

Multiplex DNA Deletion Detection and Exon Sequencing of the Hypoxanthine Phosphoribosyltransferase Gene in Lesch-Nyhan Families

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The Lesch-Nyhan (LN) syndrome is a genetically lethal human neurological disease that results from mutations that inactivate the hypoxanthine phosphoribosyltransferase (HPRT) gene. The elucidation of the complete DNA sequence of the human HPRT gene locus has enabled the construction of multiple oligonucleotide primer sets for the simultaneous *in vitro* amplification of all nine HPRT exons. The multiplex polymerase chain reaction provides a facile assay for the detection of HPRT exon deletions and the reaction products can be analyzed by direct automated fluorescent DNA sequencing to identify subtle alterations in the gene. Alterations have been identified in the HPRT genes from 15 independent LN cases, and 10 LN family studies were performed. The sequencing method uses solid supports and is sufficiently simple and sensitive to be a favored approach for LN diagnosis. LN heterozygotes can be diagnosed without reference to the affected male. In addition, these procedures will be useful for somatic mutagenesis studies.

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INTRODUCTION

The molecular analysis of mutations at the human hypoxanthine phosphoribosyltransferase (HPRT) locus that result in the Lesch-Nyhan (LN) syndrome (Stout and Caskey, 1989) has been greatly facilitated by the recent development of polymerase chain reaction (PCR)-based DNA sequencing procedures (Gibbs *et al.*, 1989a). We previously reported the use of direct automated fluorescent DNA sequencing to identify cDNA alterations in 15 independently arising HPRT deficiency patients. The cDNA sequencing studies revealed a wide spectrum of human germ line mutations, including DNA base substitutions, deletions, and RNA splice errors. The prospects for rapid detection of the

mutant alleles in relatives of the affected individuals were greatly improved, as the alterations identified in the expressed sequences from the affected male could be used to design allele-specific oligonucleotides (ASOs) to track the disease-causing mutations in other family members (Gibbs *et al.*, 1989a).

The cDNA sequencing approach suffers from two limitations. First, the precise DNA sequence changes that lead to RNA splice errors cannot be determined and must be inferred from the structure of the mutant RNA. This complicates both the interpretation of the details of the mutational event and subsequent attempts to diagnose relatives who are at risk. Second, the pattern of expression of normal and mutant HPRT alleles in female LN carriers is not always random, as would usually be expected for an X-chromosome-linked gene. Instead, blood cell lines predominantly express the normal HPRT mRNA, while the mutant alleles are characteristically underexpressed (Stout and Caskey, 1989).

A simple solution to both of these problems is to examine the HPRT gene sequences directly. However, it is not practical to perform molecular cloning analyses on a routine basis and the limited availability of DNA sequence information from regions flanking each of the nine exons has previously prevented the construction of appropriate PCR primers. Recently, the sequence of the entire human HPRT gene locus was elucidated (Edwards *et al.*, 1990) and we report the application of this information to simplify further the analysis of the genetic alterations that have arisen at the HPRT locus in LN families.

MATERIALS AND METHODS

Cell lines, oligonucleotide construction, and ASO probing procedures have been previously described (Gibbs *et al.*, 1989a). Underivatized oligonucleotide

Exon	Length	Sense Primer	Antisense Primer	Sequence Primer
1.	626	(887, 130i) TGG GAC GTC TGG TCC AAG GAT TCA	(888) CCG AAC CCG GGA AAC TGG CCG CCC	(1380) N.TGG GCC TGA ACC GGC CA
2.	572	(796, 1299) TGG GAT TAC ACG TGT GAA CCA ACC	(797) GAC TCT GGC TAG AGT TCC TTC TTC	(1381) N.CAG AAC AGC TGC TGA TGT
3.	1059	(983, 1302) CCT TAT GAA ACA TGA GGG CAA AGG	(969) TGT GAC ACA GGC AGA CTG TGG ATC	(1382) N.ACC TAC TGT TGC CAC TA
4.	334	(1147,1305) TAG CTA GCT AAC TTC TCA AAT CTT CTA G	(1011) ATT AAC CTA GAC TGC TTC CAA GGG	(905) N.TGA CTC GGT ATG AAG TGC
5.	707	(885, 1300) CAG GCT TCC AAA TCC CAG CAG ATG	(1174) GGG AAC CAC ATT TTG AGA ACC ACT	(1383) N.CTG GCT TAC CTT TAG GA
6.	441	(1012, 1303) GAC AGT ATT GCA GTT ATA CAT GGG G	(1013) CCA AAA TCC TCT GCC ATG CTA TTC	(1384) N.AGC AAT CAC TTA ATC CC
7/8.	1533	(483, 1298) GAT CGC TAG AGC CCA AGA AGT CAA G	(854) TAT GAG GTG CTG GAA GGA GAA AAC	(1385) N.CTT TTA GGT TAA AGA TGG (1386) N.GAG TGA GAA AAA GAA GC
9.	1278	(1015, 1304) GAG GCA GAA GTC CCA TGG ATG TGT	(365) CCG CCC AAA GGG AAC TGA TAG TC	(1379) N.GAT GGC CAC AGA ACT AGA

FIG. 1. Oligonucleotide primers for the multiplex amplification and direct DNA sequencing of all HPRT exons. The predicted length of each exon specific fragment is given. Exons 7 and 8 were amplified in the same 1533-bp fragment. All oligonucleotide sequences are shown 5' to 3'. Sense primers: The first number in parentheses is the code for each sense primer used for the multiplex PCR amplification. Biotinylated versions of each of the sense PCR primers were prepared for use in the solid support DNA sequencing scheme outlined in Fig. 4 (second number in parentheses). Antisense primers: The antisense primers were each used for the multiplex HPRT DNA amplification. Sequencing primers: Two types of sequencing primer were prepared. Each was in the antisense configuration and was complementary to bases between the sense and antisense PCR oligonucleotides. Separate sequencing primers were prepared for exons 7 and 8. One set of nine oligonucleotides was synthesized to enable the introduction of the M13 "universal" priming sequence onto each exon-specific DNA fragment for fluorescent DNA sequencing according to the scheme in Fig. 4. Each of these contained the sequence 5'-TGT AAA ACG ACG GCC AGT-3', which is designated N in the figure. The second type of oligonucleotide was for manual DNA sequencing and each was perfectly complementary to the amplified HPRT gene fragment and did not contain the N sequence.

