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Synthesis of normal and variant human hypoxanthine-guanine phosphoribosyltransferase in *Escherichia coli*

(B-lymphoblast; crystallization; HPRT; PCR; recombinant DNA; x-ray diffraction analysis)

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SUMMARY

Naturally occurring mutations in hypoxanthine-guanine phosphoribosyltransferase (HPRT) have been identified by amino acid sequencing, cDNA cloning, and direct nucleotide sequencing of PCR-amplified transcripts. To determine the effect these mutations have on the catalytic properties of the molecule, knowledge of the three-dimensional structure of HPRT is required. A prerequisite for this, however, is the availability of a large amount of purified product for crystallization and x-ray diffraction analysis. For these reasons we have developed an effective means of producing high levels of human HPRT in *Escherichia coli* using the expression cassette PCR. By taking advantage of a T7 polymerase/promoter system, we have expressed both normal and variant human *hprt* sequences in *E. coli*. The proteins synthesized from these sequences are immunologically and enzymatically active, and are physically indistinguishable from the HPRT in B-lymphoblasts derived from normal and three HPRT-deficient subjects.

INTRODUCTION

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is a purine salvage enzyme which catalyzes the formation of GMP and IMP from guanine and hypoxanthine, respectively, using PRPP as the phosphoribosyl donor. A partial deficiency of this cytosolic enzyme can cause hyperuricemia, hyperuricaciduria, uric acid nephrolithiases, and in extreme cases, neurologic dysfunction (Kelley et al., 1967). Manifestations of complete deficiency, or the Lesch–Nyhan syndrome, include retarded growth, spasticity, choreathetoid movements, a tendency to self-mutilate and, in most cases, mental retardation (Lesch and Nyhan, 1964; Seegmiller et al., 1967). The pathophysiologic basis for these neurologic effects remains unclear.

The primary structure of human HPRT has been defined (Wilson et al., 1982b). The mature enzyme consists of 217 aa with the N-terminal Met removed and the penultimate Ala acetylated (Wilson et al., 1982b). Regions of α-helix, β-sheet, and other secondary structure motifs have been predicted for the molecule based on this primary sequence information. Inferences on the tertiary structure of the molecule, with regards to PRPP and purine-binding sites, have been made based on comparisons of conserved structures in other PRTases and by defining

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Abbreviations: aa, amino acid(s); bp, base pair(s); DTT, dithiothreitol; ECPCR, expression cassette PCR; HPRT, hypoxanthine-guanine phosphoribosyltransferase; *hprt*, gene (DNA) encoding HPRT; IPTG, isothiopyranogalactoside; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; PRPP, 5-phosphoribosyl-1-pyrophosphate; SD, Shine–Dalgarno; SDS, sodium dodecylsulfate; wt, wild type; [ ] denotes plasmid-carrier state.

mutations in kinetic variants (Hershey and Taylor, 1986; Argos et al., 1983). Based on these types of studies, aa 130–150 and 140–210 have been predicted to be involved in purine and PRPP binding, respectively. Both areas contain stretches of aa sequence which is highly conserved from *E. coli* to mammals. As noted, substitutions in these regions are usually deleterious to enzyme function.

The actual role of these putative binding domains, however, can only be addressed by x-ray crystallographic analysis of normal and mutant enzymes, a major prerequisite of which is large amounts of purified protein. As demonstrated in this report, we have addressed this by developing a novel ECPCR method for enhanced translation of human *hprt* sequences in *E. coli*. This technique was effective in producing high levels of both normal and mutant HPRT for subsequent purification.

