sub> (phe<sub>74</sub> → leu; reference 13) and HPRT<sub>Yale</sub> (gly<sub>71</sub> → arg; reference 16). All fall within a region of predicted  $\beta$ -turn and  $\alpha$ -helix structure that is heavily conserved among phosphoribosyltransferases from *Escherichia coli*, rodents, and humans (32), and, except in the case of HPRT<sub>New Haven</sub>, the initial glycine of the gly<sub>70</sub> → gly<sub>71</sub> pair in these phosphoribosyltransferases is invariant. The mutation in HPRT<sub>Arlington</sub> is at the carboxyl end of this conserved region and like HPRT<sub>Yale</sub> is predicted to affect the hydrophilicity of the area as well as the conserved  $\beta$ -turn and  $\alpha$ -helix structure. All four variants are catalytically incompetent under normal substrate concentrations.

The substitutions in HPRT<sub>Evansville</sub> (loss of val<sub>179</sub>) and HPRT<sub>Milwaukee</sub> (ala<sub>161</sub> → ser) predict a loss in  $\beta$ -turn structure and change in hydrophilicity similar to another HPRT variant (pro<sub>174</sub> → leu) (Davidson, B. L., unpublished results). Conservation of amino acid sequence of this region, and hence  $\beta$ -turn structure, may be essential for normal enzymatic function, since each of these variants has diminished to undetectable levels of enzyme activity.

The mutation in HPRT<sub>New Briton</sub> (phe<sub>199</sub> → val) is near the carboxy terminus of the molecule where two other human mutants, HPRT<sub>Kinston</sub> (asp<sub>194</sub> → asn) and HPRT<sub>Ashville</sub> (asp<sub>201</sub> → gly), and one mouse neuroblastoma HPRT variant, NBR4 (asp<sub>201</sub> → asn), have been mapped. A common feature

2. Two cell lines, G.S. and D.B., though apparently unrelated, contain the same mutation and are therefore the same variant.

Table I. Mutations in HPRT Determined by PCR

Mutant	Cell line	Nucleotide change(s)	Putative amino acid change(s)
<b>Insertion</b>			
HPRT <sub>Chicago</sub>	D.M.	Insertion of T <sub>56</sub> *	Translation termination at asp <sub>20</sub>
<b>Deletions</b>			
HPRT <sub>Connersville</sub>	H.D.	Deletion <sub>532→609</sub> (exon 8)	Loss of phe <sub>178</sub> → asn <sub>203</sub>
HPRT <sub>Michigan</sub>	D.A.	GTT <sub>535→537</sub> deleted	Loss of val <sub>179</sub>
HPRT <sub>Evansville</sub>	B.S.	Deletion <sub>643→663</sub>	Loss of lys <sub>215</sub> → stop codon; addition of 24 amino acids (new stop site)
<b>Point mutations</b>			
HPRT <sub>Detroit</sub>	K.M.	T <sub>122</sub> → C	leu <sub>41</sub> → pro
HPRT <sub>New Haven</sub>	D.G.	G <sub>209</sub> → A	gly <sub>70</sub> → glu
HPRT <sub>Arlington</sub>	M.S., W.B.	A <sub>239</sub> → T	asp <sub>80</sub> → val
HPRT <sub>Milwaukee</sub>	J.M.	G <sub>481</sub> → T	ala <sub>161</sub> → ser
HPRT <sub>New Briton</sub>	E.C.	T <sub>595</sub> → G	phe <sub>199</sub> → val

\* The nucleotide insertion could occur at nucleotide position 56, 57, or 58.

of these variants is decreased substrate affinity demonstrated by large increases in the  $K_m$ 's for both PRPP and hypoxanthine (12, 18, 33). Secondary structure analysis predicts changes in the hydrophilicity of the protein in the area surrounding these mutations. The kinetic parameters for HPRT<sub>New Briton</sub> cannot be determined due to insufficient levels of protein (7).

Although changes in secondary structure parameters are altered in most HPRT variants, correlating changes in secondary structure to the loss of enzyme function are speculative. In the absence of known three-dimensional structures for the native and mutant proteins predictive programs of this type are one way to assess the possible impact of amino acid substitutions. Estimates of accuracy using these predictive techniques range from 50 to 70% (34, 35).

In summary, PCR is a rapid means for determining mutations in HPRT, allowing the identification of nine additional variants. Although mutations in HPRT are scattered throughout the molecule, clusters of mutations are now apparent. The location of these regions, in concert with secondary structure predictions, refines our understanding of structural features in HPRT that may be necessary for proper enzymatic function and stability.

Table II. Structural Changes in HPRT Variants

Mutant	Change in hydrophilicity	Change in secondary structure
HPRT <sub>Arlington</sub>	Decreased	$\alpha$ -Helix and random coil converted to $\beta$ -sheet
HPRT <sub>Detroit</sub>	Increased	N.P.*
HPRT <sub>Milwaukee</sub>	Increased	Loss of two $\beta$ -turns
HPRT <sub>New Briton</sub>	Increased	N.P.
HPRT <sub>New Haven</sub>	Increased	N.P.
HPRT <sub>Evansville</sub>	Decreased	Loss of two $\beta$ -turns

\* No change predicted.

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