MOLECULAR STUDIES IN TWO UNRELATED LESCH-NYHAN SYNDROME FAMILIES

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THE FACULTY OF GRADUATE STUDIES
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by

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ABSTRACT

Lesch-Nyhan Syndrome (L-N) is a rare severe neurological disorder affecting males. The primary defect is a functional absence of the purine salvage pathway enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT). Biochemical, and more recently, molecular studies have showed HPRT deficient disorders to be very heterogeneous at the protein and DNA levels.

Two unrelated families, each with two boys affected with L-N were studied using molecular and biochemical analysis. Results from probing Southern blots with HPRT cDNA indicate the HPRT structural genes are unaltered at the gross level. As well, expression of HPRT mRNA seemed to be normal in L-N fibroblasts from the two families. Measuring the L-N fibroblasts' abilities to incorporate radioactive hypoxanthine into acid insoluble material revealed quite different HPRT levels in the two families.

Southern blot analysis of familial DNA with probe 36B-2 showed that Y and D-family mothers were both heterozygous for DXS10 Taq I alleles, and that the L-N carrier/non-carrier status of the Y-family daughters as determined in this study, were consistent with those determined by conventional technologies.

No new restriction fragment length polymorphisms were found after probing DNA from family members digested with a wide variety of restriction enzymes with HPRT cDNA. Results are consistent with the HPRT lesions in the Y- and D-families being single base substitutions, or very small deletions or duplications in the coding region of the structural gene.

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1.000 INTRODUCTION:

Hypoxanthine guanine phosphoribosyl transferase (HPRT) is a cytoplasmic soluble enzyme expressed constitutively at low levels in most mammalian cells. Due to its extensive use as a selectable marker in somatic cell hybrid work, HPRT has been the focus of a great deal of research. The biochemical and molecular lesions associated with HPRT deficiency, in particular the Lesch-Nyhan Syndrome, will be discussed in this thesis.

1.100 CLINICAL AND NEUROLOGICAL ASPECTS;

The Lesch-Nyhan Syndrome (L-N) was first described in two brothers in 1964 by Michael Lesch and William Nyhan (Lesch and Nyhan, 1964). The disease is a rare cerebral palsy-like condition, with affected individuals appearing normal at birth except for excessive uric acid production (Seegmiller, 1976). Development of clinical symptoms varies greatly from family to family, but is similar for affected members of one given family. The first signs of motor weakness are apparent usually between the seventh and ninth month of age. Choreoathetoid movement, mental and physical retardation, and a bizarre form of self mutilatory behavior characterize the fully manifested syndrome. Self-destructive tendencies are exhibited as finger and lip biting, as well as banging of the head and limbs against walls.

To date the overwhelming majority of cases of L-N have been males, implying an X-linked recessive mode of transmission, which was subsequently verified when the disease locus was identified. There has however, been one report of a female with this condition (Ogasawara et al., 1982).

In the past 22 years, administration of allopurinol was the only form of drug treatment available for L-N patients. Allopurinol serves to inhibit the enzyme xanthine oxidase and thus curbs the production of uric acid (Ogawa et al.,1985), although it does nothing to alleviate the self-mutilation.

The finding that the primary defect was a virtual absence of the salvage pathway enzyme hypoxanthine guanine phosphoribosyl transferase activity (Kelley et al., 1967) did little to explain the bizzare behavior of these patients. Up to that point, HPRT was thought merely to function in nucleotide salvaging, an economical method of reintroducing guanine and hypoxanthine back into the nucleotide pool. Biochemical studies have localized highest HPRT enzyme activity in nervous tissue, specifically in the basal ganglia of the brain (Seegmiller et al., 1967).

Neurological studies indicate that dopaminergic pathways are significantly affected in L-N patients. At necropsy, there appears to be a 10-30% reduction in the function of dopamine-neurone terminals

in affected males (Goldstein et al., 1985). Self-mutilation in laboratory animals can be induced by intercerebral injection of 6-Hydroxydopamine which causes supersensitization of dopaminergic striatal receptors, followed administration of dopamine agonists such as apomorphine (Casas-Bruge, 1985).

GTP seems to modulate the activity state of dopaminergic receptors by interaction with G-protein (regulatory component of dopamine receptor complex) causing decreased affinity for dopamine agonists. Basal areas of the brain are thought to utilize the salvage pathway extensively to maintain nucleotide pools. Neurological studies have demonstrated that HPRT deficiency prevents normal GTP regulation of dopamine receptors in these parts of the brain, hence the supersensitive state and vulnerability to dopamine induced self-mutilation. Use of the dopamine receptor antagonist fluphenazine have proved hopeful in at least one L-N patient (Casas-Bruge, 1985). D-1 receptor antagonists, if given to young L-N patients may help to reduce (selfinduced biting). The alternative to this drug therapy is tooth extraction or fitting with specialized dental apparatus or protective mittens (Wurtell et al., 1984).

1.200 BIOCHEMICAL STUDIES:

The enzyme HPRT catalyzes the formation of guanylic acid and inosinic acid from guanine and hypoxanthine respectively using phosphoribosyl pyrophosphate (PRPP) as the ribose phosphate donor (FIG.1).

