Identification of a single nucleotide change in a mutant gene for hypoxanthine-guanine phosphoribosyltransferase (HPRT$_{\text{Ann Arbor}}$)

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Summary. HPRT$_{\text{Ann Arbor}}$ is a variant of hypoxanthine (guanine) phosphoribosyl-transferase (HPRT: EC 2.4.2.8), which was identified in two brothers with hyperuricemia and nephrolithiasis. In previous studies, this mutant enzyme was characterized by an increased K_m for both substrates, a normal V_{max}, a decreased intracellular concentration of enzyme protein, a normal subunit molecular weight and an acidic isoelectric point under native isoelectric focusing conditions. We have cloned a full-length cDNA for HPRT$_{\text{Ann Arbor}}$ and determined its complete nucleotide sequence. A single nucleotide change (T→G) at nucleotide position 396 has been identified. This transversion predicts an amino acid substitution from isoleucine (ATT) to methionine (ATG) in codon 132, which is located within the putative 5′-phosphoribosyl-1-pyrophosphate (PRPP)-binding site of HPRT.

Introduction

Hypoxanthine-guanine phosphoribosyltransferase (HPRT: EC 2.4.2.8) is a purine salvage enzyme that catalyzes the conversion of hypoxanthine and guanine to their respective mononucleotides, IMP and GMP. Complete deficiency of HPRT is associated with the Lesch-Nyhan syndrome, an X-linked disorder, which is characterized clinically by hyperuricemia, mental retardation, choreoathetosis, and compulsive self-mutilation (Seegmiller et al. 1967). Partial deficiency of HPRT leads to overproduction of uric acid, which results in the development of a severe form of gout and nephrolithiasis (Kelley et al. 1967).

Several mutant forms of HPRT have been isolated from patients with gout and the Lesch-Nyhan syndrome. By sequence analysis of tryptic peptides, single amino acid substitutions have been defined in four HPRT variants (Wilson et al. 1983). Southern blot analysis has indicated major gene abnormalities in a minority of Lesch-Nyhan patients. These include partial and complete deletions and internal duplication of the structural gene for HPRT (Yang et al. 1984). More recently, ribonuclease A cleavage analysis has been used to localize mutations at the HPRT locus in five Lesch-Nyhan patients (Gibbs and Caskey 1987).

HPRT$_{\text{Ann Arbor}}$ is a unique structural variant of HPRT, which was identified in two brothers, 12 and 14 years old, who presented with hyperuricemia and nephrolithiasis. A maternal uncle of these patients, who was also partially deficient

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in HPRT, suffered from a severe form of gout with early onset. In previous studies, HPRT$_{\text{Ann Arbor}}$ was shown to have: (i) a markedly decreased concentration of enzyme protein, (ii) a normal subunit, molecular weight and a more acidic isoelectric point, (iii) an increased K_m for hypoxanthine and 5′-phosphoribosyl-1-pyrophosphate (PRPP) and (iv) a normal V_{max} (Wilson et al. 1982, 1986).

The amino acid sequence of this HPRT variant could not be determined because the markedly decreased levels of enzyme protein precluded purification of sufficient quantities for characterization. In the present study, we have isolated HPRT cDNA synthesized from messenger RNA (mRNA) encoding HPRT$_{\text{Ann Arbor}}$ and have determined its full nucleotide sequence. Using this approach we have identified a single nucleotide change in codon 132 (AT*T*→AT*G*), which predicts an amino acid substitution from isoleucine (Ile) to methionine (Met).

Materials and methods

AMV reverse transcriptase was purchased from Life Science and T4 DNA polymerase was from Pharmacia. All other enzymes were purchased from New England Biolabs. Lambda gt11 arms and in vitro packaging extract for lambda DNA were obtained from Stratagene. M13 sequencing vectors and the universal primer were purchased from Bethesda Research Laboratories. [α-^{32}P]dCTP (3000 Ci/mmol), [^{35}S]dATP (1240 Ci/mmol) and nitrocellulose filters were obtained from New England Nuclear. All other reagents were of the highest quality commercially available.

