

Isolation and characterization of a full-length expressible cDNA for human hypoxanthine phosphoribosyltransferase

(Lesch-Nyhan disease/gene cloning/transfection/DNA sequence analysis)

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ABSTRACT We have cloned a full-length 1.6-kilobase cDNA of a human mRNA coding for hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) into a simian virus 40-based expression vector and have determined its full nucleotide sequence. The inferred amino acid sequence agrees with a partial amino acid sequence determined for authentic human HPRT protein. Transfection of HPRT-deficient mouse LA9 cells with the purified plasmid leads to the expression of human HPRT enzyme activity in cells stably transfected and selected for enzyme activity in hypoxanthine/aminopterin/thymidine medium.

Current methods of molecular biology, including the techniques of recombinant DNA construction and cloning, rapid nucleotide sequence analysis, the design and construction of transducing vectors, and techniques of transfection of eukaryotic cells with foreign genes, have made it possible to clone and characterize a large number of eukaryotic genes. One gene of particular interest, not only for basic studies of eukaryotic gene regulation but also for understanding of several important human genetic diseases, is the gene encoding the enzyme hypoxanthine phosphoribosyltransferase (HPRT) (1). This enzyme catalyzes vital steps in the reutilization pathway for purine biosynthesis, and its deficiency leads to forms of gouty arthritis and to the devastating Lesch-Nyhan disease (2, 3). The HPRT locus is known to be X linked in the human and other mammalian genomes, and the availability of a cloned HPRT gene would facilitate studies of the organization of a particularly interesting region of the human X chromosome and of the mechanisms of inactivation of specific and well-mapped regions of the X chromosome. Recently, we succeeded in isolating a human genomic clone containing a portion of the HPRT gene together with some intervening sequence (4). This fragment of the HPRT gene has been used to isolate a full-length cDNA clone encoding the human HPRT enzyme from a human cDNA library. Nucleotide sequence analysis of the cloned cDNA segment established that it encodes the entire HPRT protein.

METHODS

Isolation of the HPRT Gene Fragment. A genomic segment of human DNA containing sequences from the extreme 5' end of the HPRT gene together with a portion of an intervening sequence has been isolated and cloned as plasmid p6B2aE2 (4) by a combination of gene transfection into enzyme-deficient mouse cells and localization of human sequences by hybridiza-

tion with probes of middle repetitive human (*Alu*) sequence, as described (4). A 1.5-kilobase (kb) *EcoRI/BamHI* fragment derived from p6B2aE2 and free of repetitive human sequences has been subcloned into the *BamHI* and *EcoRI* sites of pBR322 and shown to hybridize to a discrete human cytoplasmic poly(A)⁺RNA approximately 1.5 kb long, presumably representing the mRNA for HPRT (4). DNA from this subcloned plasmid, called pBR1.5, was prepared by detergent lysis (5) of chloramphenicol-treated (6) transformed SF8 *Escherichia coli* (7) followed by cesium chloride/ethidium bromide ultracentrifugation (8). This DNA was cleaved with *EcoRI/BamHI* and the cloned insert was isolated by trough electroelution onto a dialysis membrane (9) after agarose gel electrophoresis. It was labeled by nick-translation (10) with [³²P]dATP (3,000 Ci/mmol; 1 Ci = 37 GBq; Amersham) in the presence of unlabeled dCTP/dGTP/dTTP to a specific activity of approximately 2 × 10⁸ cpm/μg and was thereafter used in hybridization studies to screen a human cDNA library.

Preparation and Screening of a Human cDNA Library. The preparation of the complete human cDNA library and the sublibraries containing different size cDNA inserts is described in detail elsewhere (11). Plasmid DNA representative of the complete and sublibraries was transformed into actively growing cultures of *E. coli* SF8 (OD₆₀₀ = 0.6) by the method of Lerach *et al.* (12). Transformed colonies were selected by growth overnight at 37°C on Penassay base agar (Difco Laboratories) containing ampicillin at 25–50 μg/ml (Sigma) in 400-cm² Petri dishes (Nunc Bio-assay plates) at one dish per transformation. Approximately 300,000 transformants per library were grown and replica plated twice with velvet. *E. coli* SF8 containing the cloned HPRT fragment (p6B2aE2) was plated as a positive control.