primers were not subjected to further purification following deprotection and lyophilization. Biotinylated primers were prepared using the Applied Biosystems Inc. (ABI) Aminolink chemistry. After deprotection and lyophilization, 0.2 μ mol of the amino-modified oligonucleotide was dissolved in 200 μ l of 150 mM sodium carbonate/bicarbonate, pH 9.0, mixed with 1 mg of D-biotin-*N*-hydroxysuccinimidester (Boehringer Mannheim) dissolved in 100 μ l of *N,N*-dimethylformamide, and held at ambient temperature for 14 h. Crude labeling reactions were simultaneously desalted and excess biotin was removed with oligonucleotide purification cartridges (ABI).

DNA Templates for PCR Amplification

Genomic DNA was prepared by a slight modification of the procedure of Miller *et al.* (1988). Blood cells or harvested lymphoblastoid or fibroblast cell cultures were rinsed in PBS, and cell pellets were resuspended in approximately 2 vol of 10 mM Tris $\cdot$ HCl, 400 mM NaCl, 2 mM Na₂EDTA, pH 7.5. SDS and proteinase K were added to 0.8% and 100 μ g/ml, respectively, and the mixture was incubated at 55°C for 2 h. The sample was then cooled to room temperature and a one-third volume of saturated NaCl (approximately 6 M) was added. Precipitated proteins and cellular debris were removed by centrifugation. The supernatant was transferred to a fresh tube and the nucleic acids were recovered by addition of 2.0 vol of ethanol at room temperature and either spooling or centrifugation.

PCR Amplification

Amplification of individual HPRT exons was carried out with a slight modification of the method of Kogan *et al.* (1987), usually with 250 ng of genomic DNA as template. Templates were mixed with 50 pmol of each PCR primer (Fig. 1) in a total volume of 50 μ l containing 6.7 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 5 mM 2-mercaptoethanol (BME), 6.8 μ M EDTA, 67 mM Tris $\cdot$ HCl, pH 8.8 at 25°C, 1.5 mM of each deoxyribonucleotide triphosphate, 2.5 units of *Taq* DNA polymerase (Ampli*Taq*; Perkin-Elmer/Cetus), and 10% dimethyl sulfoxide (DMSO). The reactions were overlaid with mineral oil and heated to 94°C for 4.5 min and 25 cycles of DNA polymerization (68°C, 1–3 min), denaturation (94°C, 30 s), and annealing (55–60°C, 30 s). The final 68°C incubation was for 7 min. All PCRs were carried out in a Perkin-Elmer/Cetus thermocycler.

Multiplex Amplification Reactions

The simultaneous amplification of all nine HPRT exons was achieved using eight pairs of oligonucleotides in a single PCR. DNA (250 ng) was mixed with 16 different primers in a total volume of 50 μ l containing 6.7 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 5 mM BME, 6.8 μ M EDTA, 67 mM Tris $\cdot$ HCl, pH 8.8 at 25°C, 1.5 mM of each deoxyribonucleotide triphosphate, 4.0 units of *Taq* DNA polymerase, and 10% DMSO. The final primer amounts were 10 pmol (887, 888, 1012, 1013), 16 pmol (1015, 365, 983, 969), 18 pmol (483, 854), 20

pmol (885, 1174, 796, 797), and 25 pmol (1147, 1011). The reactions were overlaid with mineral oil and heated to 94°C for 4.5 min followed by 25 cycles of DNA polymerization (68°C, 2 min), denaturation (94°C, 30 s), and annealing (61°C, 50 s). The final 68°C incubation was for 7 min. For convenience, multiplex kits were constructed by aliquoting all ingredients except the template DNA and the *Taq* polymerase into individual reaction tubes and storing them at -70°C (Chamberlain *et al.*, 1988).

Manual Direct DNA Sequencing

The preparation of DNA templates and the manual DNA sequencing reactions were performed by a modification of previously described procedures, using ³²P 5'-end-labeled primers and the modified T7 DNA polymerase (Sequenase; United States Biochemicals) (Gibbs *et al.* 1989a,b). To generate single DNA strands, approximately 1.0% of either a single exon amplification reaction or a multiplex PCR was used, without purification, to initiate 30 rounds of a second thermocycling reaction. These single-strand-producing reactions (SSPRs) were identical to the first PCRs, except that only one primer was added (30 pmol), the annealing and extension times were doubled, and they were routinely performed on a 100-μl scale. The oligonucleotide in the SSPR was in the sense opposite to that of the DNA sequencing primer to be used. The preferred buffer for the SSPRs was 1.5 mM MgCl₂, 250 μM dNTP, 50 mM KCl, and 10 mM Tris · HCl at pH 8.8 ("minimal buffer") because the SSPR products were collected by ammonium acetate/ethanol precipitation and the use of the DMSO-containing buffer occasionally resulted in a "carryover" of salt or protein which interfered with the DNA sequencing. The sequencing reactions using radioactively labeled primers and the analysis of the products were performed as previously described (Gibbs *et al.*, 1989b).

