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Genetic basis of hypoxanthine guanine phosphoribosyltransferase deficiency in a patient with the Lesch–Nyhan syndrome ($HPRT_{Flint}$)

(Recombinant DNA; cDNA cloning; RNase A mapping; secondary structure prediction; hyperuricemia; gout; phage λgt11 vector)

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SUMMARY

The molecular basis for complete hypoxanthine guanine phosphoribosyltransferase (HPRT) deficiency has been determined in a patient with Lesch–Nyhan syndrome. A B-lymphoblastoid cell line derived from this patient expresses normal amounts of HPRT mRNA yet no detectable immunoreactive protein as determined by radioimmunoassay. These findings suggest either a decreased rate of translation or accelerated degradation due to enhanced proteolytic susceptibility. cDNAs synthesized from this patient's RNA have a single nucleotide (nt) substitution, a C → A transversion at nt 222. RNase A cleavage analysis confirms the presence of a mutation at this position within mRNA isolated from lymphoblasts from patient A.C. This transversion predicts a phenylalanine to leucine replacement at amino acid position 73 in the translated protein. We have designated this mutant $HPRT_{Flint}$. The mutation in $HPRT_{Flint}$ disrupts a strongly conserved region among PRTases from *Escherichia coli*, rodents and man, suggesting an important role for this region for the normal function of HPRT. Since it is unlikely that this amino acid substitution alters the translational rate, we hypothesize that disruption of the secondary structure within this region renders $HPRT_{Flint}$ more susceptible to proteolysis.

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Abbreviations: aa, amino acid(s); A.C., patient initials; bp, base pair(s); CRM, immunologically cross-reactive material; GMP, guanosine monophosphate; HPRT, hypoxanthine guanine phosphoribosyltransferase; IMP, inosine monophosphate; nt, nucleotide(s); RIA, radioimmunoassay.

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INTRODUCTION

Hypoxanthine guanine phosphoribosyltransferase (HPRT; EC 2.4.2.8) is a purine salvage enzyme that catalyzes the conversion of hypoxanthine and guanine to IMP and GMP, respectively. Human HPRT mutants were isolated over 25 years ago and became the basis for development of a DNA-mediated transformation system (Szybalska and Szybalski, 1962; Scangos and Ruddle, 1981). Partial deficiency of this enzyme results in hyperuricemia and a severe form of gout (Kelley et al., 1967). Complete HPRT deficiency causes the Lesch–Nyhan syndrome, a disease manifested by hyperuricemia, hyperuricaciduria, uric acid renal stones and severe neurological abnormalities (Lesch and Nyhan, 1964; Seegmiller et al., 1967).

The determination of mutations responsible for HPRT deficiency in man has been limited in the past to the study of mutations which do not greatly affect the intracellular enzyme concentration. The identification of the complete amino acid sequence of the normal enzyme (Wilson et al., 1982a) and the determination of amino acid substitutions in four mutant proteins (Wilson et al., 1986) have led to a preliminary understanding of the roles of certain domains with respect to the catalytic function of HPRT (Argos et al., 1983).

To extend our understanding of the molecular basis of HPRT-deficient states, we have undertaken the characterization of mutant forms of human HPRT. HPRT-deficient subjects are a phenotypically heterogeneous population with regard to enzyme activity and concentration. This phenotypic variability reflects the occurrence of new and independent mutations at the HPRT locus (Yang et al., 1984; Wilson et al., 1986). More than 75% of these mutants have normal amounts of HPRT specific mRNA. Furthermore, in all but five cases described to date, transcript size is grossly normal (Wilson et al., 1986; Yang et al., 1984). These characteristics, together with available techniques for cDNA cloning, provide the necessary rationale and tools for the identification of mutations which lead to dysfunctional HPRT.

A subject (A.C.) with the Lesch–Nyhan syndrome, has been previously characterized. Erythrocytes and lymphoblasts from A.C. had undetectable HPRT activity (<0.1 mU/mg or <0.7% of control values). Immunoreactive protein, as determined by RIA using polyclonal anti-human HPRT, was also undetectable (<1.5 ng CRM/mg or <0.5% of control values). HPRT-specific mRNA from A.C. lymphoblasts was grossly normal with respect to size and amount on Northern-blot analysis (Wilson et al., 1986). We have identified a single nucleotide substitution in HPRT cDNA clones derived from A.C. mRNA. The resultant amino acid change replaces phenylalanine with leucine in a region of the protein strongly conserved in *E. coli*, rodent and human phosphoribosyltransferases (Hershey and Taylor, 1986). These data suggest an important role for Phe-73 in maintaining an enzymatically active protein.