The colonies on the replica plates were transferred to Whatman 541 paper, amplified on chloramphenicol plates overnight at 37°C by the method of Gergen *et al.* (13), denatured and fixed to the filters, and screened for HPRT cDNA sequences by hybridization with the gel-purified insert from pBR1.5 containing the HPRT fragment free of repeat sequences. The filter replicas of the random colony arrays from each library were prehybridized for 4 hr at 60°C in 30% formamide/0.5 M NaCl/20 mM Hepes, pH 7.4/0.1 mM EDTA/10 mM NaH₂PO₄/10 mM Na₄P₂O₇ containing denatured and sonicated herring sperm DNA at 500 μg/ml (buffer A) and then hybridized to the ³²P-labeled nick-translated pBR1.5 insert fragment overnight at 60°C in buffer A/10% dextran sulfate. The filter sheets were

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; kb, kilobase(s); HAT, hypoxanthine/aminopterin/thymidine; bp, base pair(s).

then washed twice with 0.30 M NaCl/0.030 M Na citrate, pH 7.4, and then with 60 mM NaCl/6 mM Na citrate, pH 7.4, once at 60°C and twice at room temperature. Filters were dried and autoradiographed on flashed Kodak XAR-5 film with an intensifying screen at -70°C.

Areas showing a putative positive signal on duplicate filters were identified, scraped with a toothpick onto 1 ml of Penassay broth, plated at various concentrations from the broth, and grown overnight. Single colonies were transferred with a toothpick into arrays, grown overnight, and rescreened on Whatman 541 paper as described above.

DNA "minipreps" (14) were prepared from apparently positive colonies derived from the one original positive area from the transformation with sublibrary 4 (11), which contains inserts of 1.5–2.0 kb of DNA. The cDNA inserts were screened with ³²P-labeled nick-translated purified insert DNA from pBR1.5 by blotting agarose gels containing whole DNA or *Bam*HI digests onto nitrocellulose according to Southern (15), treatment with HCl (16), and hybridization as described above for the cDNA library, with the addition of 5-fold concentrated Denhardt's additives (17) to the prehybridization and hybridization buffers.

All manipulations involving recombinant DNA were carried out in accordance with National Institutes of Health and institutional guidelines and requirements.

Transfection of Enzyme-Deficient Cells. The isolated full-length cDNA plasmid was introduced into enzyme-deficient mouse LA9 cells grown in Dulbecco's modified Eagle's medium/10% calf serum by calcium phosphate-mediated transfection as described (4, 18), and the resulting HPRT-positive cells were selected by growth in hypoxanthine/aminopterin/thymidine (HAT) selective medium (19). Colonies were picked through cloning cylinders and grown in the selective medium.

Gel Assay for HPRT Enzyme Activity. After isoelectric focusing in polyacrylamide gels, cytoplasmic extracts of trans-

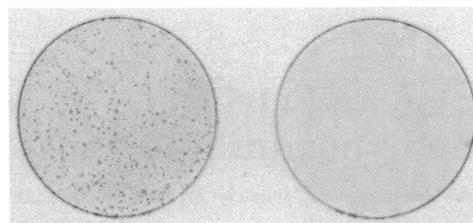


FIG. 2. Transfection of mouse LA9 cells with the HPRT clone p4aA8 (Left) and a control clone, p4aB8 (Right). Approximately 10^6 HPRT-deficient LA9 cells per plate were transfected with 1 μ g of plasmid together with 20 μ g of LA9 carrier DNA, grown in HAT medium after transfection, and stained with Giemsa.

ected and control cells were assayed for HPRT activity by the method of Johnson *et al.* (20).