Construction of cDNA library

Ebstein-Barr virus transformed lymphoblast cell lines derived from patient K.C. (Wilson et al. 1982) were grown in RPMI 1640 medium containing 10% fetal calf serum. The lymphoblasts were lysed with 6.0 M guanidium isothiocynate and total RNA was isolated by ultracentrifugation of the cell lysates in 5.7 M CsCl (Maniatis et al. 1982). Poly (A)$^+$mRNA was purified by oligo-d(T) column chromatography (Aviv and Leder 1972) and was electrophoresed in a 0.8% agarose-formaldehyde gel for Northern blot analysis (Fuscoe et al. 1983).

Oligo-d(T) primed poly (A)$^+$mRNA was reverse transcribed to synthesize the first cDNA strand. Two methods of second strand synthesis were used. In method A, the RNA:cDNA heteroduplex formed during first strand synthe-

sis was digested with RNase H and the second strand synthesized by *Escherichia coli* DNA polymerase and *E. coli* DNA ligase (Gubler and Hoffman 1983). In method B, after RNase H digestion of the first strand heteroduplex, the second strand was synthesized by AMV reverse transcriptase (Polites and Marotti 1986). The double-stranded cDNA synthesized by both methods was blunt-ended with T4 DNA polymerase and ligated to Eco RI linkers. After digestion with Eco RI, the cDNA was purified by sepharose CL4-B column chromatography and ligated to Eco RI digested lambda gt11 arms (Young and Davis 1983) using conditions recommended by the supplier. The *E. coli* (strain Y1088) were infected with recombinant phages.

Screening of recombinant phages

Two kinds of probes were used for screening the cDNA libraries. One was prepared from a 955-base pair (bp) HPRT cDNA, which contains the entire HPRT coding sequence (kindly provided by C.T. Caskey). The second probe was a 270-bp Taq I fragment which spans the 5'-noncoding sequences of the HPRT cDNA. Both DNA probes were labeled with $[\alpha^{-32}P]dCTP$ by the hexadeoxynucleotide priming method (Feinberg and Vogelstein 1984).

Phage plaques (20000 plaques/plate) were transferred to nitrocellulose filters and screened by in situ hybridization (Benton and Davis 1977).

Sequence analysis of cDNA

Recombinant lambda phages derived from single clones were grown in *E. coli* strain Y1088 and purified by centrifugation to equilibrium in a CsCl gradient (Maniatis et al. 1982). Phage DNA was digested with Eco RI and subjected to electrophoresis in a 0.8% agarose gel. Eco RI inserts were extracted from the gel with DEAE cellulose paper (Dretzen et al. 1981). The inserts were then subcloned into the Eco RI site of the plasmid vector pUC18. Several fragments of cDNA digested with appropriate restriction endonucleases were subcloned into the single-stranded phages M13mp18 and M13mp19 for sequence analysis. DNA sequencing was carried out by the dideoxynucleotide chain termination method using M13 universal primer (Biggin et al. 1983).

