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A 13 base pair deletion in exon 1 of $HPRT_{Illinois}$ forms a functional GUG initiation codon

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Abstract. More than 50 mutations in the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus have been described, yet only 2 alter the AUG initiation codon. One, variant $HPRT_{1151}$, results in Lesch-Nyhan syndrome (LNS), and the other, $HPRT_{Illinois}$, results in partial HPRT deficiency. Although previously undetectable, we used a sensitive gel assay to demonstrate that $HPRT_{Illinois}$ is not only active, but has a native Mr indistinguishable from normal. Confirmatory evidence of activity and native Mr is demonstrated following transfection of HPRT cells with expression plasmids containing cDNA sequences representing $HPRT_{Illinois}$. These data provide support for the hypothesis that patient RT, or variant $HPRT_{Illinois}$, is spared manifestations of the LNS as a result of translation at the newly formed GUG initiation codon.

Introduction

Hypoxanthine-guanine phosphoribosyltransferase (HPRT; EC 2.4.2.8) is a purine salvage enzyme that catalyzes the conversion of hypoxanthine and guanine to inosine monophosphate and guanosine monophosphate, respectively. Complete deficiency of HPRT enzyme activity (< 0.1%) is phenotypically expressed as the Lesch-Nyhan syndrome (LNS) and is characterized by choreoathetosis, spasticity, and, in many cases, a tendency to self mutilation by biting of the fingers and lips (Lesch and Nyhan 1964). Partial deficiency, or the Kelley-Seegmiller syndrome (KSS), is characterized by hyperuricemia, precocious gout, and uric acid nephrolithiasis (Kelley et al. 1972). The genetic abnormalities responsible for these HPRT deficiency states have been defined and represent a heterogeneous group of missense and nonsense point mutations, deletions, insertions, and other genomic rearrangements (Gibbs et al. 1989; Davidson et al. 1989b; Rossiter et al. 1991).

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Among individuals phenotypically characterized with severe KSS is patient RT, or HPRT variant $HPRT_{Illinois}$. Genetic analysis of the HPRT cDNA from RT identified a 13 base pair (bp) deletion within the first exon (Gibbs et al. 1989). This deletion spans nucleotides –12 to +1 and results in the juxtaposition of a G to the remaining UG of the initiation codon. The consequence of this is the replacement of the AUG start codon with a GUG codon. Thus, the approximately 1% of residual activity in RT may be the result of either alternative initiation codon usage, or initiation at a downstream, in frame, AUG. We provide evidence in this report that translation of $HPRT_{Illinois}$ mRNA occurs by inefficient translational initiation at the GUG codon formed as a result of the 13-bp deletion and ablation of the normal AUG start codon.

Materials and methods

Cell lines and preparation of lysates

The mutations of the HPRT gene that characterize WE and RT ($HPRT_{Illinois}$) have previously been described (Gibbs et al. 1989). B-lymphoblast cell lines from WE and RT were produced by transformation with Epstein-Barr virus and maintained in RPMI media containing 10% fetal calf serum and 2 m*M* glutamine (Wilson et al. 1982). Cell line WE contains no detectable HPRT mRNA by RNA blot analysis or RNase A mapping experiments (Wilson et al. 1986). B-lymphoblast cell line N333 was derived from a normal human male (Human Mutant Cell Repository, Camden, N.J.). Cell lysates were prepared as previously described and concentrated by ultrafiltration (Centricon 10, Amicon). Protein concentrations were determined by the method of Lowry (BCA Protein Assay Reagent, Pierce).