EXPERIMENTAL AND DISCUSSION

(a) cDNA cloning and recombinant analysis

B-lymphoblastoid cells were established from lymphocytes isolated from patient A.C. as previously described (Wilson et al., 1982b). RNA was isolated

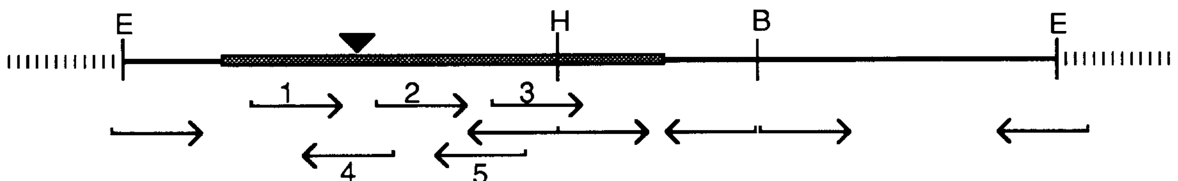

Fig. 1. Strategy for sequencing mutant cDNA clones. The entire cDNA was sequenced in both directions using HPRT-specific oligomers (arrows 1 → 5) or the M13 universal primer (unnumbered arrows). The nucleotide sequences of HPRT-specific primers 1 through 5 are listed in Table I. The site of the mutation is indicated by a triangle. Stippled bar, HPRT-coding sequence; single line, HPRT-untranslated sequence; vertical dashes, vector sequence; B, *Bal*I site; E, *Eco*RI site; H, *Hin*dIII site. The direction of the arrows corresponds to sequencing direction.

TABLE I

HPRT specific oligodeoxynucleotide primers

Oligodeoxy-nucleotide	Sequence (5′ → 3′)	Priming site[a] (nt)	Strand[b]
1	AGTGATGATGAACCAG	31– 47	–
2	CACTGAATAGAAATAGT	251–267	–
3	GATATAATTGACACTGG	403–419	–
4	CCCCTGTTGACTGGTCA	337–321	+
5	AGTCCTGTCCATAATTA	138–122	+

[a] Corresponds to the nucleotides of sense strand of HPRT cDNA where the A of the ATG start codon is position 1. Note that primers 4 and 5 hybridize to the sense strand and are therefore the complement of the HPRT cDNA sequence.

[b] The strand to which the primer anneals is represented as either sense (+) or antisense (–).

using guanidium isothiocyanate (Chirgwin et al., 1979) and then subjected to oligo-d(T) column chromatography to obtain poly(A)$^+$ RNA (Aviv and Leder, 1972). Approximately 10 μg of poly(A)$^+$ RNA were used to synthesize an oligo-d(T)-primed library (Okayama and Berg, 1982; Gubler and Hoffman, 1983), which was cloned into λgt11 (Young and Davis, 1983).

Recombinant phages were screened with an [α-^{32}P]dCTP-labeled *Msp*I-*Taq*I (160 nt) fragment of normal HPRT cDNA containing portions of exons 1 and 3 and all of exon 2 (Brennand, 1983). Four HPRT-positive recombinants were isolated: pHPAC1, pHPAC2, pHPAC8 and pHPAC12. Restriction endonuclease mapping and nucleotide sequencing revealed that pHPAC8 and pHPAC12 were full-length, containing 654 nt of coding sequence, 590 nt of 3′-untranslated sequence and 118 and 120 nt of 5′-untranslated sequence, respectively. pHPAC1 contained only 52 nt of 5′-untranslated sequence.

(b) Sequencing of mutants

The dideoxynucleotide sequencing strategy for pHPAC1, pHPAC8, and pHPAC12 is depicted in Fig. 1, and used both HPRT-specific primers and the M13 universal primer (Bethesda Research Laboratories; Biggin et al., 1983; Bonthron et al., 1985). The positions of the HPRT nucleotide sequence to which these primers anneal and the strands to which they anneal are shown in Table I.

Sequencing of pHPAC8 showed two differences from normal HPRT sequence, a C → A transversion at nt 222 and a G → A transition at nt 658. Sequencing of clones pHPAC12 and pHPAC1 revealed only the C → A transversion at 222, indicating that the G → A transition is probably a cloning artifact. Consistent with this conclusion, RNase cleavage analysis also failed to detect the transition at nt 658 (not shown).