EXPERIMENTAL AND DISCUSSION

(a) Construction of an expression cassette

The PCR primers used to generate the expression cassette for *hprt* were synthesized at the Oligonucleotide Synthesis Core Facility, University of Michigan. The 5′ primer contains an *Eco*RI site, a T7 phage gene *10* (T*n10*) leader sequence (Olins et al., 1988), an SD consensus sequence, and 20 bp of normal human *hprt* coding sequence beginning with the A of the ATG start codon (Fig. 1). The 3′ primer consists of an *Eco*RI site 3′ of the terminal 15 bp of HPRT-coding sequence (Fig. 1). Five μg of each primer, 100 ng of *hprt* template in M13mp18, and 1 mM dNTPs, were combined in 1 × *Taq* buffer (2 mM $MgCl_2$/10 mM KCl/10 mM Tris pH 8.3/1 mM DTT) and heated to 94°C for 3 min. After the addition of 1 unit *Taq* DNA polymerase (Ampli-Taq, Perkin Elmer/Cetus, Norwalk, CT) the mixture was subjected to five cycles of 94°C, 1 min, 22°C, 1 min, 72°C, 2 min and then 30 cycles of 94°C, 1 min, 72°C, 2 min. The PCR reaction products were cut with *Eco*RI (Bethesda Research Laboratories) and purified on a Qiagen™ tip-5. The *hprt* expression cassettes for mutant sequences were made by performing the PCR on cDNAs generated by reverse transcribing 1 μg total RNA isolated from B-lymphoblast cell lines derived from patients K.C., P.C., and D.B., using previously described techniques (Table I; Davidson et al., 1989b). Conditions for amplifying and purifying mutant sequences were the same as those used for the wt template.

Expression cassettes for normal and mutant sequences were cloned into *Eco*RI-cut pSP72 (Promega, Madison, WI) downstream from the T7 promoter, and used to transform *E. coli* DH5α (Bethesda Research Laboratories). All expression plasmids were subjected to dideoxynucleotide sequencing to insure authenticity of sequence. The wt clone pSPSDE3, and variant clones pSPPC24, pSPKC4 and pSPDB1 (representing $HPRT_{Ashville}$, $HPRT_{Ann\ Arbor}$, and $HPRT_{London}$, respectively) were then used to transform BL21(DE3) for high-level production of human HPRT (Olins et al., 1988). The plasmids are described in detail in Table II.

(b) Time course of production of human HPRT in *Escherichia coli*

Fig. 2 is an SDS-PAGE gel of aliquots from a time course of induction of BL21(DE3)[pHPSDE3]. The production of a 24-kDa protein is evident in as little as 30 min post induction, and continues for up to 8 h (lanes 2–7). When the induction is allowed to continue for 24 h, there is little change in the relative amount of protein produced as compared to the 8 h time point (data not shown). As seen in lanes 8 and 9, there is no concomittant increase in the expression of a 24-kDa protein when pSP72 in BL21(DE3) is used; only the T7 polymerase subunits are induced. Note that expression of these subunits in the HPRT-expressing plasmids appears to be repressed, presumably due to the enhanced translation.

Immunoblotting was done using anti-human HPRT antibody to identify the overexpressed 24-kDa protein. As evident in lane 1 of Fig. 3, low levels of human HPRT are expressed before the addition of IPTG. This is presumably due to the fact that the *lac*UV5 promoter driving transcription of the T7 polymerase gene is not tightly repressed, even in the absence of IPTG. However, when IPTG is added, there is a large increase in the level of HPRT expressed by pHPSDE3 (lanes 2–4). Human HPRT is also evident in lane 6, which is BL21(DE3)[pHPSDE5]. This construct contains human *hprt* cloned in the opposite orientation to the T7 promoter. In this context, low-level synthesis of HPRT may occur as a result of transcription off an upstream pro-

AGA **GAA TTC** AAT AAT TTT GTT **TAA CTT** TAA GAA **GGA** GAT ATA TCC
Eco RI Epsilon SD

ATG GCG ACC CGC ACC CGC AGC CCT GGC GTC
Human *hprt* Sequence

Fig. 1. Expression cassette for expressing human *hprt* in *E. coli*. The cassette contains an *Eco*RI cloning site, the ε (epsilon) sequence from T7 bacteriophage gene *10* (Olins et al., 1988), and an SD sequence. The sequences which overlap with human *hprt* cDNA sequences are indicated (bold).