Direct Fluorescent DNA Sequencing

Aliquots of approximately 1% of a multiplex PCR were taken to initiate a second series of nine PCRs for the introduction of both a biotin residue and the "universal" M13 DNA priming sequence onto DNA fragments, each containing a single HPRT exon (Fig. 4). Each biotinylated primer was identical to the sense oligonucleotide used in the initial multiplex PCR, and the primers with the universal tails were complementary to bases between the multiplex primers. Conditions for each of the biotin-containing PCRs are described in Table 1. The biotinylated DNA strands were captured by mixing the PCR products with 40 μg of prewashed streptavidin-coated magnetic beads (Dyna) at room temperature for 30 min in a total volume of 80 μl with mild agitation. The supernatant was removed

TABLE 1
Conditions for PCR Amplification of HPRT Exon
Fragments with Biotinylated and
"Universal" Primers

Exon number	Primer pair	Annealing temp (°C)	Extension time (min)	Number of cycles
1	1301/1380	61	0.5	20
2	1299/1381	61	1.0	14
3	1302/1382	62	1.0	18
4	1305/905	61	1.0	15
5	1300/1383	61	1.0	18
6	1303/1384	59	1.5	18
7	1298/1385	59	1.5	18
8	1298/1386	59	1.5	18
9	1304/1379	64	1.5	18

and the unbound strand eluted by treatment with 150 μl of 0.15 M NaOH for 5 min at room temperature. The beads were washed twice in 10 mM Tris · HCl, pH 7.5, 1.0 mM EDTA and resuspended in 24 μl of H₂O. The fluorescent DNA sequencing reactions were performed using the material bound to the beads exactly as previously described (Gibbs *et al.*, 1989a), except that commercially available fluorescent M13 universal primers (ABI) were used and the 65°C heat denaturation step following the polymerization reaction was omitted. Instead, the unincorporated nucleotides were washed from the material bound to the beads with H₂O and then the products from all four base-specific sequencing reactions were combined. The sequencing reaction products were eluted from the beads in 3 μl of formamide at 37°C for 30 min and the sample was loaded directly on an 8% denaturing acrylamide gel on an ABI 370A fluorescent sequencer. The gel was electrophoresed at 27 W.

Sequence Data Analysis

DNA sequences that were obtained from ³²P-based methods were recorded into a personal computer with the aid of a sonic digitizer. Automated sequences were directly identified by the ABI Version 1.3 software. All sequence was downloaded into a Unix-based computer network and analyzed with the Baylor College of Medicine "Molecular Biology Information Resource" DNA sequence analysis package developed in the laboratory of Dr. Charley Lawrence (Department of Cell Biology).

RESULTS

Amplification of Individual HPRT Exons

Oligonucleotide primers were constructed for the amplification of individual HPRT exons, based on the DNA sequence of the entire HPRT locus (Edwards *et*

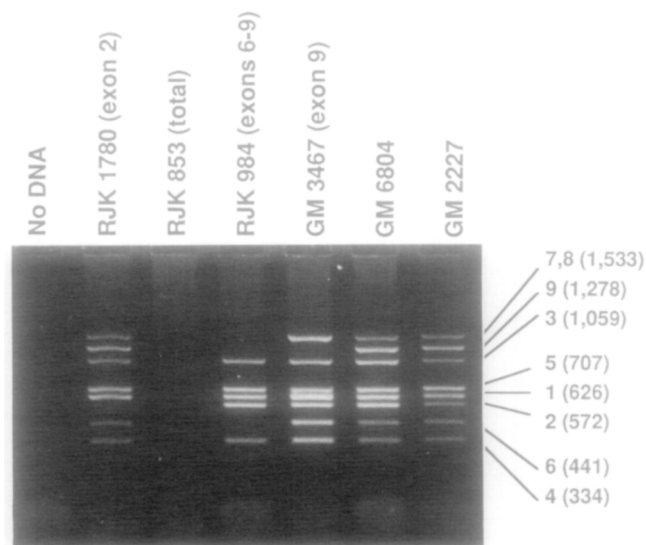


FIG. 2. Multiplex amplification of the HPRT locus. All nine exons of the human HPRT gene were amplified on eight separate DNA fragments using 16 oligonucleotide primers in a single PCR reaction. The lane containing no DNA was a control to ensure that there was no spurious amplification due to endogenous contaminating sequences. Four examples of complete or partial HPRT gene deletions are shown. The cell lines GM 6804 and GM 2227 represent the normal multiplex pattern of eight bands. These cell lines contain intragenic duplications and inversions, respectively, that do not perturb the normal amplification pattern. The exons contained within the individual fragments and the size of the amplification units are given on the right. The 1.4% agarose gel shown here was loaded with approximately 15% of the products of each amplification reaction.

al., 1990). The rationale for primer design was to generate DNA fragments that included both the exon (coding) sequences and the immediately flanking bases. Exons 7 and 8 were routinely amplified on a single fragment, as they are spaced a mere 170 bases apart. Throughout the course of the study approximately 35 different exon-flanking oligonucleotides were constructed and, with a single exception, every pair that was tested generated the expected size amplification product, once the appropriate amplification conditions were identified. One primer was constructed to be complementary to a region within an *Alu* repeat, and several attempts to identify satisfactory exon-specific PCR conditions with this oligonucleotide were unsuccessful.

Multiplex HPRT Amplification

The development of conditions for multiplex PCR of all nine HPRT exons required the sequential addition of individual primer pairs into a single reaction, followed by testing to determine whether conditions needed to be modified to enable approximately equal amplification of the different fragments. In a departure from previous approaches (Chamberlain *et al.*, 1988)

the concentration of each primer set in the reaction was adjusted to compensate for uneven signal strength. At each step, different annealing and extension temperatures and times were tested to identify reproducible amplification conditions. All primers were designed to conform as closely as possible to the general formula of 24 bases with a 50% GC content, although the requirement to generate products that could be easily resolved by size often restricted the final choice of oligonucleotide. Suitable multiplex conditions could not be identified for several primers that functioned adequately for the amplification of a single exon.

