Identification of mutations leading to the Lesch–Nyhan syndrome by automated direct DNA sequencing of in vitro amplified cDNA

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ABSTRACT The Lesch–Nyhan (LN) syndrome is a severe X chromosome-linked disease that results from a deficiency of the purine salvage enzyme hypoxanthine phosphoribosyltransferase (HPRT). The mutations leading to the disease are heterogeneous and frequently arise as de novo events. We have identified nucleotide alterations in 15 independently arising HPRT-deficiency cases by direct DNA sequencing of in vitro amplified HPRT cDNA. We also demonstrate that the direct DNA sequence analysis can be automated, further simplifying the detection of new mutations at this locus. The mutations include DNA base substitutions, small DNA deletions, a single DNA base insertion, and errors in RNA splicing. The application of these procedures allows DNA diagnosis and carrier identification by the direct detection of the mutant alleles within individual families affected by LN.

Inactivation of the human hypoxanthine phosphoribosyltransferase (HPRT) gene (1) leads to the Lesch–Nyhan syndrome (LN), an X chromosome-linked, genetically lethal, neurological disease (2). Partial HPRT activity results in a severe form of gouty arthritis (3). Both conditions are characterized by elevated levels of uric acid in serum, and LN is also associated with choreoathetosis, spasticity, mental retardation, and a bizarre form of self-mutilation.

Approximately 15% of LN patients have major gene rearrangements that can be detected by Southern analysis (4). The majority of cases have normal size and amounts of HPRT mRNA when analyzed by Northern analysis (4, 5), but ~35% of these reveal molecular lesions when assayed by ribonuclease digestion of RNA-RNA heteroduplexes (6). Only a small percentage produce detectable HPRT protein (5). Overall, these data indicate a heterogeneity of mutations in the disease, with a preponderance of single DNA base changes. In combination with a paucity of informative polymorphic DNA markers at the HPRT locus, the mutational heterogeneity has considerably hampered the application of DNA-based techniques to LN family analysis.

Recent developments in DNA technology have greatly increased the simplicity and speed with which single base changes in human DNA can be identified. In particular, the development of the polymerase chain reaction (PCR) (7–9) has provided a procedure to enrich for a specific cDNA so that DNA sequence analyses may be performed without the need for time-consuming library construction and screening (10). Fluorescently labeled DNA oligonucleotides have been combined with dideoxynucleotide termination DNA sequencing and real-time laser gel scanners to automate DNA sequencing of cloned materials (11). When a DNA sequence change is identified, the mutant allele can be detected by differential hybridization to allele-specific oligonucleotides (ASOs) (12). We have adapted and extended these procedures to detect LN mutations by automated direct DNA sequencing of PCR-amplified HPRT cDNA. These strategies now permit simplified diagnoses of the mutations in the majority of LN affected families.

MATERIALS AND METHODS

Cell Lines. B lymphoblasts were transformed by Epstein–Barr virus and maintained in RPMI medium supplemented with 20% fetal calf serum (GIBCO). Cell lines RJK 888 and RJK 906 were obtained from the American Tissue Culture Collection (GM 7092 and GM 1899, respectively). The cell lines RJK 949 and RJK 951 were derived from patients with partial HPRT deficiency (patients J.M. and R.T.; see ref. 5), while all other cell lines were HPRT total deficiency cases.

Oligonucleotides. Oligonucleotide primers for PCR amplification and manual DNA sequencing (Fig. 1) were manufactured on an Applied Biosystems Inc. (ABI) 380B DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis, electroelution, and passage through a NENSORB column (DuPont). Oligonucleotide primers for automated fluorescent DNA sequencing were dye labeled at the 5′ terminus by the Aminolink method (13). Oligonucleotide 058 (Fig. 1) was synthesized on an ABI 381A DNA synthesizer at 1.0 μmol scale (standard cycle, version 1.23 software) using ABI cyanonyethyl phosphoramidites and Aminolink II (14). The four dye–triethyl amon milk acetate esters (TAMRA–NHS, FAM–NHS, ROX–NHS, JOE–NHS; ABI catalogue nos. 400 810, 400 811, 400 812, 400 813, respectively) were each dissolved to 8% (wt/vol) in anhydrous dimethyl sulfoxide (15). Following NH3 deprotection (55°C, 12 hr) four aliquots (4.0 OD560 units each) of oligonucleotide 058 were concentrated to dryness and then dissolved in 25 μl of 0.5 M sodium carbonate/bicarbonate, pH 9.0, mixed with 6 μl of one of the dyes, and held at ambient temperature in the dark for 16 hr. Crude reaction mixtures were desalted with Sephadex-G 25M [Pharmacia PD-10; 0.1 M N-hydroxylysuxcinimide (NHS) (TEAA) (pH 7.0),] and dye primers were isolated on an ABI 152A separations system fitted with an Aquapore RP 300 cartridge (220 × 4.6 mm, 7 μm; catalogue no. 400 514) using a linear gradient from 8% acetonitrile in 0.1 M TEAA (pH 7.0) to 20% acetonitrile over 24 min and a flow rate of 1.0 ml/min. In each case, 0.4–0.8 OD560 units (16) were recovered.