TABLE I

HPRT: aa substitutions and physical properties of HPRT variants

Cell line	Variant	The aa substitutions	Physical properties	Ref[a]
D.B.	London	$Ser^{110} \rightarrow Leu$	increased anodal migration on SDS–PAGE	A
K.C.	Ann Arbor	$Ile^{132} \rightarrow Met$	normal migration on SDS–PAGE and nondenaturing PAGE	B
P.C.	Ashville	$Asp^{201} \rightarrow Gly$	decreased anodal migration on nondenaturing PAGE	C

[a]A, Davidson et al. (1988); B, Fujimon et al. (1988); C, Davidson et al. (1989a).

TABLE II

Plasmids of variant *hprt* cDNAs

Variant[a]	The nt substitution[b]	Plasmid[c]
wt		pSPSDE3
D.B.	$C^{329} \rightarrow T$	pSPDB1
K.C.	$T^{399} \rightarrow G$	pSPKC4
P.C.	$A^{602} \rightarrow G$	pSPPC24

[a]See Table I for details regarding variants.
[b]Substitution of nt with respect to wt sequence.
[c]The plasmid backbone is pSP72 (Promega, Madison, WI).

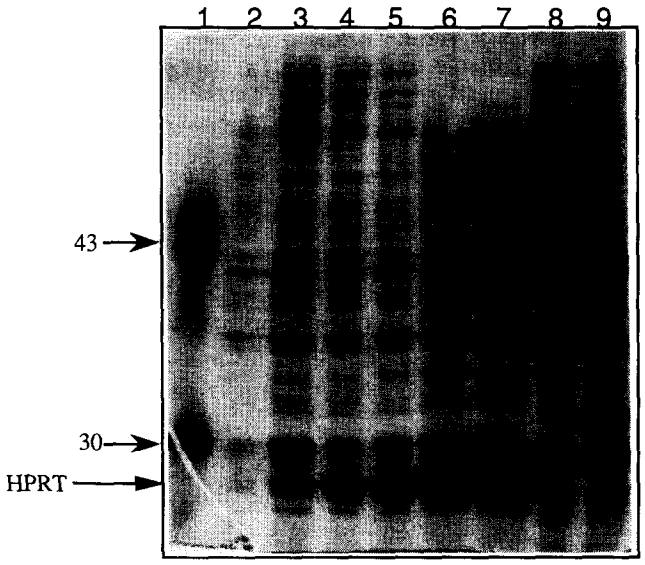

Fig. 2. Time course of synthesis of human HPRT in *E. coli*. Human HPRT was produced by the addition of 0.4 mM IPTG to cultures of BL21(DE3)[pHPSDE3] when A_{600} reached 0.4. Aliquots (1 ml) were removed at 0, 30 min, 1, 2, 4, and 8 h (lanes **2**–**7**, respectively), centrifuged 3 min, 14 000 × *g*, washed in 1 ml Hanks Balanced Salt Solution (HBSS, Gibco) divided into two 500 ml aliquots and centrifuged again for 3 min at 14 000 × *g*. One cell pellet was resuspended in Laemmli SDS-PAGE buffer and a portion analyzed by 0.1% SDS-12% PAGE. Note the rapid expression of human HPRT (large arrow). IPTG was also added to cultures of BL21(DE3)[pSP72]. These lysates were analyzed 1 and 8 h post induction (lanes **8** and **9**). Lane **1**, M_r markers. Molecular size (in kDa) are indicated on the left margin.

moter (for example β-lactamase) in conjunction with the strong translational enhancer sequences. In the parent plasmid alone (lane 6) no HPRT is seen even 8 h post-induction.