The multiplex assay was tested for the detection of HPRT gene deletions using several cell lines that had previously been characterized by Southern analysis using cDNA probes (Fig. 2). For most applications 250 ng of genomic DNA was used as the multiplex template, although we successfully screened a series of crude cell isolates that were obtained from "dead" freezer cell stocks (data not shown). A reaction with no added DNA template was carried out in parallel with the multiplex amplifications to control for the possible introduction of exogenous contaminating sequences that might give rise to spurious amplification products (Chamberlain *et al.*, 1988, 1989). The preparation of premixed multiplex kits was a great advantage for reducing problems of contamination, as it reduced the overall number of manipulations that were necessary.

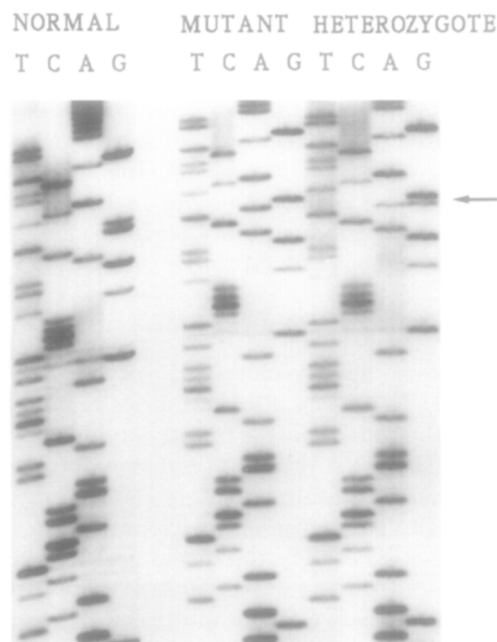


FIG. 3. Manual direct DNA sequence analysis and heterozygote detection. A C to T transition was identified in patient RJK 1930 (reverse strand shown here) by manual direct DNA sequencing of PCR-amplified exon 7 DNA. The mutant allele was also identified in his mother by the presence of two bases at a single position in the autoradiograph (arrow).

Manual DNA Sequence Analysis

The DNA sequence analysis of individual HPRT exons was carried out using a slight modification of our previously published procedures (Gibbs *et al.*, 1989a,b). Single-stranded template was generated by a thermocycling reaction that used one of the original PCR primers and a small aliquot of an HPRT exon amplification reaction. The sequencing was then performed using a ^{32}P -end-labeled primer that was complementary to a sequence within the amplified fragments and between the original PCR primers. All primers listed in Fig. 1 yielded clear readable sequence that enabled unambiguous determination of HPRT exon coding sequences and the neighboring RNA splice acceptor and donor sites. An example of a manual sequence analysis of a single DNA base substitution leading to LN is shown in Fig. 3.

Fluorescent DNA Sequencing Using Solid Supports

The automated DNA sequencing methods were adapted from previously described procedures using biotinylated primers and streptavidin linked to solid supports (Hultman *et al.*, 1989; Mitchell and Merrill, 1989). The PCR scheme that is illustrated in Fig. 4 enabled the simultaneous introduction of a biotin residue at the 5' terminus and a universal sequencing primer binding site at the 3' end of a DNA fragment.

The biotinylated strand was then captured onto commercially available magnetic beads and the bound single-stranded DNA was used as template in a fluorescent DNA sequencing reaction. The fluorescent sequencing reactions with the solid supports yielded clear and unambiguous sequence through each of the nine HPRT exons. The outputs from the automated sequencer did not require any manual editing (base calling) before comparison with the normal sequence to identify mutation sites (Fig. 5). The largest of the human HPRT exons is 184 bases long, and therefore the longest sequence read that was necessary to encompass a complete exon and its surrounding splice sites did not exceed 250 bases. The material sequenced from solid supports routinely provided more than 300 bases of sequence, and thus throughout the study the length of sequence read was not an issue.

HPRT Germ Line Mutations

DNA alterations in the HPRT genes of 15 different HPRT deficiency cases are described here (Fig. 6). These include 5 single DNA base substitutions and 6 intragenic DNA deletions. One of the DNA deletion cases (RJK 894) is the same patient designated HPRT_{EVANSVILLE} by Davidson and colleagues (1989), and our study therefore confirms their report. RNA splice donor and acceptor sites that were predicted to be altered in 4 LN patients from previous cDNA se-

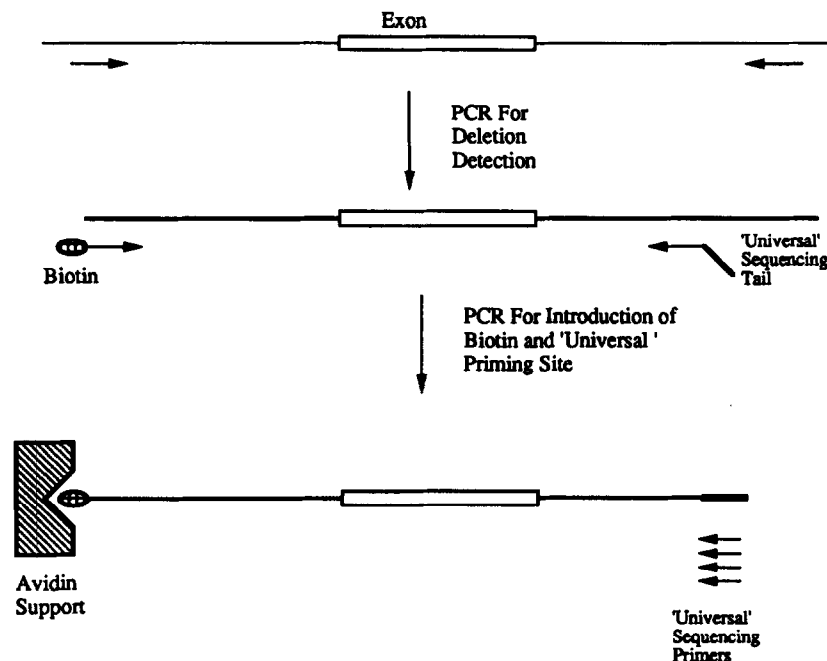


FIG. 4. DNA sequencing scheme utilizing solid supports. Each HPRT exon was first amplified using flanking oligonucleotide primers in a multiplex PCR. The reaction products were analyzed by gel electrophoresis to identify DNA deletions and then a small aliquot was taken to initiate a second PCR with one biotinylated primer and one universal sequence tailed primer. Single DNA strands containing the biotin residue were captured on magnetic beads coated with streptavidin, and then universal fluorescent sequencing primers were used to prime an dideoxy-chain termination sequencing reaction.