Isoelectric focusing

Normal lymphoblasts (WI-L2, MGL-8, N333) and lymphoblasts from subject KC were lysed by six cycles of freezing and thawing. Membrane-free extracts, obtained by centrifugation at $100000\,g$ for 45 min, were dialyzed in 10 mM Tris, pH 7.4, with 50 mM NaCl overnight.
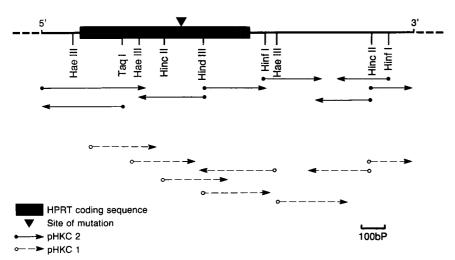
Isoelectric focusing was performed in polyacrylamide slab gels (2 mm × 13 cm × 16 cm) containing 6.0% acrylamide, 0.16% N, N'-methylene bisacrylamide, 5% glycerol and 5% ampholites (pH 5–7, LKB). The gel was prefocused for 2 h and samples were loaded by wicking from filters for 1 h. Focusing was complete after an additional 2 h at 10 W. HPRT activity was localized by incubation of the gels with substrate solution containing 100 mM Tris, pH 7.4, 10 mM MgCl$_2$, 10 mM 5'-phosphoribosyl-1-pyrophosphate (PRPP), and 72 μM $[8^{-14}C]$hypoxanthine (45 mCi/mmol) for 30 min. The gel was then blotted with PEI cellulose, which binds IMP but not hypoxanthine, washed and exposed to Kodak XAR film (Zannis et al. 1980).
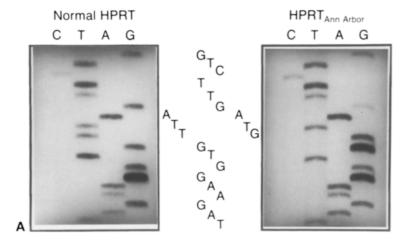
Results
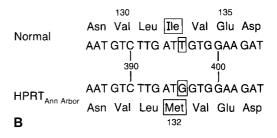
cDNA cloning and sequence analysis

Messenger RNA encoding HPRT$_{Ann\ Arbor}$ was indistinguishable in its size and amount from mRNA derived from normal lymphoblasts by Northern blot analysis (data not shown). The cDNA library synthesized by method A contained approximately 5×10^5 recombinant phage plaques. Four plaques from this library hybridized to the 950-bp HPRT probe. The sizes of the cDNA inserts from these clones were determined by Southern blot analysis after Eco RI digestion of phage DNA. The largest cDNA from this library was 1.3 kb. This clone was designated pHKC1. Method B yielded a library containing approximately 4×10^5 recombinants. Of these clones 19 hybridized to the 955-bp HPRT cDNA: 8 of these that also hybridized to the 270-pb Taq I HPRT probe were selected for insert sizing. The largest cDNA from this library, 1.45 kb, was designated pHKC2.

The nucleotide sequences of pHKC1 and pHKC2 were determined as described in Materials and methods. The sequencing strategy employed is diagrammed in Fig. 1. pHKC1 contained 559 nucleotides of the coding sequence (which is 55
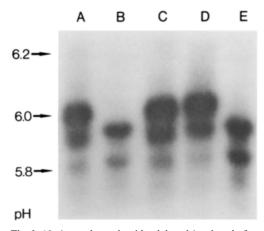

Fig. 1. Restriction map and sequencing strategy of pHKC1 and pHKC2. The *arrows* indicate directions and the ranges of sequence determination

HPRT coding sequence
▼ Site of mutation
●——▶ pHKC 2
○——▶ pHKC 1

100bP

Fig. 2. A Comparison of the nucleotide sequences of normal HPRT and HPRT$_{Ann\ Arbor}$. Sequences from base position 388 to 405 are shown with the transversion identified at position 396. **B** The amino acid substitution predicted by the nucleotide change demonstrated in **A**. (Amino acid 1 is the initial methionine)

Fig. 3. Native polyacrylamide slab gel isoelectric focusing of HPRT. The enzyme was detected radiochemically by an in situ activity stain. Lymphoblast extracts from normal and mutant cell lines were focused. *Lane A*, WIL-2 (30 μg protein); *lanes B, E*, KC (100 μg protein); *lane C*, MGL-8 (30 μg protein); *lane D*, N333 (30 μg protein)

nucleotides short of the entire coding sequence), and 592 nucleotides of the 3' noncoding sequence. pHKC2 contained 119 nucleotides of 5' noncoding sequence, the entire coding sequence and 592 nucleotides of 3' noncoding sequence.

A single nucleotide substitution — a T to G transversion at nucleotide position 396 — was identified (Fig. 2A). This transversion alters codon 132 (AT*T*→AT*G*) and predicts an isoleucine to methionine substitution at this position (Fig. 2B). This nucleotide change does not alter a restriction recognition site within the HPRT gene. The remainder of the sequence, including 5'- and 3'-noncoding regions, was identical to normal HPRT cDNA (Jolly et al. 1983; Patel et al. 1986).