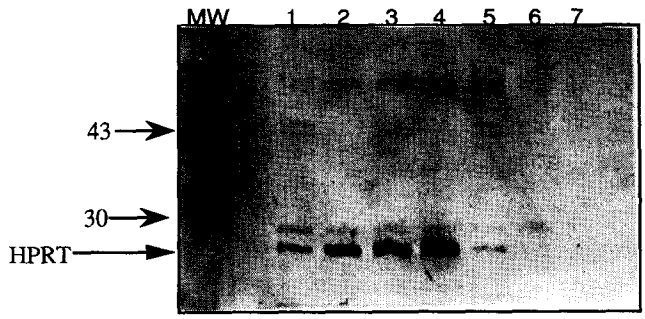

Fig. 3. Immunoblot of human HPRT produced in *E. coli*. Cultures of BL21(DE3)[pHPSDE3] were analyzed for the presence of human HPRT at 0, 30 min, 1, and 2 h post induction (lanes **1**–**4**). Protein was blotted onto PVDF membranes (Millipore), blocked 2 h at room temperature with TNS/0.5% Tween-20 (TT buffer; TNS is 20 mM Tris pH 7.5/500 mM NaCl), washed (all washes and antibody dilutions were done in TT buffer unless otherwise indicated), incubated overnight with anti-human HPRT antibody at 4°C, washed, incubated with goat-anti-rabbit antibody conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) for 2 h at room temperature, washed in TNS, and incubated with NBT/TIBS substrate (Kirkegaard and Perry Laboratories). Lane **5** is of BL21(DE3)[pSPSDE5] lysate in which the *hprt* cDNA is cloned antisense to the T7 promoter in pSP72. Lane **6** is lysate from BL21(DE3)[pSP72]. Cultures of BL21(DE3)[pSPSDE5] and BL21(DE3)[pSP72] were harvested 4 h after induction. Lane 7 contains 4 ng of purified human HPRT, the lower limit for this detection system. Gel as in Fig. 2. MW, prestained molecular size markers (Amersham). Molecular sizes as in Fig. 2. Large arrow points to the human HPRT.

(c) Purification of human HPRT from *Escherichia coli*

Large-scale cultures (500-ml aliquots) of BL21-(DE3)[pHPSDE3] were grown, and the cells harvested and lysed as described. Xanthine was added to a concentration of 1 mM before column loading to reduce the binding of *E. coli* xpt/gpt to the affinity sites. Bound human HPRT was eluted with buffer A containing 1 mM GMP, and the fractions containing the peak of human HPRT collected and concentrated. As seen in the SDS-PAGE gel in Fig. 4 (lane 2), the eluate contains more than 95% pure human HPRT. Initially, a molecular sieve column was included in the purification scheme, and was run before the affinity column. However, this did not significantly increase the purity of the final product (data not shown).

(d) Production of mutant HPRT in *Escherichia coli*

cDNAs were generated from three previously characterized human HPRT variants, $HPRT_{Ann\ Arbor}$,