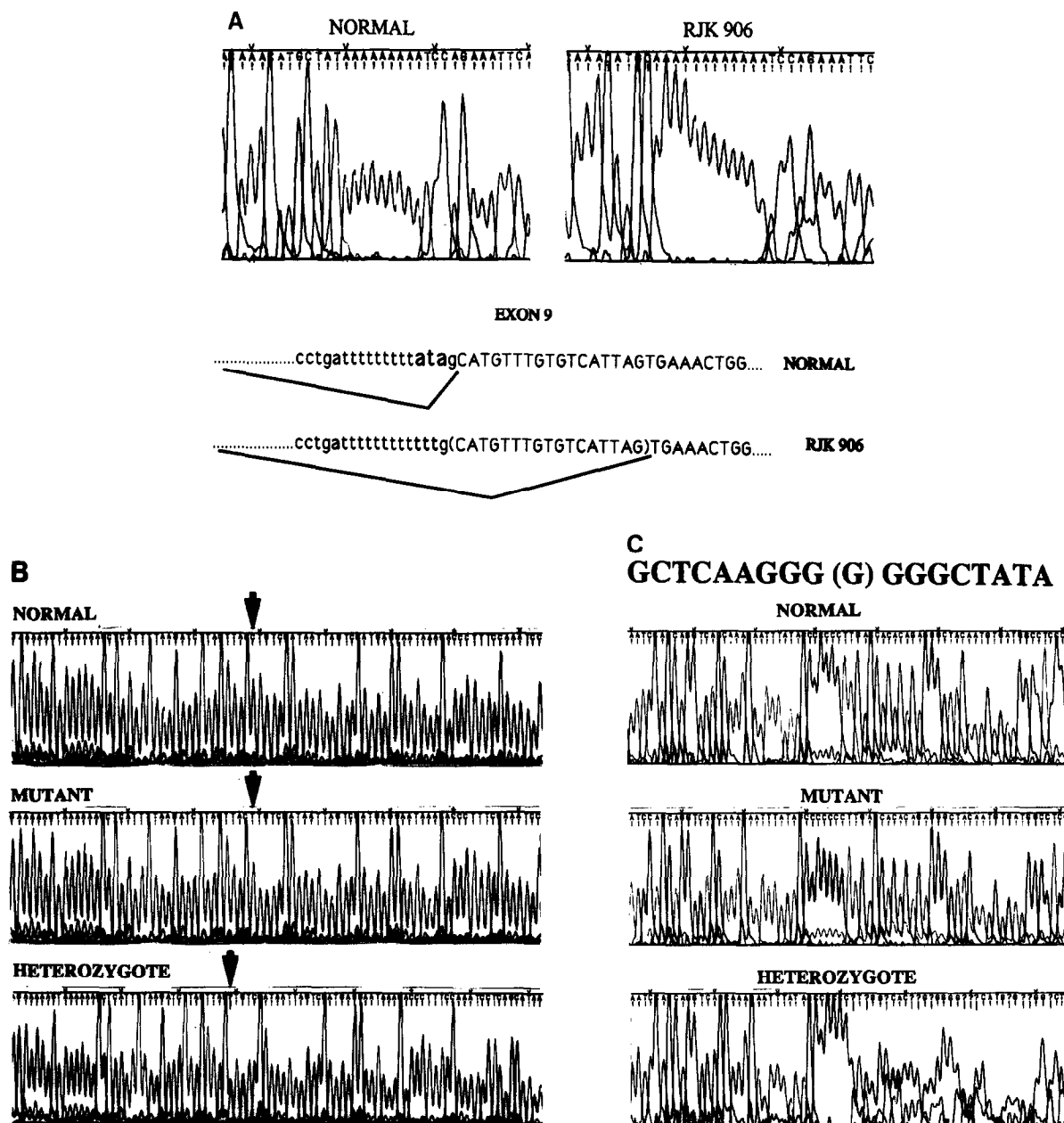


FIG. 5. Examples of direct automated fluorescent DNA sequencing of PCR-amplified HPRT gene fragments using the solid support strategy outlined in Fig. 4. All sequence profiles show the reverse (antisense) strand. The original display of these data is in four colors. (A) Altered boundary of exon 9 in patient RJK 906. The normal and mutated sequences (sense) are shown below. Intron bases are in lowercase and exon sequences in uppercase letters. Utilization of a cryptic splice acceptor site ATTAG was previously demonstrated in this patient by HPRT cDNA sequencing (7). (B) Single DNA base substitution in exon 2 of patient RJK 2163. The G to A transition in the sense strand is shown here as a C to T alteration. The mother of patient RJK 2163 clearly shows the presence of both the normal and the mutant bases at this position, indicating that she is a carrier of this allele. (C) Single DNA base insertion in exon 3 of patient RJK 866. This insertion of an extra G was previously identified by cDNA sequencing studies (7). A female carrier of the mutation was diagnosed by the solid support sequencing. The computer could not identify bases beyond the site of the insertion because of the frameshift of one allele.

quencing studies (Gibbs *et al.*, 1989a; R.A.G., P.N.N., unpublished observations) were also identified. In all cases the entire HPRT peptide coding base regions were characterized. In addition, every mutation was confirmed independently by sequencing both the cDNA and the gene alterations, by manual vs automated se-

quencing, or by the hybridization of ASOs and examination of relatives of the affected male.