Western blot analysis

Western blot analysis or RT cells lysates for HPRT immunoreactive material were performed using standard methods (Wilson et al. 1982). Increasing amounts of total protein from RT lymphoblast cell lysates and known amounts of partially purified human erythrocyte HPRT were subjected to electrophoresis in parallel on 12% polyacrylamide gels containing 1% sodium dodecyl sulfate (SDS). Protein was transferred to PVDF paper (Millipore) using a semi-dry blotter apparatus (Biorad). The membrane was blocked by incubation with 1% nonfat dry milk in TBS/Tween-20 (0.02 *M*

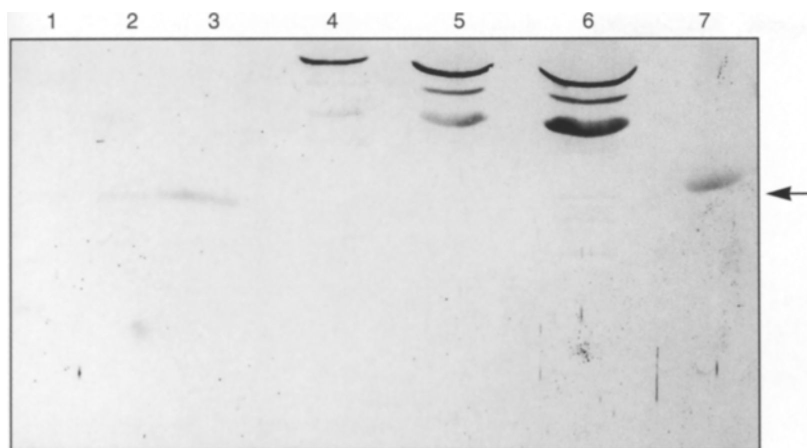

Fig. 1. Western blot analysis of whole cell lysates obtained from RT lymphoblasts for HPRT immunoreactive material. Increasing amounts of whole cell lysates from RT lymphoblasts were loaded onto a 12% polyacrylamide gel containing SDS, subjected to electrophoresis and transferred to PVDF membrane (Millipore). The membrane was blocked for 2 h with TBS/Tween-20 (0.02 *M* Tris, 0.5 *M* NaCl, 0.05% Tween-20, pH7.5) containing 1% bovine serum albumin. The membrane was treated with rabbit anti-human HPRT antibody for approximately 16 h. After four washes in TBS/Tween-20 the membrane was treated with goat anti-rabbit antibody conjugated to alkaline phosphatase (Bio-Rad), washed, and reacted with BCIP/NBT reagents (Gibco BRL). *Lanes 1–3* show increasing amounts of purified human erythrocyte HPRT: *lane 1* 20 ng; *lane 2* 50 ng; *lane 3* 200 ng. *Lanes 4–6* show increasing amounts of RT lymphoblast lysates: *lane 4* 50 µg; *lane 5* 100 µg; *lane 6* 200 µg. *Lane 7* pre-stained 30-kDa molecular weight markers (Amersham). *Arrow* indicates the location of human HPRT protein

Tris, 0.5 *M* NaCl. 0.05% Tween-20, pH7.5), then incubated for 4 h with rabbit anti-human HPRT antibody (diluted 1:20), and washed four times in TBS/Tween-20. The membrane was then incubated with goat anti-rabbit antibody (diluted 1:100) conjugated to alkaline phosphatase (Kirkegaard), washed in TBS/Tween-20, and reacted with BCIP/NBT reagents (Gibco BRL).

Enzyme activity analysis

Cell lysates were fractionated by nondenaturing 6% polyacrylamide gel electrophoresis (PAGE), and enzyme activity assayed directly as previously described with the following modifications (Davidson et al. 1989a). Briefly, the gels were reacted with a mixture containing 1 m*M* [8–^{14}C] hypoxanthine (NEN; 57 mCi/mmol), 15 m*M* PP-ribose-P, and 30 m*M* MgCl, for 2 h at 37°C, washed, and exposed to Kodak XAR5 film. The amounts of IMP produced during the in situ assays were determined directly from the gels by quantification of particle emission using an array detector (Betagen), and the amount of HPRT activity present was quantified as a function of levels of substrate turnover and were expressed as cpm [8–^{14}C] IMP/µg of whole cell protein.