Cloning of p4aA8 cDNA Fragments into M13. Plasmid p4aA8 was treated with *Bam*HI and electrophoresed on a preparative 1% agarose gel in Tris acetate buffer (21). Subclones in M13 mp8 were generated by the method of P. Deininger (personal communication). Briefly, the 1.6-kb *Bam*HI fragment from p4aA8 was electroeluted (9), ligated into a multimer, and sonicated to give random shear fragments of 200–800 base pairs (bp), which were ligated into the *Sma*I-cut phosphatase-treated M13 mp8 vector. The ligation mixture was used to transfect *E. coli* JM 101 (22).

DNA Sequence Analyses. Single-stranded template was prepared from white M13 plaques and used for sequence determination by the dideoxy chain-termination methods of Messing *et al.* (22) and Sanger *et al.* (23) using an M13 pentadecamer primer (New England BioLabs).

HPRT Amino Acid Sequence Determination. Authentic human HPRT enzyme was purified as described by Johnson *et al.* (24) and tryptic peptides were prepared and purified by HPLC (25). The amino acid sequences of several of the peptides were determined by microanalysis using a modified spinning cup method (26–28).

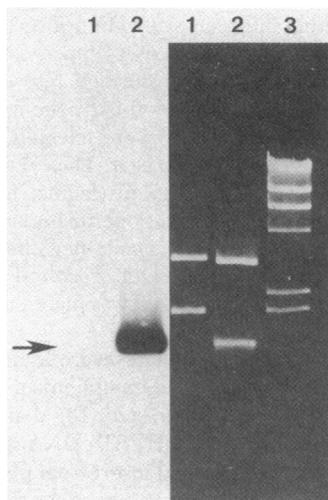


FIG. 1. Structure of the HPRT cDNA plasmid (p4aA8). Purified plasmid DNA was digested to completion with *Bam*HI, electrophoresed on a 1% agarose gel, stained, and photographed, and the DNA was blotted onto nitrocellulose paper. The paper was hybridized to the ³²P-labeled insert from pBR1.5 in 10% formamide/0.5 M NaCl/0.1 mM EDTA/50 mM HEPES, pH 7.4, at 60°C, washed, and exposed to x-ray film. (Left) X-ray film of the hybridized blot. (Right) Photograph of the ethidium bromide-stained gel. Lanes: 1, p4aB8 (a randomly picked control plasmid); 2, p4aA8 (the HPRT cDNA plasmid); 3, *Hind*III-digested bacteriophage markers (from top to bottom): 23.6, 9.6, 6.6, 4.3, 2.3, 2.0, and 0.55 kb (29). Arrow, the heavily hybridizing insert fragment from the HPRT cDNA plasmid.

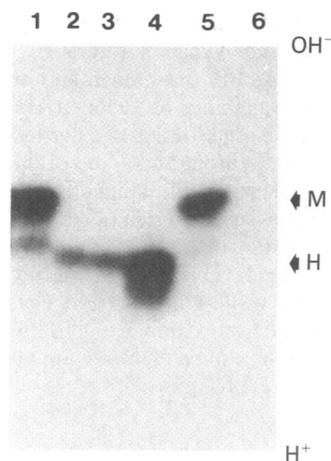


FIG. 3. Gel assay for HPRT activity of transfected mouse LA9 cells. Polyacrylamide isoelectric focusing gels loaded with cell lysates from different cell types were assayed for enzyme activity, and results were detected by autoradiography. OH⁻ and H⁺, alkaline and acid ends of the gel; M and H, major mouse and human HPRT activities. (Both human and mouse HPRT also display a minor band of activity at a lower pI.) Lanes: 1, 3T6 mouse cells; 2, a line of cells derived from LA9 cells transfected to HAT resistance with p4aA8 and making HPRT that has human isoelectric focusing properties; 3, as in lane 2 but a separately derived cell line; 4, HeLa (human) cells; 5, 3T6 mouse cells; 6, LA9 HPRT⁻ cells, the parent of the cell lines in lanes 2 and 3.