cDNA Synthesis. Five micrograms of total cellular RNA (16) was mixed with 10 μg of random hexamer primers (pd[N6]; Pharmacia) on ice, in a total vol of 16.5 μl containing 14.0 units of ribonuclease inhibitor (RNasin; Pharmacia) and 4 μl of 5× Pol buffer (250 mM Tris-HCl, pH 8.3 (37°C)/40 mM

detected LN mutations (12). We have adapted and extended these procedures to detect LN mutations by automated direct DNA sequencing of PCR-amplified HPRT cDNA. These strategies now permit simplified diagnoses of the mutations in the majority of LN affected families.

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; LN, Lesch–Nyhan syndrome; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide.

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**Fig. 1.** Oligonucleotide primers for PCR amplification and direct DNA sequence analysis of the human HPRT cDNA (5’ to 3’). Lowercase letters indicate noncomplementary bases that can facilitate DNA cloning. X indicates an alphabetic amine group introduced at the 5’ terminal of each oligonucleotide 58-mer to enable fluorescent dye conjugation. The complementary positions of each primer to the HPRT cDNA are shown below. Primers beneath the bold line were used for PCR and DNA sequencing; those above were used for DNA sequencing only.

MgCl2/150 mM KCl/50 mM diethetol]. The mix was heated to 95°C for 1 min, chilled on ice, and briefly spun in a microcentrifuge to collect the condensate. A further 14 units of RNasin, 2.0 μl of a 25 mM mix of all four deoxyribonucleotide triphosphates, and 2.0 μl of Moloney murine leukemia virus reverse transcriptase (Pharmacia; 12,000 units/ml) were added to bring the total vol to 20.0 μl. Reaction mixtures were incubated at 37°C for 1 hr. The RNA was hydrolyzed by addition of 30 μl of 0.7 M NaOH/45 mM EDTA and incubation at 65°C for 10 min, and the cDNA was collected by ethanol precipitation.

**In Vitro DNA Amplification.** PCR was carried out essentially according to Kogan et al. (9) using either 10% of the products of one reverse transcriptase reaction or 100–500 ng of genomic DNA prepared on an ABI 340A DNA extractor as template. Templates were mixed with 100 pmol of each PCR primer in a total vol of 100 μl containing 6.7 mM MgCl2, 16.6 mM (NH4)2 SO4, 5 mM 2-mercaptoethanol, 6.8 μM EDTA, 67 mM Tris-HCl (pH 8.8; 25°C), bovine serum albumin (80 μg/ml), 1 mM each deoxyribonucleotide triphosphate, and 10% dimethyl sulfoxide, heated to 95°C for 7 min and briefly centrifuged before addition of 5 units of Taq DNA polymerase (Perkin–Elmer/Cetus) and 30 cycles of DNA polymerization (65°C; 1–3 min), denaturation (92°C; 30 sec), and annealing (37°C–55°C; 30 sec). The final 65°C incubation was for 7 min.

**Manual Direct DNA Sequencing.** PCR products were partially purified by phenol and ether extraction and NENsorb affinity column chromatography. The fragments were resuspended in 30 μl of H2O and 1 μl (=50 ng of DNA) was taken to initiate a second PCR containing only one of the original primers, which was in opposite sense to the DNA sequencing primer to be used. Apart from the absence of one PCR primer, the single strand-producing reactions were identical to the two-primer PCR protocol. Reaction products were concentrated by NH4OAc/ethanol precipitation and dissolved in a final vol of 10 μl. DNA sequencing primers were radiolabeled at the 5’ terminus as described (17). Unincorporated nucleotide was removed by passage through a NENsorb column and the dried products were resuspended in 12 μl of H2O.