The C → A transversion at nt 222 predicts a Phe → Leu change at aa 73 (Fig. 2). The normal abundance of HPRT mRNA in patient A.C. suggests that one effect of this nucleotide substitution is at the level of the primary structure of the protein since no immunoreactive protein is detectable in lymphoblasts derived from this patient (Wilson et al., 1986). Phe-73 is strongly conserved among various phosphoribosyltransferases when aligned to give maximal overlap of predicted secondary structures (Table II). Analysis (Chou and Fasman, 1984)

			73		
	Tyr	Lys	[Leu]	Phe	Ala
$HPRT_{Flint}$	TAT	AAA	TT[A]	TTT	GCT
			222		
	TAT	AAA	TT[C]	TTT	GCT
Normal	Tyr	Lys	[Phe]	Phe	Ala

Fig. 2. Mutant nucleotide (nt 214–228) and aa (71–75) sequence of $HPRT_{Flint}$ compared to normal. The C → A transversion at nt 222 (box) causes a Phe → Leu substitution at aa 73 (box).

TABLE II

Conservation of Phe-73 among various phosphoribosyltransferases[a]

Phosphoribosyl-transferases	Amino acid residues from position 69 to 76[b]								Reference[c]
	69	70	71	72	73	74	75	76	
$HPRT_{Flint}$	Gly	Gly	Tyr	Lys	**Leu**	Phe	Ala	Asp	
Human HPRT	Gly	Gly	Tyr	Lys	Phe	Phe	Ala	Asp	**A**
Hamster HPRT	Gly	Gly	Tyr	Lys	Phe	Phe	Ala	Asp	**B**
Mouse HPRT	Gly	Gly	Tyr	Lys	Phe	Phe	Ala	Asp	**C**
Human APRT	Gly	Phe	Leu	—	Phe	Gly	Pro	Ser	**D**
Mouse APRT	Gly	Phe	Leu	—	Phe	Gly	Pro	Ser	**E**
E. coli APRT	Gly	Phe	Leu	—	Phe	Gly	Ala	Pro	**F**
E. coli XGPRT	Gly	Gly	Leu	Val	Pro	Gly	Ala	Leu	**G**
E. coli OPRT	—	Gly	Ile	Glu	Phe	—	—	Asp	**H**

[a] Sequences are aligned to give maximal overlap of predicted secondary structure (Chou and Fasman, 1978)

[b] Residue numbers correspond to the amino acid sequence of the mature human enzyme isolated from erythrocytes (Wilson et al., 1983)

[c] **A**, Wilson et al. (1982b); **B,C**, Konecki et al. (1983); **D**, Wilson et al. (1986); **E**, Dush et al. (1985); **F**, Hershey and Taylor (1986); **G**, Pratt and Subramani (1983). **H**, Poulsen et al. (1983).

of the region spanning aa residues 69 to 76 predicts an α-helical region directly adjacent to a β-turn. Leu, which has a stronger propensity toward α-helical formation than does Phe, could result in the extension of a helix into what normally exists as a turn. Furthermore, it is likely that the replacement of the strongly conserved aromatic side chain at this position affects appropriate packing to accommodate