RESULTS

Approximately 300,000 colonies of the cDNA library were transferred from nutrient-containing plates to filters and hybridized with the nick-translated insert from plasmid pBR1.5,

isolated previously (4), thought to contain a portion of the HPRT coding sequence and some HPRT intervening sequence. One reproducibly hybridizing colony was found on duplicate filters, and it was picked, colony purified, and grown to prepare large

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GGGGGGGGGGGGGGTCTTGCTGCGCCTCCGCCCTCCTCCTCTGCTCCGCCACCGGCTTCCCT
 10          20          30          40          50          60
CCTCCTGAGCAGTCAGCCCGCGCGCCGGCCGGCTCCGTTATGCGGACCCGCGAGCCCTGGC
 70          80          90          100          110          120
val val ile ser asp asp glu pro gly tyr asp leu asp leu phe cys ile pro asn his
GTCGTGATTAGTGATGATGAACCAGGTTATGACCTTGATTTATTTTGCATACCTAATCAT
 130          140          150          160          170          180
tyr ala glu asp leu glu arg (val phe ile pro his gly leu ile) met (asp arg thr glu
TATGCTGAGGATTTGGAAAAGGGTGTTTATTCCTCATGGACTAATTTATGGACAGGACTGAA
 190          200          210          220          230          240
arg leu ala arg asp val met) lys glu met gly gly his his ile val ala leu cys val
CGTCTTGCTCGAGATGTGATGAAGGAGATGGGAGGCCATCACATTTGTAGCCCTCTGTGTG
 250          260          270          280          290          300
leu lys gly gly tyr lys phe phe ala asp leu leu asp tyr ile lys ala leu asn arg
CTCAAGGGGGGCTATAAAATCTTTGCTGACCTGCTGGATTACATCAAAGCACTGAAATAGA
 310          320          330          340          350          360
asn ser asp arg ser ile pro met thr val asp phe ile arg leu lys (ser tyr cys asn
AATAGTGATAGATCCATTCCTATGACTGTAGATTTTATCAGACTGAAGAGCTATTGTAAAT
 370          380          390          400          410          420
asp gln ser thr gly asp ile lys) val ile gly gly asp asp leu ser thr leu thr gly
GACCAGTCAACAGGGGACATAAAAGTAAATTTGGTGGAGATGATCTCTCAACTTTAACTGGA
 430          440          450          460          470          480
lys (asn val leu ile val glu asp ile ile asp thr gly lys) thr met gln thr leu
AAGAATGTCTTGATTTGTGGAGAATATAATTTGACACTGGCAAACAATGCAGACTTTGCTT
 490          500          510          520          530          540
ser leu val arg gln tyr asn pro lys met val lys val ala ser leu leu val lys arg
TCCTTGGTTCAGGCAGTATAATCCAAAGATGGTCAAGGTCGCAAGCTTGTCTGGTGAAAAGG
 550          560          570          580          590          600
thr pro arg ser val gly tyr lys pro asp phe val gly phe glu ile pro asp lys phe
ACCCACGAAAGTGTGGATATAAGCCAGACTTTTGTGGATTTGAAATTCAGACAAAGTTT
 610          620          630          640          650          660
val val gly tyr ala leu asp tyr asn glu tyr phe arg (asp leu asn his val cys val
GTTGTAGGATATGCCCTTGACTATAATGAAATACCTTCAGGGAATTTGAATCATGTTTGTGTC
 670          680          690          700          710          720
ile ser glu thr gly lys ala lys) tyr lys ala ***
ATTAGTGAAAACCTGAAAAGCAAAAATACAAAAGCCTAAGATGAGAGTTCAAGTTGAGTTTG
 730          740          750          760          770          780
AAACATCTGGAGTCTTATTGACATCGCCAGTAAAATTAATCAATGTTCTAGTTCTGTGGCC
 790          800          810          820          830          840
ATCTGCTTAGTAGAGCTTTTGTGATGATCTTCTAAGAAATTTTATCTGTTTGTACTTTA
 850          860          870          880          890          900
GAAATGTCAGTTGCTGCATTCCTAAACTGTTTATTTGCACATATGAGCCTATAGACTATCA
 910          920          930          940          950          960
GTTCCCTTTGGGCGGATTTGTTGTTTAACTTGTAAATGAAAAAATTTCTCTTAAACCACAGC
 970          980          990          1000          1010          1020
ACTATTGAGTGAAACATTTGAACCTCATATCTGTAAAGAAATAAAGAGAGATATATTAGTTT
 1030          1040          1050          1060          1070          1080
TTTAAATGGTATTTTAAATTTTATATATGTCAGGAAAGAAATAGAAGTGATGAATATTGTT
 1090          1100          1110          1120          1130          1140
AATTATACCACCGTGTGTTAGAAAAGTAAGAAGCAGTCAATTTTTCACATCAAAGACAGCA
 1150          1160          1170          1180          1190          1200
TCTAAGAAGTTTTGTTCTGTCTGGAATTAATTTTAGTAGTGTTCAGTAATGTTGACTGT
 1210          1220          1230          1240          1250          1260
ATTTTCCAACCTTGTTCAAATTAATTAACAGTGAATCTTTGTTCAGCAGTTCCTTTTAAATG
 1270          1280          1290          1300          1310          1320
CAAATCAATAAATTCCTCAAAAATTTAAAAA
 1330          1340          1350