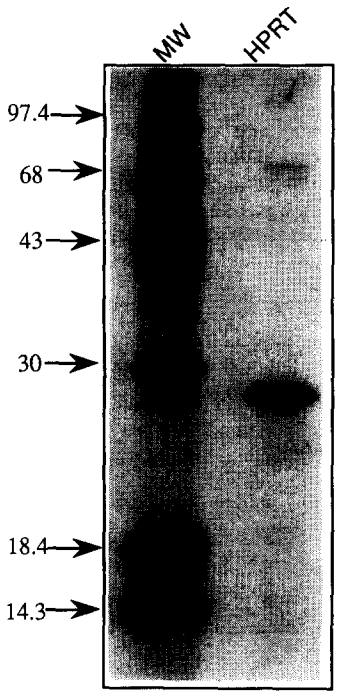

Fig. 4. Purified human HPRT isolated from *E. coli*. For purification, single colonies were placed in 3 ml of LB broth containing 30 μg ampicillin/ml, allowed to grow to A_{600} of 0.4–0.6, and transferred to 500 ml LB broth. The culture was allowed to reach A_{600} of 0.4, after which IPTG was added to 0.4 mM final concentration. Inductions ranged from 4 h to overnight for large scale cultures. After induction the cells were harvested, washed in buffer A, and resuspended in buffer A containing 10% glycerol. The suspension was lysed by French press, debris pelleted, and the remaining lysate brought to 1 mM xanthine. The lysate was then passed over a GMP-agarose affinity column (Sigma Biochemicals). The column was washed with buffer A (buffer A is 50 mM Tris pH 7.4/25 mM KCl/10 mM $MgCl_2$/1 mM DTT), and human HPRT eluted with 1 mM GMP in buffer A. Those fractions containing human HPRT were pooled, concentrated, and analyzed for purity by 0.1% SDS-12% PAGE followed by Coomassie staining. Molecular size in kDa are indicated on the left margin.

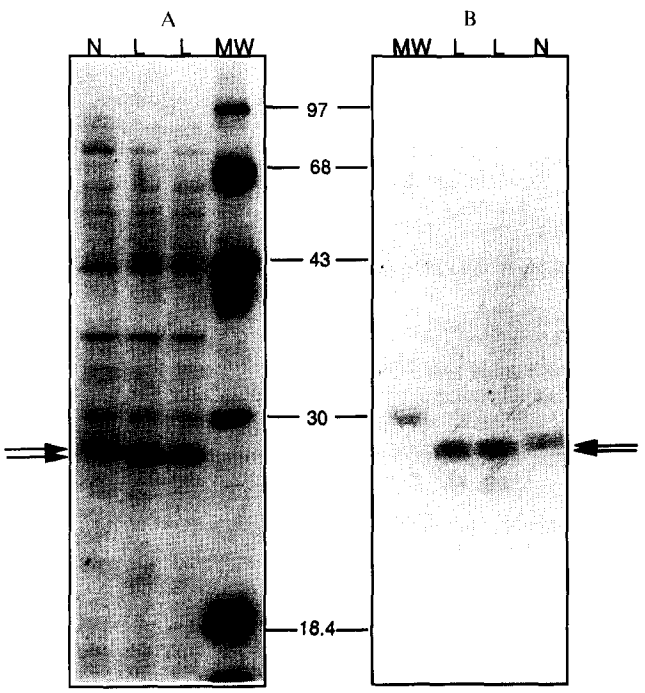

Fig. 5. Expression of $HPRT_{London}$ in *E. coli*. (Panel **A**) Cultures of BL21(DE3)[pSPDB1] and BL21(DE3)[pSPSDE3] were induced, and lysates analyzed by SDS-PAGE (as in Fig. 2). The arrows denote the differences in migration between normal (N) and London (L) HPRTs. MW, rainbow markers (Amersham). Molecular sizes in kDa are indicated. (Panel **B**) Immunoblot of the SDS polyacrylamide gel in panel **A**.

$HPRT_{Ashville}$, and $HPRT_{London}$ (Table I), by reverse transcribing RNA isolated from B-lymphoblasts derived from patients K.C., P.C., and D.B., respectively, followed by ECPCR. The resultant expression cassettes were then cloned into pSP72 and transformed into BL21(DE3) as described. Colonies were induced for production of HPRT, and analyzed by SDS-PAGE and nondenaturing PAGE followed by activity gel assay. As noted in Table I, $HPRT_{London}$ migrates more anodally than does normal HPRT in SDS-PAGE of B-lymphoblast lysates. This physical property is reproduced in pHPDB1, the expression cassette representing this variant (Fig. 5A). Immunoblotting confirmed that the faster migrating species was HPRT (Fig. 5B). The HPRT produced from pSDKC4 and pSDPC21 exhibited normal migration on SDS-PAGE following induction with IPTG, consistent with the corresponding variant protein from B-lymphoblasts (data not shown).