Heterozygote Detection

Individuals who were at risk as LN carriers were tested using both ASOs and direct DNA sequencing.

	CELL LINE	MUTATION
A.	RJK 1930	CAG → TAG GLN (108) → STOP
	RJK 2079	CAT → GAT HIS (203) → ASP
	RJK 2163	AGG → AAG ARG (44) → LYS
	RJK 2185	GAC → TAC ASP (176) → TYR
	RJK 2188	GAC → AAC ASP (193) → ASN
B.	RJK 894	22bp deletion GGA AAA GCA (AAA TAC AAA... ...GCC TAA GAT GAG A)GT TCA AGT TGA
	RJK 1747	2bp deletion GCT ATT (GT)g tga gta
	RJK 1894	3bp deletion CCA CGA AGT (GTT) GGA TAT AAG
	RJK 1939	1bp deletion ATG GAC TAA T(T)A TGG ACA
	RJK 2019	1bp deletion AAT GTC (T)TG ATT G
	RJK 2108	40bp deletion CGA GA(T GTG ATG... 25 bases... .TAG CCC TC)T GTG TGC TCA AGG
C.	RJK 888	..exon 8 ..GAT TTG AAT gta agt aat ↓ ..exon 8 ..GAT TTG AAT gta agt aat
	RJK 906	gat ttt ttt tta tag CAT GTT TGT GTC...exon 9 ↓ gat ttt ttt ttt ttg CAT GTT TGT GTC...exon 9
	RJK 1934	exon 7 ... AGC CAG ACT gta agt gaa ↓ exon 7 ... AGC CAG ACT gta agt gaa
	RJK 1760	ttt ctt ttt cag ATT AGT... exon 2 ↓ ttt ctt ttt cag ATT AGT... exon 2

FIG. 6. Molecular alterations in 15 independently arising Lesch-Nyhan patients. (A) Single DNA base substitutions. One base substitution leads to a premature chain termination and four alterations gave rise to missense mutations. The codon number is shown in parentheses. (B) DNA deletions. The normal bases are listed and the deleted segments are contained in parentheses. The normal translational stop codon is shown in bold type, and bases that are reiterated at the deletion boundaries and therefore possibly involved in the deletion mechanism are underlined (7). RJK 2019 is the mother of an affected male (see text). (C) Mutations in RNA splice sites. The normal sequences are given above the mutant, and the bases involved in the alteration are underlined. Altered cDNAs were identified in each case (6, 7; R.A.G. and P.N.N., unpublished observations.).

The ASOs were constructed using the mutation information that was derived from the analysis of the affected male or from the direct sequencing of HPRT gene fragments amplified from a female carrier. The ASOs were then hybridized to PCR-amplified HPRT gene sequences as previously described (Gibbs *et al.*, 1989a) or used as allele-specific PCR primers (Gibbs *et al.*, 1989c). Approximately one-half of the families described here were analyzed by ASOs.

Both the manual and the automated DNA sequencing enabled the direct detection of HPRT deficiency heterozygotes. When heterozygotes were sequenced with ³²P-labeled primers, single DNA base substitutions appeared as a second base at one position on an autoradiograph (Fig. 3). The presence of each allele was generally clear, although artifacts at other positions in the autoradiograph might have confused the analysis if the exact site of the mutation was not known a priori from the sequence of the affected male. In contrast,

the sequence from the fluorescent primers using the solid supports rarely contained any background noise that generated computer miscalls, except at the positions predicted to be heterozygous from examination of other family members. The example illustrated in Fig. 5B shows the computer ? tag where a C and T residue are present in equal amounts of the predicted DNA base position in an LN carrier.

Direct DNA sequence analysis of heterozygotes that contain alleles with base deletions or insertions is also possible (Fig. 5C). Patient RJK 866 contained an extra G residue within a string of six G residues that are normally present within HPRT exon 3 (Gibbs *et al.*, 1989a). When a heterozygote with this allele (mother of RJK 866) was analyzed, a normal sequence was seen up until the repeated bases. Following the repeat, the computer called a series of ?s due to the frameshifting of one allele and the presence of mixed bases at many positions. Interestingly, we were not able to identify this carrier by ASO analysis, presumably because the reiterated bases complicated the base pairing. A similar analysis of mixed sequences was used to identify the mutation in RJK 2019, who was the mother of an affected male who was unavailable for study (Fig. 6B).

Family Studies

Ten independent families were examined to identify heterozygous female carriers and to diagnose LN (Fig. 7). Both allele-specific probing and direct DNA sequencing were used in these studies. In almost every case mutant and normal alleles could be easily discriminated by ASOs, but the direct sequencing assay became the preferred approach because it was simpler to perform and did not require the synthesis of additional oligonucleotides.

DISCUSSION

Multiplex DNA amplification provides a simple, rapid, and reliable alternative to Southern analysis for the detection of deletion mutations in an X-linked gene (Chamberlain *et al.*, 1988, 1989; Xu *et al.*, 1989; Ballabio *et al.*, 1989). The identification of multiplex primers and conditions for the simultaneous amplification of all human HPRT exons means that PCR can now be used in the routine diagnosis of LN patients. Successful amplification of the eight fragments described here requires all 16 oligonucleotide binding sites to be present, and the loss of one or more of the complementary sequences is revealed as the absence of the corresponding band by agarose gel electrophoresis. Nondeletion mutations, including insertions and rearrangements, may also result in an altered multiplex pattern if the normal sequence or orientation of priming site(s) is perturbed. Approximately 15% of LN patients reveal alterations