Isoelectric focusing

Dialyzed lymphoblast extract from normal cell lines (WIL-2, MGL-8 and N333) demonstrated the same isoelectric focusing patterns with three major bands of activity at pH 6.00, pH 5.90, and pH 5.80 (Fig. 3). HPRT$_{Ann\ Arbor}$ also showed multiple bands of activity, but the isoelectric points of this mutant enzyme were pH 5.95, pH 5.80, and pH 5.70. These isoelectric focusing patterns of normal and mutant HPRT did not change after treatment of cell extracts with 4.5 M and 0.35 M

β-mercaptoethanol prior to focusing or by using nondialyzed extract instead of a dialyzed one (data not shown).

Discussion

Previous studies have identified structural and functional abnormalities of mutant HPRT proteins from erythrocytes, fibroblasts, and cultured lymphoblasts derived from HPRT-deficient patients with detectable enzyme activity. These studies have suggested marked heterogeneity of mutations in the structural gene for HPRT (Chinault and Caskey 1984; Wilson et al. 1986; Gibbs and Caskey 1987).

Single amino acid substitutions have been identified in four variants from three gouty and one Lesch-Nyhan patient by comparative mapping of the tryptic peptides and analysis of amino acid sequences (Wilson et al. 1983). Since more than two-thirds of HPRT-deficient patients have less than 5% residual HPRT immunoreactive protein (Wilson et al. 1986), the study of mutations by analysis of residual active protein cannot be performed in most HPRT-deficient patients using amino acid sequencing techniques.

Southern blot analysis of genomic DNA derived from Lesch-Nyhan patients has demonstrated gross alterations of the HPRT gene in a small minority of those studied (Yang et al. 1984; Wilsons et al. 1986). These include deletion of the entire HPRT gene in one subject, partial gene deletions in two subjects, and an internal duplication of exons 2 and 3 in another (Yang et al. 1984). Furthermore, 90% of HPRT mutants have HPRT mRNA of normal size and quantity (Wilson et al. 1986). These observations suggest that the majority of HPRT mutations are the result of single nucleotide changes or small DNA deletions or rearrangements.

More recently, ribonuclease A (RNase A) cleavage analysis has been used to detect small gene abnormalities that cannot be identified by Southern blot analysis (Myers et al. 1985; Winter et al. 1985; Gibbs and Caskey 1987). Gibbs and Caskey (1987) localized the putative sites of mutations in HPRT mRNA of five Lesch-Nyhan patients using this technique. This technique can reveal abnormalities in less than half of HPRT-deficient subjects because of the failure of RNase A to recognize and cleave certain mismatches in RNA:RNA or RNA:DNA hybrids (Myers et al. 1985; Winter et al. 1985; Gibbs and Caskey 1987). Furthermore, while this analysis can localize mutations in HPRT mRNA, it cannot be used to de-

termine the exact nucleotide changes except when allele-specific oligonucleotides (ASO) are available that allow deduction of certain nucleotide substitutions (Caskey 1987). In general, however, construction of an ASO requires prior knowledge of the mutation itself. ·

Cloning mutant forms of HPRT DNA sequences is a logical approach to the detection, localization, and characterization of mutations at the nucleotide level. Since the human HPRT gene is large (approximately 44 kb) and complex (eight introns; Patel et al. 1986), genomic cloning is impractical in most cases. Therefore, in cases where sufficient mRNA is present, mutant cDNA cloning is the method of choice.