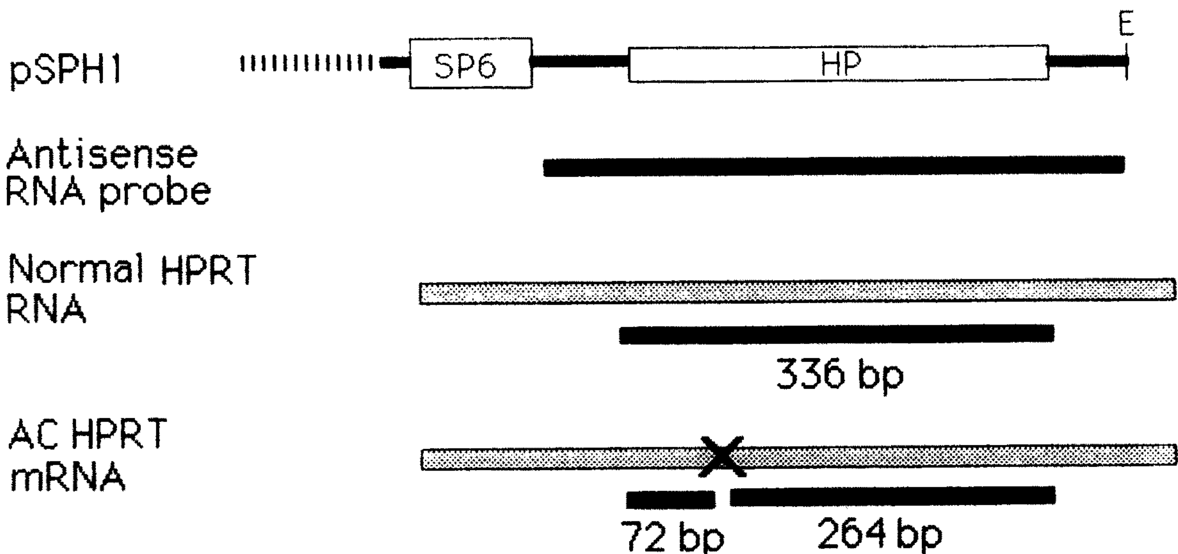

Fig. 3. Map of plasmid pSPH1 used in RNase A mapping analysis. The pSP64 plasmid was cleaved with *Hin*dIII + *Sal*I and ligated to the 336-bp *Hin*dIII-*Xho*I fragment of normal HPRT cDNA sequence. The resultant recombinant plasmid, pSPH1, was linearized with *Eco*RI (E). Transcription with SP6 RNA polymerase using the SP6 promoter results in the production of the antisense strand of HPRT sequence. The probe, hybridized to normal RNA or to RNA of patient A.C., is cleaved with RNase A and RNase T1, giving either a 336-bp fragment (completely protected) or 72- and 264-bp fragments, respectively. The actual cleavage products are shown in Fig. 4. HP, HPRT sequences; SP6, SP6 promoter; vertical dashes and single line, vector sequences; blackened bar, RNA probe; stippled bar, mRNA sequences.

the preceding turn. There are numerous examples of the impact of single amino acid substitutions on the rate of folding and/or assembly of proteins in vivo (Beasty et al., 1986). Thus, the impact of this mutation may also be at the levels of both secondary and tertiary structures.

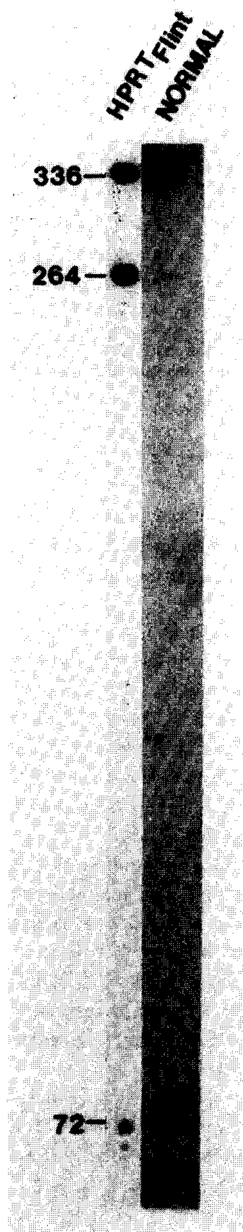

Fig. 4. RNase A mapping of $HPRT_{Flint}$. RNase cleavage products of approx. 72 bp and 264 bp (see Fig. 3) are seen in RNA from patient A.C., compared to the completely protected fragments in the adjacent lane (normal). RNase A and RNase T1 digestion (Gibbs and Caskey, 1986) were followed by two ethanol precipitations. The RNase-digested RNA was then electrophoresed in a 6% denaturing polyacrylamide gel containing 8 M urea. The gel was dried and autoradiographed for 12 to 16 h.

(c) RNase A mapping

RNase A mapping of A.C. mRNA was done using pSPH1 which is the pSP64 plasmid (Promega Biotech) into which the *Xho*I-*Hin*dIII fragment representing bp 149 to 485 of normal human HPRT cDNA has been cloned (Fig. 3). RNA transcripts were made using [α-^{32}P]GTP and hybridized to 100 μg of total RNA, as described by Gibbs and Caskey (1987).

A mismatch between the cDNA clones of $HPRT_{Flint}$ and normal HPRT mRNA was confirmed using this approach (Figs. 3 and 4). The 336-bp fragment spanning the area of the mutation was cleaved when hybridized to RNA isolated from lymphoblasts derived from patient A.C., yielding the cleavage products of 72 and 264 bp, appropriate sizes for a mutation site at bp 222 (Fig. 4). RNase cleavage was incomplete as noted by the presence of the 336-bp fragment. No cleavage products were seen when a labeled RNA probe spanning bp 658 was used (not shown), providing further evidence that the transition at 658 in pHPAC8 is a cloning artifact.

(d) Conclusions

Cloning of mutant forms of HPRT cDNA from deficient subjects is a rapid method for determining those structural alterations which render the enzyme inactive and/or unstable. This is the first report of a mutation in this region of the HPRT molecule being responsible for a complete absence of enzyme protein. The high degree of evolutionary conservation of amino acid sequence in this region, as well as the profound impact of this mutation within that region, suggest an important role for this sequence in the maintenance of normal protein stability and activity.

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