Dideoxynucleotide termination sequencing reactions were initiated by mixing 5.0 μl of DNA template, 3.0 μl of labeled primer, and 2.0 μl of 5× Pol buffer in a standard 1.5 ml microcentrifuge tube, heating to 95°C for 10 min, and spinning to collect condensation. The primer-template mixture (2.5 μl) was dispensed into each of four tubes to which 2.0-μl aliquots of an appropriate DNA sequencing/termination mix was added. Each mixture contained 80 μM dATP, 80 μM dCTP, 80 μM dGTP, 80 μM dTTP, the appropriate deoxyribonucleotide triphosphate (8.0 μM), and 1.0–1.5 units of Sequenase per μl (modified T7 DNA polymerase, United States Biochemical). Reaction mixtures were incubated at 50°C for 40 min, spun to collect condensation, mixed with 3.0 μl of sequencing “stop” buffer (95% formamide/20 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol), heated to 80°C for 3 min, and then analyzed by standard electrophoresis and autoradiography.

**Automated Direct DNA Sequencing.** Products from two single-strand-producing PCRs were resuspended in a vol of 24 μl. Dye-labeled primers (1 μM) were added to annealing reaction mixtures as follows: A, 3 μl of template/1 μl of JOE58/1 μl of 5× Pol; C, 3 μl of template/1 μl of FAM58/1 μl of 5× Pol; G, 9 μl of template/3 μl of TAMRA58/3 μl of 5× Pol; T, 9 μl of template/3 μl of ROX58/3 μl of 5× Pol. Mixes were heated to 85°C for 2 min and cooled to room temperature over 30 min. After a brief centrifugation, dideoxynucleotide termination/Sequenase mixes were added to each tube (A, 4 μl; C, 4 μl; G, 12 μl; T, 12 μl) and the reaction mixtures were incubated at 50°C for 5 min, heated to 68°C for 10 min, pooled, and precipitated by addition of 6 μl of 3 M NaOAc (pH 5.2) and 170 μl of ethanol. After an 80% ethanol wash, the samples were briefly dried under vacuum and redissolved in 12 μl of formamide. Each sample (3–5 μl) was electrophoresed on a constant 20 W on an 8% polyacrylamide gel using an ABI 370 automated DNA sequencer. Fluorescence analyses and base calling were performed with the manufacturers Version 1.3 software. Corrections for altered electrophoretic mobilities of the individual dye-labeled primers (11) were made by labeling one sample with all four colored primers and terminating in a single nucleotide. Data from this file were incorporated into the final analyses by the ABI software.

**ASO Probe Analysis.** Oligonucleotide probes complementary to either the normal HPRT cDNA sequence (5’-TAA TGA CAC AAA CAT G-3’) or to the mutant allele identified in cell line RJK 1727 (5’-TAA TGA CAC AAA CAT G-3’) were 32P labeled as described (17). DNA from family members was amplified by PCR, resolved on a 1% agarose gel, transferred to Zetaprobe membrane, and sequentially hybridized to the oligonucleotide probes. Filters were washed to a final stringency of 2.0× SSC (0.3 M NaCl/0.03 M sodium citrate) at 42°C.

**RESULTS**

In **Vitro HPRT cDNA Amplification.** The amplification of a 938-base-pair fragment (primers 581/365) containing the entire peptide coding region of the human HPRT cDNA (651 nucleotides) was achieved by using the equivalent of 0.5 μg of total cellular RNA as starting material. The identity of the full-length cDNA amplification product (Fig. 2) was verified by hybridization to HPRT cDNA probes, by the altered size of products generated from a case with a known cDNA deletion and by direct DNA sequence analysis (see below). Shorter regions of the HPRT cDNA could be amplified by other combinations of PCR primers shown in Fig. 1. All cDNA amplifications were run in parallel with controls where no reverse transcriptase had been used in the cDNA synthesis reactions to ensure that there was no contaminating HPRT cDNA template introduced from exogenous sources.

**Direct DNA Sequence Analysis of PCR Products.** The direct DNA sequence analysis of amplified HPRT cDNA was facilitated by a strategy of asymmetric priming, whereby a double-stranded PCR product was converted into a partially single-stranded mixture by the priming of multiple rounds of DNA synthesis by a single oligonucleotide (18). The DNA
sequencing primers were each complementary to DNA sequences between the PCR primers. Manual reactions were each generally able to provide 200–220 bases of DNA sequence, and therefore the oligonucleotide primers 194, 195, and 196 could provide complete coverage of the peptide coding region. Other primers shown in Fig. 1 were occasionally required to clarify particular regions. Each of the mutations described here was identified by sequencing an entire 651-base peptide coding segment, with the exceptions of RJK 855, RJK 888, and RJK 951, where the analyses were restricted to regions surrounding previously identified RNase A cleavage sites (6). Examples of manual direct DNA sequence analyses are shown in Fig. 3.