Preparation and amplification of HPRT cDNAs

Total cellular RNA was isolated from 1×10^6 RT lymphoblasts or 1×10^6 normal lymphoblasts and amplified as previously described (Davidson et al. 1989b). Polymerase chain reaction (PCR) primers HP3E and HP5E were used to amplify both normal HPRT and $HPRT_{Illinois}$ cDNAs. The sequence of the 3′ primer (HP3E) is 5′CCG GC*G AATTC*A GAT GTT TCC AAA CTC AAC TTG3′, while the sequence of the 5′ primer (HP5E) is 5′CCG GC*G AAT TC*C TGA GCA GTC AGC CCG CGC GCC3′. Each primer has 11 bp of flanking sequence within which is an *Eco*RI site (italics) in sequence to facilitate subcloning. Primer HP5E anneals to nucleotides –37→–14 of the antisense strand, while HP3E anneals to nucleotides +690→+668 of the sense strand. The resulting partial length HPRT cDNA fragment contains the entire protein coding region. PCR amplification conditions were denaturation at 94°C for 1 min followed by extension at 72°C for 3 min.

Construction of expression plasmids and transfection into HPRT-deficient cell line WE

PCR fragments were digested with *Eco*RI, purified, and cloned into the eukaryotic expression vector pSG5 (Stratagene). Recombinant clones were identified by restriction enzyme mapping and nucleotide sequencing of plasmid minipreps using standard methods (Sambrook et al. 1989).

WE lymphoblasts were used for all plasmid transfections. Approximately 1×10^8 WE lymphoblasts were transfected with 50 µg of pSG5HPRT in DEAE dextran in T250 Falcon tissue culture flasks (Ausubel et al. 1990). For $pSG5HPRT_{Illinois}$ 3×10^8 WE lymphoblasts were transfected with 150 µg of plasmid in 3 T250 flasks. Untransfected WE and N333 (normal) lymphoblasts served as controls. Cell lysates were isolated 48 h after transfection, concentrated, and assayed for HPRT activity in situ following nondenaturing PAGE.

Results and discussion

To address the discrepancy between the severe systemic deficiency of HPRT activity and the clinical manifestations in patient RT, we attempted to define the relationship between the mutant genotype and phenotype. Using rabbit polyclonal antisera to human HPRT we were able to detect small amounts of immunoreactive material present in pooled cell lysates obtained from RT lymphoblasts (Fig. 1). Additionally, $HPRT_{Illinois}$ comigrates with purified normal HPRT providing evidence that the two proteins have similar apparent molecular weights (subunit Mr).

Previously, HPRT activity in B-lymphoblast lysates obtained from patient RT was undetectable by routine radioisotopic assay on unconcentrated cell lysates (Wilson et al. 1986). However, by using a more sensitive radio-

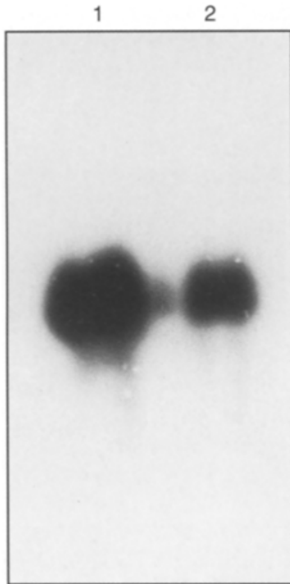

Fig. 2. HPRT enzyme activity and electrophoretic mobility of membrane-free extracts from normal lymphoblasts and lymphoblasts derived from patient RT. Protein from cell extracts subjected to electrophoresis through a 6% nondenaturing polyacrylamide gel and assayed in situ for HPRT activity as described. *Lane 1* normal lymphoblast (N333) lysate (106.9 cpm [8–^{14}C] IMP/μg whole cell protein); *lane 2* RT lymphoblast lysate (0.37 cpm [8–^{14}C] IMP/μg whole cell protein)