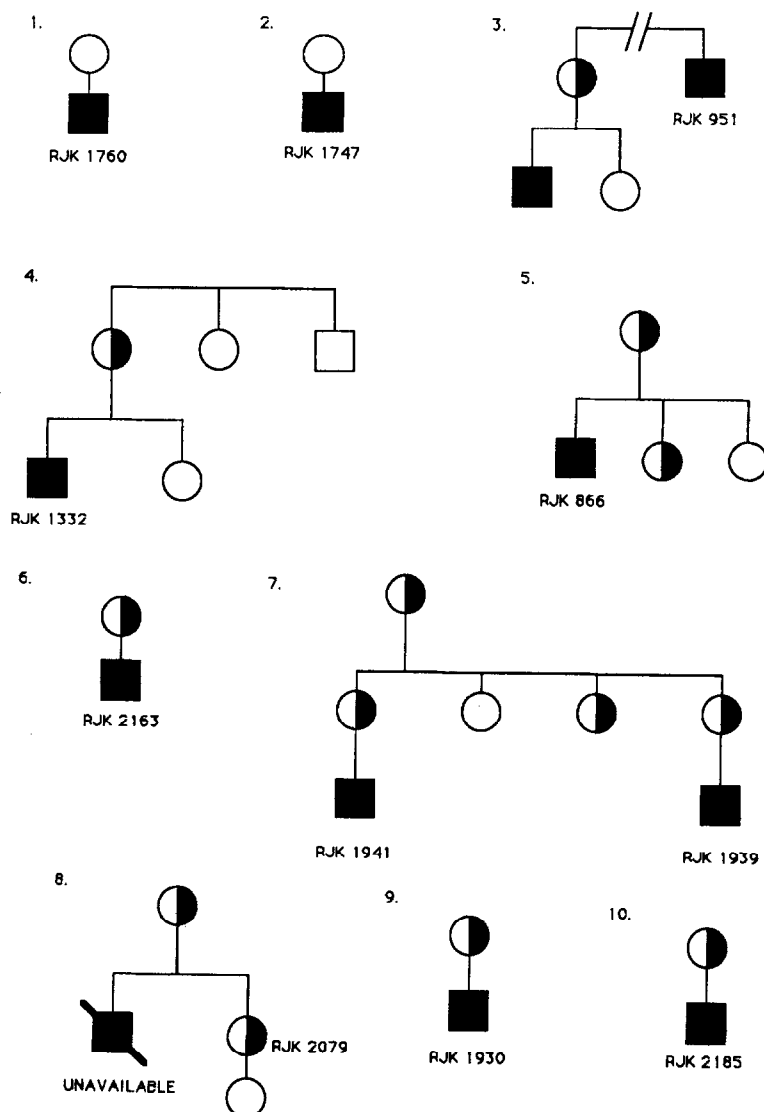


FIG. 7. Ten Lesch-Nyhan family studies. Relatives of LN males from this and our previous study (7) were tested for the presence of mutant alleles by ASO hybridization and/or direct DNA sequencing. Two *de novo* mutation events were identified (families 1 and 2). In family 8 the affected male was not available for study and the mutation was identified directly in the carrier females. In the remaining pedigrees only the individuals who were tested are shown.

by Southern analysis using radioactive HPRT cDNA probes, and it is anticipated that, with the exceptions of an intragenic duplication case and an inversion with one boundary in the locus, all these individuals could have been diagnosed by the multiplex PCR.

The majority of LN mutations involve only one or a few DNA bases, and characterization of these alterations requires more detailed analysis of the multiplex amplification products. There are now a large number of procedures for detection of single DNA base differences that could be adapted for the LN diagnosis using the multiplex products (Gibbs, 1990). We have favored a DNA sequence-based approach because of the relatively small size of the gene, because of the desire to

design allele-specific assays to test relatives of affected males, and because the precise DNA base changes are of interest. The manual sequence analysis of the amplified regions proved to be straightforward using our previously described protocols and enabled detection of individual DNA base changes and the direct visualization of heterozygotes (Fig. 3). Fluorescent DNA sequencing with custom fluorescent DNA sequencing primers that were complementary to sequences between the initial PCR primers also allowed detection of individual base changes and LN carrier diagnosis (data not shown).

We wished to avoid the need to synthesize a separate dye-labeled sequencing primer set for each of the am-

plified HPRT exons while still retaining an unambiguous output from the fluorescent reactions, so that the signal from two bases at the same position in an LN heterozygote could be reliably distinguished from background noise. This was achieved by the introduction of solid supports for the amplified fragments. Oligonucleotides were constructed with a biotin residue at the 5' terminus that permitted the capture of one strand of the amplified DNA onto a commercially available streptavidin-coated magnetic bead. Rare earth magnets simplified the manipulation of the beads so that washing to remove unwanted material took a fraction of the time needed for the usual ethanol precipitations. A universal sequence was introduced via the 5' terminus of one PCR primer and was used as a priming site for the sequencing reactions.

In general, the quality of the sequence obtained from fragments linked to solid supports was higher than that obtained by our previous protocols. Although there seems little advantage to the biotin system in terms of the length or fidelity of the sequence, the sharpness and resolution of the individual base peaks markedly improved the identification of heterozygotes where one normal and one altered DNA base could be easily discerned at a single position. This is an important advantage because it means that it is not necessary to have access to an affected individual to diagnose a related female carrier (e.g., family 8 in Fig. 7.). Overall, we now prefer the direct DNA sequencing approach for the family studies because there is no need to construct ASOs for each new mutation that is identified.

The LN cases that we have characterized add to the current catalog of human HPRT germ line mutations (Gibbs *et al.*, 1989a; Davidson *et al.*, 1989). The five single DNA base substitutions and six intragenic deletions are each characteristic of the wide range of mutation types that are found in this disease. The intronic mutations are of interest as they represent the first report of DNA base alterations that may lead to altered HPRT splice patterns. In each of the four cases the mutations generated sequences that differ from consensus 5' and 3' splice sites (Shapiro and Senapathy, 1987) and diminished the patient HPRT levels sufficiently to produce the LN syndrome. In RJK 888 and RJK 1934 the G to A transition at the fifth position in the usual splice donor site abolished the splicing of the preceding exon, and none of the normal mRNA was detectable by PCR. In RJK 906 no normal message was observed but the downstream cryptic splice site ATTAG was used (Gibbs *et al.*, 1989a) with approximately the same efficiency as the normal site, as judged by measurement of the steady-state HPRT mRNA levels by ribonuclease protection (Gibbs and Caskey, 1987). The mutation in RJK 1760 also resulted in cryptic splice site utilization, as additional HPRT

mRNA forms were recovered along with those lacking exon 2 (data not shown).