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FIG. 4. Nucleotide sequence of human HPRT cDNA and inferred amino acid sequence. The cDNA sequence was determined by dideoxy sequence analysis of M13 mp8 recombinant clones. Fifty-three independent cloned fragments having a random distribution within the cDNA were analyzed and their sequences were assembled according to their overlapping regions. All of the DNA sequence was confirmed on independent clones and, for about 85% of the cDNA fragment, both strands of the sequence were analyzed. Verified amino acid sequences of isolated peptides from purified human HPRT are shown in parentheses. We have assumed that translation begins at the first available AUG codon (at base 86) and terminates at the first chain-termination codon in the single open reading frame (i.e., at the UAA codon marked by asterisks at base 740). A potential poly-adenylation site, A-A-T-A-A, occurs at base 1,334.

amounts of plasmid DNA. Digestion of this purified plasmid (p4aA8) DNA with *Bam*HI yielded an insert of approximately 1.6 kb (Fig. 1). The cloning vector has two *Bam*HI sites that flank the cloned cDNA segment. If the cloned cDNA segment does not contain a *Bam*HI restriction site, digestion with *Bam*HI will excise the complete cDNA sequence flanked at the 5' end by about 100 bp derived from simian virus 40 DNA and the G-C joint and at the 3' end by 100–200 bp containing the A-T joint and simian virus 40 DNA.

Transfection of mouse HPRT-deficient LA9 cells with the HPRT cDNA p4aA8 resulted in the appearance of HAT-resistant colonies at a frequency of approximately 1 per 10^3 cells when 1 μ g of plasmid and LA9 carrier DNA was used (Fig. 2). Transfection of these cells with a control plasmid picked at random from the library (p4aB8) failed to produce any HAT-resistant colonies. Lysates from two HAT-resistant colonies were tested for HPRT activity by an isoelectric focusing gel assay (Fig. 3). Both colonies contained enzyme activities that cofocused with the major band of authentic human HeLa marker enzyme, as opposed to the major and minor bands of enzyme activity derived from mouse 3T6 cells.

Fragments of the isolated 1.6-kb insert were cloned into M13 mp8 and their sequences were determined. The nucleotide sequence of the insert spanning the G-C to the A-T tailing regions is presented in Fig. 4 together with the inferred amino acid sequence of the encoded polypeptide and the tryptic and cyanogen bromide peptides whose sequences were determined independently. In the five peptides (a total of 56 amino acids), there are no disagreements between the amino acid sequences and the nucleotide sequences determined.