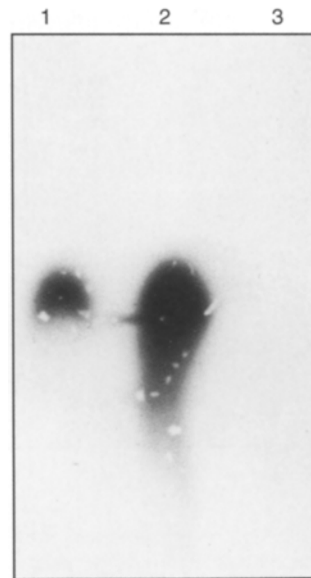

Fig. 3. Enzymatic activity and native electrophoretic mobility of expressed HPRT in HPRT mRNA negative WE lymphoblasts transiently transfected with pSG5HPRT$_{Illinois}$ or pSG5HPRT. Membrane-free cell extracts from transfected WE cells were subjected to electrophoresis through a 6% nondenaturing polyacrylamide gel and assayed in situ for HPRT activity as described. Cpm were determined using a Betagen. *Lane 1* WE lymphoblasts transfected with pSG5HPRT$_{Illinois}$ (4.9×10^{-3} cpm [8–^{14}C] IMP/μg whole cell protein; *lane 2* WE lymphoblasts transfected with pSG5HPRT (0.30 cpm [8–^{14}C] IMP/μg whole cell protein); *lane 3* WE lymphoblasts as a negative control (no detectable cpm). Note the similar migration pattern of HPRT in both pSG5HPRT$_{Illinois}$ and pSG5HPRT transfected cells

chemical in situ gel assay we were able to detect HPRT enzymatic activity present in RT lymphoblast lysates. As is evident in Fig. 2, HPRT$_{Illinois}$ is capable of catalyzing substrate turnover. The HPRT activity present in the lysates was expressed as a function of substrate turnover. Enzyme activity of HPRT$_{Illinois}$ was 0.37 cpm [8–^{14}C] IMP/μg whole cell protein, representing 0.34% of control values (activity in normal lymphoblasts was 106.9 cpm [8–^{14}C] IMP/μg whole cell protein). This correlates closely with the 0.8% level of immunoreactive protein previosly reported (Wilson et al. 1986). Notably, the migration of HPRT$_{Illinois}$ and normal HPRT are indistinguishable under nondenaturing conditions.

To further establish that the 13 bp deletion and resultant initiation codon mutation is capable of promoting translation of a functional HPRT, the cDNAs representing normal and HPRT$_{Illinois}$ were cloned into an expression plasmid and the normal and mutant sequences were confirmed by nucleotide sequencing. Both pSG5HPRT and pSG5HPRT$_{Illinois}$ were then transfected into the HPRT mRNA negative lymphoblast cell line WE and examined using a transient expression assay. After transfection the cells were harvested, and HPRT activity assessed by in situ activity gel of whole cell lysates. The results of this experiment are shown in Fig. 3. WE cells transfected with pSG5HPRT or pSG5HPRT$_{Illinois}$ expressed HPRT protein with a migration pattern indistinguishable from HPRT present in normal lymphoblasts. The amount of HPRT activity present in the pSG5HPRT$_{Illinois}$ transfectants when expressed as a function of substrate turnover per microgram total protein, was 1.6% of the activity present in cells transfected with pSG5HPRT.

Definitive verification of the translation initiation site used by HPRT$_{Illinois}$ mRNA would require that purified preparations of the HPRT$_{Illinois}$ protein be subjected to peptide sequence analysis (Prats et al. 1989). Because cultured RT lymphoblasts produce extremely low levels of mutant HPRT protein, we were unable to directly isolate sufficient amounts of purified HPRT$_{Illinois}$ protein for unambiguous peptide sequence analysis using either polyclonal rabbit antisera, or by the use of GMP agarose column chromatography. Attempts to isolate stable transfectants in WE lymphoblasts transfected with pSG5HPRT$_{Illinois}$ by selection in media supplemented with hypoxanthine, aminopterin and thymidine (HAT) were also unsuccessful. This was probably due to the extremely low levels of active HPRT$_{Illinois}$ that are produced by the transfected clones.