**Automated Direct DNA Sequencing.** The automated DNA sequence analysis of the amplified HPRT cDNA was performed with a modest scaling up of the manual sequencing reactions. Twenty-one-base dye-labeled oligonucleotide primers (TAMRA058, FAM058, ROX058, JOE058) were constructed for the DNA sequence analysis of a 387-base fragment that was amplified from the HPRT cDNA by primers 244/243. An example of the analysis shows a 245-base segment in a sample derived from RJK 1784, including a T to C transition located 160 bases from the 3' terminus of the sequencing primer (Fig. 4). A contiguous 205 bases were correctly identified in this sequence when one position that was posted as uncertain (?) by the computer software was manually edited, without reference to the normal HPRT cDNA sequence.

**HPRT cDNA Sequences.** The position and the individual sequence changes that were identified in 15 HPRT cDNAs are shown in Fig. 5. The mutations include 8 DNA base substitutions, 3 DNA deletions, 1 single base insertion, 1 compound mutation with 5 previous bases being replaced by 6 others, and 2 cDNA deletions that involve exon boundaries.

**ASO Probe Analysis.** The cDNA sequence information from RJK 1727 was used to design ASO probes for the analysis of the G to A transition in other family members.

Primer complementary to HPRT exons seven (no. 352) and nine (no. 246) were used to amplify an 1795-base HPRT gene fragment from DNA extracted from lymphoblastoid cells. Next, the PCR products (Fig. 6A) were hybridized to oligonucleotide probes complementary to either the normal (Fig. 6B) or mutant (Fig. 6C) sequences. The oligonucleotide hybridization pattern revealed that only the affected male carried the mutant allele.

**DISCUSSION**

In addition to the 15 cases reported here, there are, to our knowledge, 5 other human germ-line HPRT mutations for which the cDNA sequence alterations have been directly identified or inferred. These include three cases of HPRT deficiency that have been characterized by amino acid sequencing (1) and one identified by denaturing gradient gel electrophoresis and Maxam–Gilbert sequencing of PCR amplified DNA (19). Davidson and colleagues (20–22) have used conventional cloning and sequencing techniques to identify single DNA base changes in three HPRT deficiency cases including two of the patients we report here (RJK 892, RJK 896). Our identification of these mutations confirms their studies.

The missense HPRT mutations are of particular interest as they may reveal sites within the peptide that have an important functional role. Two of the single amino acid substitutions [RJK 896 (Val to Asp), RJK 1784 (Leu to Ser)] are within a 20-amino acid region that is a putative catalytic domain highly conserved among different phosphoribosyltransferases (23). In addition, the Cys at position 205 in human HPRT is conserved in the murine and hamster HPRT proteins (23), but a mutation is found at that position in RJK 1727 (Cys to Tyr) and in two 6-thioguanine-resistant Chinese hamster cell lines identified by Vrieling et al. (Cys to Arg, Cys to Phe) (24). The clues to the functional significance of the other amino acid substitutions described here are not so obvious.

A 3-base deletion in RJK 855 is characterized by a short repeat sequence immediately adjacent to the deletion breakpoint, similar to those implicated in the genesis of deletions at the hamster adenosine phosphoribosyltransferase locus (25). A possible mechanism for the GTT deletion is therefore strand slippage, where the GTT residues immediately preceding the breakpoint are mispaired and recognized as the bases that are ultimately deleted during replication. Strand slippage may also have given rise to the insertion of an extra G residue within a sequence of six consecutive Gs in RJK 866. The dinucleotide deletion in RJK 1332 does not appear to be associated with either a nearby repeat or any likely stable secondary structures. In contrast, the 13-base deletion

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**Fig. 2.** In vitro amplification of HPRT cDNA. A 938-base-pair DNA fragment including the 651-base peptide coding region of the human HPRT mRNA was amplified from cDNA generated by random hexamer-primed reverse transcription of total cellular RNA; 1/20th of the reaction mixture is shown analyzed on an ethidium bromide-stained 1% agarose gel (lane 2). The molecular weight markers (lane 1) are HaIl-digested X'174 DNA.