Lysates from induced cultures of pSDDB1, pSDKC4 and pSDPC21 were also run on native gels and then assayed in situ for HPRT activity. As expected, protein synthesized from pSDKC4 had normal migration while pSDPC21 was less anodal than normal (Fig. 6). Again, these properties are consistent with those of the B-lymphoblast proteins.

The use of the T7 bacteriophage gene *10* translational enhancer sequences upstream from the required SD sequence in the expression cassette greatly improved the expression of T7 promoter-driven sequences (Fig. 1). When expression cassettes were made containing only the normal *hprt* cDNA construct plus SD sequences, the production of HPRT was minimal (data not shown). Indeed, the presence of human HPRT was visualized only by nondenatured PAGE/activity assay in lysates from these cultures, and not by SDS PAGE. Additionally, we saw only variable levels of low expression when this cassette was cloned downstream from a heat-inducible *tac* promoter (data not shown). However, the *tac* promoter system was not tried in conjunction with the strong translational enhancer sequences.

An additional consideration when using strong translational enhancer sequences is the 'leakiness' of the promoter driving T7 polymerase. As evident in Fig. 3, human HPRT was synthesized, albeit at low levels, even in the

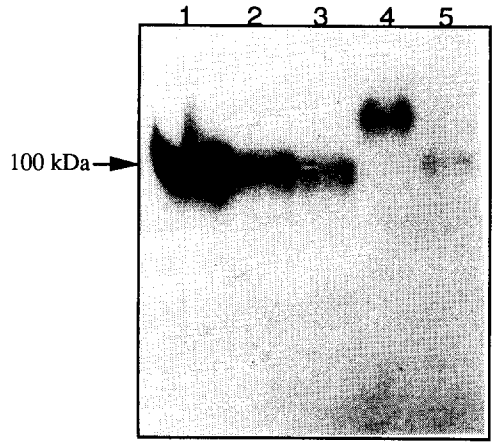

Fig. 6. Analysis of synthesised proteins by native 6% PAGE and activity assay. Cultures containing the variant *hprt* expression cassettes were induced for 4 h, harvested, and aliquots resuspended in lysis buffer (20 μg lysozyme/ml in 10 ml $MgCl_2$/20 mM KCl/50 mM Tris pH 7.4), incubated for 30 min at room temperature, and centrifuged at 14 000 × *g* for 5 min. Non-denatured lysates were run on 6% polyacrylamide gels and assayed in situ for human HPRT activity using previously described techniques (Davidson et al., 1989a). Lanes: **1**, 5 μg of protein from GM558 lysate, a B-lymphoblast line derived from a normal male; **2–5**, *E. coli* BL21(DE3) cells carrying: pSPDB1, pSPKC4, pSPPC24, and pSPSDE3, respectively (described in Table II).

absence of IPTG. If the gene of interest is deleterious to cell viability such an enhancer may be counterproductive. Based on this, a variety of combinations of translation enhancer/promoter systems may need to be tried to maximize protein production.

The one-step purification scheme for obtaining human HPRT is a dramatic improvement over that required to isolate purified human HPRT from erythrocyte lysates (Wilson et al., 1982b), making this an attractive alternative for obtaining large amounts of the purified enzyme for crystallographic analysis. Furthermore, one of the variant proteins chosen for this study, $HPRT_{London}$, has been purified in small amounts from erythrocytes using a GMP-affinity column (Wilson et al., 1983). Crystallization and analysis of these two proteins alone will enable us to understand the elevated (sixfold) K_m for hypoxanthine in this variant.

(e) Conclusions

ECPCR done with expression cassettes containing strong translational enhancer sequences enabled us to synthesize and purify active human HPRT in *E. coli*. In addition, variant sequences were expressed, with the resultant proteins exhibiting physical properties indistinguishable from their B-lymphoblast counterparts. These results complete an important preliminary step towards understanding the enzymology of HPRT, namely, producing large quantities of the protein for crystal growth, diffraction analysis, and three-dimensional structure determination.

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