Despite the absence of peptide sequence data, alternative explanations for the partial HPRT activity observed in patient RT are limited. One hypothesis is that translational initiation in HPRT$_{Illinois}$ mRNA begins at a downstream AUG codon producing a truncated HPRT protein. However, the Lesch-Nyhan patient HPRT$_{1151}$ has a G-to-A transition at nucleotide +3 that alters the AUG start codon to AUA (Tarlé et al. 1991; Table 1). Presumably, HPRT$_{1151}$ mRNA would be capable of producing functional HPRT by translational initiation at the next in frame AUG codon (nucleotide +120). Analysis of cell lysates obtained from HPRT$_{1151}$ lymphoblasts using the in situ activity gel assay failed to reveal evidence for the formation

Table 1. HPRT mutations of the initiation codon and clinical phenotypes

Variant	Partial 5′ HPRT mRNA flanking sequence[a]	Mutation	Phenotype
$HPRT_{Normal}$	CCCGCGCGCCGGCCGGCUCCGUU AUG GCG ACC	Normal	Normal
$HPRT_{Illinois}$	CCCGCGCGCC GUG GCG ACC	Deletion $-12 \rightarrow +1$	KSS
$HPRT_{1151}$	CCCGCGCGCCGGCCGGCUCCGUU AUA GCG ACC	$G_3 \rightarrow A$	LNS

LNS, Lesch-Nyhan syndrome; KSS, Kelley-Seegmiller syndrome
[a] Sequences are expressed in 5′–3′ format

of a catalytically active truncated HPRT protein (data not shown).

Initiation at a downstream in-frame AUG in RT is also not consistent with the results reported here. If this were the case, we would expect $HPRT_{Illinois}$ to be truncated by 40 amino acids. Assuming a mean amino acid residue weight of 113, this would result in a minimum decrease of 18,000 between the truncated $HPRT_{Illinois}$ and normal tetrameric HPRT. However, both $HPRT_{Illinois}$ and the native tetramers comigrate (Figs. 2, 3). Thus our results indicate that $HPRT_{Illinois}$ is the result of translation at the in-frame GUG at base pair 1′, the result of a 13 bp deletion with subsequent juxtaposition of a G to the remaining UG of the normal start codon.

Translational initiation at non-AUG codons is rare, but has been reported to occur in a variety of eukaryotic systems (see Kozak 1991, for review; also Curran and Kolakofsky 1988; Giorgi at al. 1983). Often translational initiation at non-AUG codons occurs in conjunction with initiation events at AUG codons of the same mRNA such as has been described for the human fibroblast growth factor gene (Prats et al. 1989; Florkiewicz and Sommer 1989). Precedence also exists for the use of alternative initiation codons in genes encoding enzymes of purine biosynthesis. Taira et al. (1990) have described a testes-specific isoform of the phosphoribosylpyrophosphate synthetase gene that exclusively uses an ACG codon for translational initiation in vitro; however, definitive evidence for the use of this codon in vivo has not yet been reported.

Although the GUG codon that initiates translation in $HPRT_{Illinois}$ has not been previously described in humans, GUG initiation codon usage is not unprecedented in eukaryotes, particularly in consideration of the context of the surrounding sequence. The deletion in $HPRT_{Illinois}$ produces the sequence CGC GCC *GUG* G (Table 1). Studies in eukaryotes have shown that translational initiation is most efficient when the start codon is in the context GCC (A/G)CC *AUG* G (Kozak 1986, 1987). The mutation in $HPRT_{Illinois}$ recapitulates this consensus sequence −4 to +1 with respect to the GUG codon. Additionally, the GC-rich downstream sequences present in HPRT mRNA may contribute to a favorable context for alternative initiator codon usage in $HPRT_{Illinois}$ (Kozak 1989). Of the six alternative initiator codons identified, GUG is the most efficient, producing up to 3%–5% of the amount of protein produced by the usual AUG codon (Kozak 1989, 1990). Although in eukaryotes there have been more reports of ACG and CUG initiation codon usage, initiation at GUG has been documented for *Drosophila melanogaster* choline acetyltransferase mRNA (Sugihara et al. 1990).

In summary, this is the first demonstration of a functional GUG initiation codon in humans, and interestingly, the capability of the GUG codon to drive translation has spared patient RT severe neurological disease. Because of the low levels of $HPRT_{Illinois}$ produced by RT lymphoblasts, purification and a formal kinetic analysis of this mutant protein was not possible. However, our data supports the hypothesis that $HPRT_{Illinois}$ is catalytically normal, with the partial deficiency due to diminished amounts of HPRT protein production. Presumably, this is due to inefficient translation initiation at the newly formed GUG start codon.

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