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**Fig. 3.** Manual direct DNA sequence analysis of in vitro amplified HPRT cDNA. (a) Dinucleotide deletion in RJK 1332 (*). (b) A to T substitution in RJK 896 (reverse strand). (c) A 6-base insertion in RJK 1210 (reverse strand).
Deletions in cDNA that involve exon boundaries can arise from errors in RNA splicing. The deleted bases in RJK 888 correspond to the entire eighth HPRT exon, while the RJK 906 mutation encompasses the 5′-most 17 bases of exon nine. These mutations may be the result of single DNA base changes that disrupt 3′ splice acceptor sites (27). In RJK 906, the splice donor recognizes a downstream sequence (TTAG)

![Diagram](image.png)

**Fig. 5.** Altered DNA sequences identified in HPRT cDNAs from 15 HPRT-deficiency patients. (Upper) (A) Single DNA base substitutions; codons are numbered from the methionine initiation codon. (B) DNA deletion cases with the deleted bases shown in parentheses. The breakpoints in RJK 855 are ambiguous and may be 1 base 5′ to those shown. (C) Insertion of a single G residue. The insertion is ambiguous and could have been any of the seven G residues shown. (D) RJK 1210; normal bases that are deleted are shown above in parentheses, a 6-base insertion is shown below. (E) Potential RNA splicing errors. Introns sequences are shown in lowercase letters and 17 bases deleted in RJK 906 are shown in parentheses. (Lower) Distribution of the mutations in the HPRT cDNA. bp, Base pairs.
The amplified fragments within are resolved on one LN family. The amplified fragments were resolved by agarose gel electrophoresis (A) and then hybridized to oligonucleotide probes corresponding to normal (B) or mutant (C) alleles, revealing that the mother of this case was not a LN carrier. Lane M, molecular weight standards.

as a cryptic splice acceptor. In RJK 888, a complete exon is skipped and the potential cryptic acceptor site within exon eight (GTAG) is not utilized, and therefore this mutation may be within the adjacent 5' splice donor site.

The mutant cDNA information can be used to analyze individual families affected by LN. ASO probing of lymphoblastoid DNA showed the mother of patient RJK 1727 not to carry the mutant allele that had been identified by direct sequence analysis. Together with the observation that the allele is absent in three half-sisters of the affected, this suggests that the mutation has arisen de novo in the maternal germ line and that future offspring have only the small risk associated with germ-line mosaicism of inheriting the LN mutation. Similar family analyses may be carried out on most of the remaining LN cases. The exceptions to this are the RNA splicing variants, RJK 888 and RJK 906, for which the precise gene alterations cannot be inferred from the altered cDNA. To precisely define the mutations in these cases it will be necessary to sequence the regions immediately flanking the affected exons.

The automated direct sequencing of PCR amplified cDNA described here represents a considerable simplification of methods for analysis of LN mutations. The PCR amplification overcomes the limitations imposed by the low abundance of HPRT mRNA, which is further reduced in some of the LN cases described here (R.A.G. and P.-N.N., unpublished observations). RNA heteroduplex mapping previously identified and localized individual LN mutations, but DNA sequence analysis is required to define the exact nucleotide alterations. Direct sequencing has the dual advantages of obviating the cloning of PCR products into a DNA sequencing vector and eliminating the requirement to sequence multiple isolates to avoid DNA polymerase errors. When all steps are carried out sequentially, an analysis can be carried out in 2 days. Much of the labor is devoted to the DNA sequencing reactions, the manipulation of polyacrylamide gels for autoradiography, and the entry of the sequence data into the computer. Therefore, the adaptation of automated DNA sequencing techniques substantially reduces the total number of manipulations required. An additional and somewhat unexpected advantage of the automated sequencing was the ease with which data from a position >200 bases from the DNA sequencing primer could be obtained. The manual sequencing usually required two separate gels to resolve all of the available sequence, even with the use of a buffer gradient system. The automated sequencing required the equivalent of 2-3 times more template to be loaded per lane than the radioactive gels; however, this disadvantage was offset by the ease with which the template could be produced. Our overall conclusions therefore are that the automated fluorescent DNA sequencing not only is faster and easier to perform but also generates more DNA sequence data from the PCR amplified HPRT cDNA.

Construction of additional dyes-labeled oligonucleotide primers should permit multiple automated sequence analysis of the entire HPRT peptide coding region. However, with additional knowledge of the normal sequence surrounding each of the individual HPRT exons, it may be possible to focus the analysis directly on the gene. Diagnosis of LN will then be independent of the expression of the mature HPRT mRNA.

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