DISCUSSION

Three important prerequisites were satisfied for the successful cloning of full-length human HPRT cDNA. First, a probe containing HPRT coding sequence was isolated by a combination of transfection of human DNA into enzyme-deficient mouse cells and identification of the human sequences in such transfected cells by their hybridization to human repetitive (*Alu*) sequences or to total human DNA. Second, a cDNA library, preferably one likely to contain a full-length cDNA segment was prepared by using the procedure outlined previously (30), which has been further developed to allow expression of full-length cDNA sequences in mammalian cells (11). The simian virus 40–pBR322-based cloning vector contains a simian virus 40 early region promoter and intron 5' to the cDNA segment and a polyadenylation signal 3' to the cDNA segment. These permit the expression of inserted cDNA sequences. In this instance, they permitted a direct test of the function of putative HPRT cDNA sequences in transfection assays and enabled us to identify and confirm full-length HPRT sequences. Third, it happened that pBR1.5 hybridizes to the 5' end of the cDNA clone (this was shown by hybridization to M13 subclones) and hence its use as a hybridization probe selectively detected full-length or nearly full-length cDNA clones. This correlates with the observation that mouse sequences exist in this subcloned plasmid and at one end of the original genomic clone (p6B2aE2) and, hence, implies that the site of 5' linkage of the exogenous human HPRT gene to mouse sequences is cloned in these plasmids.

When individual size classes of the cDNA library were hybridized with a fragment between positions 142 and 1,221 in the nearly full-length HPRT cDNA (Fig. 4), additional positive clones were found in the various sublibraries. The frequency of HPRT-positive clones in the library was about 2×10^{-5} or equivalent to approximately 2–5 copies of HPRT mRNA per

cell. We estimate the frequency of full-length cDNA clones relative to the total number of positive clones in the complete library to be about 5%.

The sequence of the cDNA insert between the G-C and A-T linkers has been fully determined. The cDNA sequence is just under 1,350 nucleotides long, in good agreement with the size (approximately 1,600 bp) predicted from the RNA blots hybridized to pBR1.5 (4). It is not known whether some 5' sequences are missing in the cDNA clone, but the 85-bp 5'-nontranslated region is in the usual size range for eukaryotic mRNA leader sequences. The coding sequence occupies 654 bp, slightly less than half of the full length of the cDNA, leaving a 3'-noncoding tail of approximately 600 nucleotides. The organization is typical of eukaryotic mRNA sequences, in which the major noncoding region is usually 3' to the coding sequences (31).

The single open frame encodes a polypeptide 218 amino acids long, indicating a molecular weight of approximately 24,600. Post-translational removal of the NH₂-terminal methionine would result in a protein 217 amino acids long, with a molecular weight of 24,450. The purified human HPRT enzyme has been estimated to have a molecular weight of 24,000–26,000 (32, 33). In addition, the partial amino acid composition of the purified enzyme is in complete agreement with our inferred amino acid composition. Most impressively, the inferred amino acid sequence is in complete agreement with the full amino acid sequence of the human erythrocyte HPRT enzyme recently determined by Wilson *et al.* (34). This sequence indicates that the NH₂-terminal methionine is indeed cleaved in the fully processed mature enzyme.

The protein has no obvious large hydrophobic or hydrophilic domains but instead has small alternating regions of hydrophobic and hydrophilic regions, suggesting a compact and globular structure. We predict that human HPRT is not a membrane constituent.

Caskey and co-workers (35, 36) have used a different approach in their isolation of a cDNA clone of the mouse HPRT gene, taking advantage of cells overproducing HPRT (35, 36). They have determined the sequence of mouse HPRT cDNA, and comparison of the inferred mouse and human amino acid sequences shows only eight differences, and the lengths of the polypeptides are identical.

We expect that this cDNA clone will be useful for studying the organization of normal and mutant human HPRT genes, for examining the mechanisms of X-chromosome inactivation, for studying the fate and expression of HPRT cDNA in transfected HPRT-deficient mouse and human cells, and for examining the distribution and expression of HPRT sequences in mice after introduction of the gene into the male pronucleus of fertilized mouse eggs.

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