In this study, we have isolated the cDNA encoding HPRT$_{Ann Arbor}$. Comparison of the primary structures of six phosphoribosyltransferase (HPRTases) that use as a substrate has revealed considerable amino acid sequence homology with positions 129–141 in the human HPRT protein. This highly conserved region is probably a part of the PRPP binding site (Hershey and Taylor 1986). The mutation in HPRT$_{Ann Arbor}$ falls within this region and offers an explanation for the altered kinetic parameters of this mutant enzyme. Additionally, human HPRT protein has a βαβ secondary structure in residues 1 to 120, which is characteristic of a nucleotide-binding fold. This type of structure has also been implicated in PRPP binding (Argos et al. 1983).

In previous kinetic studies of HPRT$_{Ann Arbor}$, this mutant enzyme exhibited a normal V$_{max}$, a 2-fold-increased K$_m$ for PRPP and a 10-fold increased K$_m$ for hypoxanthine (Wilson et al. 1986). Thus, the Ile to Met substitution at 132 appears to exert an effect on the binding of both substrates without altering the catalytic activity of the enzyme. HPRT$_{Munich}$ (Ser→Arg103) and HPRT$_{London}$ (Ser→Leu109) have an increased K$_m$ for hypoxanthine and a normal K$_m$ for PRPP (Wilson et al. 1983). The region between residues 60–114 has been suspected to contribute to the formation of the hypoxanthine-binding site (Argos et al. 1983; Hershey and Taylor 1986). However, the mutation in HPRT$_{Ann Arbor}$ is far from the putative hypoxanthine-binding site, and is located within the putative PRPP-binding site proposed by Hershey and Taylor (1986).

We can propose two hypotheses to explain the increased K$_m$ for hypoxanthine in HPRT$_{Ann Arbor}$. First, secondary structural change at residue 132 may have a marked effect on the folding of the protein resulting in a change of the tertiary or quartenary structure with reduction in affinity for hypoxanthine. Second, human HPRT catalyzes the formation of IMP predominantly through an ordered BiBi kinetic mechanism with PRPP serving as the first substrate (Giacomello and Salermo 1978). A mutation within the PRPP binding site may thus affect the formation of PRPP – enzyme complex resulting in alteration of affinity for hypoxanthine.

HPRT$_{Ann Arbor}$ from lymphoblasts was previously shown to have one immunoreactive isoelectric species with a more acidic isoelectric point compared to the normal enzyme (Wilson et al. 1982). Several investigators reported that HPRT from lymphoblasts has one isoelectric point, while enzyme from erythrocytes has multiple isoelectric points, resulting from extensive post-translational modification (Ghancas and Milman 1977; Johnson et al. 1982; Wilson et al. 1982). In this study, we used a narrower pH range (pH 5–7) than was used in previous reports (pH 3.5–10). Under these conditions, lymphoblast HPRT also has multiple isoelectric points. Additionally, treatment with 4.5 M urea did not change the isoelectric

focusing pattern for the normal or mutant protein suggesting that the pattern is not a result of binding of small molecules by the enzyme (Zannis et al. 1980).

The concentrations of HPRT protein applied to the gel were 150–500 μg/ml. At these concentrations, the enzyme electrofocuses as a dimer (Johnson et al. 1979). Therefore, lymphoblast HPRT appears to be composed of two major types of subunits with different isoelectric points. These findings suggest that HPRT from lymphoblast cell lines also undergoes post-translational modification.

The predicted amino acid substitution in HPRT$_{Ann Arbor}$ is neutral with respect to charge. Thus, its variant isoelectric points cannot be explained by a charge difference between isoleucine and methionine. The small magnitude of the pI shifts in HPRT$_{Ann Arbor}$ suggest that the substitution alters the environment of other ionizable groups resulting in minor changes in their pKs. Alternatively, the amino acid substitution may affect one of the several post-translational modifications the enzyme undergoes.

Since the amount of mRNA encoding HPRT$_{Ann Arbor}$ is not decreased in the cell, the decreased intracellular concentration of the enzyme may be caused by either the dysfunction of mRNA (decreased synthesis of protein) or the instability of the translation product (accelerated degradation of protein). A complete understanding of the relationship between the single nucleotide change and the decreased intracellular enzyme concentration will require additional studies, such as in vitro translation and in vivo turnover of the enzyme.

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