The LN family studies were performed both to offer diagnosis and carrier assessment for family members at risk for LN and to identify the origin of the individual mutations. Thirteen carriers were identified in the 10 families that were studied, while the mutant alleles were not detected in blood samples from 2 LN mothers. The maternal age at conception in these two families was 25 and 27 years, precluding any obvious maternal age effects. We previously reported one new HPRT mutation identified by direct DNA sequencing and ASO analysis (Gibbs *et al.*, 1989a) which brings the frequency of *de novo* mutations among the LN patients we have studied to 3/11 (~30%), which is close to the theoretical 1/3 predicted by Haldane's rule (Haldane, 1935).

The methods described here represent a further simplification of procedures for the analysis of mutations at the human HPRT locus. In addition to improving the prospects for the molecular diagnosis of LN families, the multiplex amplification and DNA sequencing will find wide application in studies of somatic mutation in this gene.

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REFERENCES

1. BALLABIO, A., RANIER, J., CHAMBERLAIN, J. S., AND CASKEY, C. T. (1989). Screening for steroid sulphatase deletions via multiplex DNA amplification. *Hum. Genet.*, in press.
2. CHAMBERLAIN, J. S., GIBBS, R. A., RANIER, J., NGUYEN, P. N., AND CASKEY, C. T. (1988). Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res.* **16**: 11,141-11,156.
3. CHAMBERLAIN, J. S., GIBBS, R. A., RANIER, J., AND CASKEY, C. T. (1989). Multiplex PCR for the diagnosis of Duchenne muscular dystrophy. In "PCR Protocols: A Guide to Methods and Applications" (M. Innis, D. Gelfand, D. Sninsky, and T. White, Eds.), pp. 272-281, Academic Press, San Diego.
4. DAVIDSON, B. L., TARLE, S. A., PALELLA, T. D., AND KELLEY, W. N. (1989). Molecular basis of hypoxanthine-guanine phosphoribosyltransferase deficiency in ten subjects determined by direct sequencing of amplified transcripts. *J. Clin. Invest.* **84**: 342-346.
5. EDWARDS, A., VOSS, H., RICE, P., CIVITELLO, A., STEGEMANN, J., SCHWAGER, C., ZIMMERMANN, J., ERFLE, H., CASKEY, C. T.,

- AND ANSORGE, W. (1990). Automated DNA sequencing of the human HPRT gene *Genomics* **6**: 593-608.
6. GIBBS, R. A., AND CASKEY, C. T. (1987). Identification and localization of mutations at the Lesch-Nyhan locus by RNase A cleavage. *Science* **236**: 303-305.
 7. GIBBS, R. A., NGUYEN, P. N., MCBRIDE, L. J., KEOPF, S. M., AND CASKEY, C. T. (1989a). Identification of mutations leading to the Lesch-Nyhan syndrome by automated direct DNA sequencing of *in vitro* amplified cDNA. *Proc. Natl. Acad. Sci. USA* **89**: 1919-1923.
 8. GIBBS, R. A., NGUYEN, P. N., AND CASKEY, C. T. (1989b). Direct DNA sequencing of complementary DNA amplified by the polymerase chain reaction. In "Methods in Molecular Biology" (C. Mathews, Ed.), Humana Press, London, in press.
 9. GIBBS, R. A., NGUYEN, P. N., AND CASKEY, C. T. (1989c). Detection of single DNA base differences by competitive oligonucleotide priming. *Nucleic Acids Res.* **17**: 2437-2448.
 10. GIBBS, R. A. (1990). Detection of point mutations. *Trends Biotechnol.*, in press.
 11. HALDANE, J. B. S. (1935). The rate of spontaneous mutation of a human gene. *J. Genet.* **31**: 317-26.
 12. HULTMAN, T., STAHL, S., HORNES, E., AND UHLEN, M. (1989). Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucleic Acids Res.* **17**: 4937-4946.
 13. KOGAN, S. C., DOHERTY, M., AND GITSCHER, J. (1987). An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. *N. Engl. J. Med.* **317**: 985-990.
 14. MILLER, S. A., DYKES, D. D., AND POLESKY, H. F. (1988). A simple salting out procedure for extracting DNA from human cells. *Nucleic Acids Res.* **16**: 1215.
 15. MITCHELL, L. G., AND MERRIL, C. R. (1989). Affinity generation of single-stranded DNA for dideoxy sequencing following the polymerase chain reaction. *Anal. Biochem.* **178**: 239-242.
 16. SHAPIRO, M. B., AND SENAPATHY, P. (1987). RNA splice junctions of different classes of eukaryotes: Sequence statistics and functional implications in gene expression. *Nucleic Acids Res.* **15**: 7155-7174.
 17. STOUT, J. T., AND CASKEY, C. T. (1989). Hypoxanthine phosphoribosyltransferase deficiency: The Lesch-Nyhan syndrome and gouty arthritis. In "The Metabolic Basis of Inherited Disease" (C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. V. Valle, Eds.), pp. 1007-1028, McGraw-Hill, New York.
 18. XU, Z., YU, Y., HSIE, A. W., CASKEY, C. T., ROSSITER, B., AND GIBBS, R. A. (1989). Deletion screening at the hypoxanthine phosphoribosyltransferase locus in Chinese hamster cells using the polymerase chain reaction. *Teratog. Carcinog. Mutagen.* **9**: 177-187.