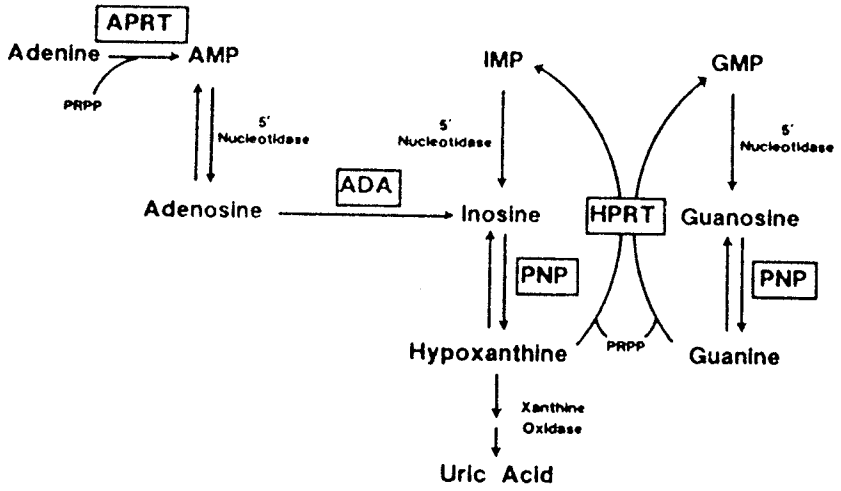


FIG. 1 Salvage Pathway for Free Purines in Mammalian Cells (Stout and Caskey, 1985)

APRT- adenine phosphoribosyltransferase
ADA- adenine deaminase
HPRT- hypoxanthine phosphoribosyltransferase
PNP- purine nucleoside phosphorylase

Salvaging of free bases provides an energy inexpensive alternative to de novo nucleotide synthesis in cells. Partial absence of enzyme activity allows hypoxanthine to be broken down to xanthine and finally uric acid which may precipitate in the joints giving rise to a gouty arthritic condition.

Biochemical studies of this enzyme are important for several reasons: 1) to define biochemical properties of the active enzyme; 2) to determine the nature of the lesions in mutant HPRT enzymes; 3) to develop a suitable method of prenatal diagnosis and carrier detection for females at risk for being L-N heterozygotes.

Estimates from polyacrylamide gel electrophoresis studies regarding the molecular weight of the enzyme subunits, ranged from 24,000 to 27,000 daltons (Olsen et al., 1974, and Holden et al., 1978). Sequence analysis using purified enzyme indicated 24,470 to be the exact molecular weight of the 217 amino acid subunit (Wilson et al., 1982b). The native enzyme is now known to be a tetramer of four identical subunits (Johnson et al., 1979). Human erythrocyte HPRT undergoes at least two posttranslational modifications, acetylation of the N-terminal alanine and partial deamidation of asparagine 106, that result in electrophoretic heterogeneity on polyacrylamide gels (Wilson et al., 1982b).

Before the advent of recombinant DNA techniques, and efficient

enzyme purification procedures, mutant enzymes were characterized according to biochemical properties and kinetics. These types of studies reinforced the notion that HPRT-ve disorders were heterogeneous in nature. Determination of biochemical characteristics such as electrophoretic mobility, relative enzyme level,heat stability, Michaelis-Menten constants (Km) for hypoxantine, guanine, and PRPP, and end product inhibition of mutant HPRT enzymes suggested molecular heterogeneity in Lesch-Nyhan syndrome. (Richardson et al., 1973, and Page et al., 1982). Depending on the particular mutant enzyme, one or more of these parameters was altered.

Wilson and Kelley have led the way in identifying point mutations in various mutant HPRT enzymes isolated from L-N or gout patients. Purification of the enzyme from erythrocytes and lymphoblasts (Wilson et al.,1981, and 1982a) along with protein sequencing revealed the precise mutations in five mutant HPRT enzymes (Wilson et al., 1983c). The same investigators also determined the complete amino acid sequence of the normal enzyme (Wilson et al., 1982b). Using analogies to well-described tertiary structures of other dinucleotide binding enzymes, a three-dimensional model of the nucleotide binding domain of HPRT was constructed (Wilson et al., 1983c). Subsequently point mutations were mapped topographically with respect to the protein domains. Other investigators have reported a extensive variation in enzyme properties of L-N fibroblasts and CHO

cells resistant to 8-azaguanine respectively (Willers et al., 1984, and Plagemann et al., 1985).

Clinically the development of reliable assays for HPRT activity is important for two main reasons: 1) assignment of carrier status for HPRT deficiency; 2) prenatal diagnosis for fetuses of obligate carriers for HPRT deficiency. These tests utilize the ability to select both for and against cells expressing the enzyme, and microassays for HPRT.

Migeon et al (1968) had previously demonstrated that females heterozygous for HPRT deficiency have two clonal populations of fibroblasts, those expressing HPRT, and those negative for enzyme expression, depending on which X-chromosome was inactivated. Subsequently, (Migeon, 1970) developed a simple and rapid method for carrier detection that eliminated the need to analyze individual cell clones. The growth of fibroblasts from L-N heterozygotes in 6-thioguanine made it possible to demonstrate the existence of 6-TG resistant (HPRT-ve) and sensitive (HPRT+ve) cells. This method was extended to intrauterine diagnosis of male fetuses of females at risk for carrying the L-N mutation. Assaying HPRT activity in hair roots provided a simpler method yet for carrier detection (Silvers et al., 1972,). However the authors suggest that the latter method be used as a complementary approach as opposed to an alternative to the fibroblast selection method. Assays that rely on measurment of HPRT activity in tissue lysates have been the subject of controversy due to a lack of

correlation between clinical severity with relative enzyme levels.

HPRT levels were invariably higher in intact cells as opposed to cell lysates, probably due to *in vitro* instability of the mutant enzymes (Page *et al.*, 1982). Measuring HPRT in intact cells seems to give a much better correlation between clinical severity and relative enzyme level.

Prenatal diagnosis of Lesch-Nyhan is now possible in the first trimester by assaying HPRT activity in chorion biopsy homogenates (Gibbs, 1984). Using amniocentisis, diagnosis of a L-N fetus could not be made before 18-19 weeks.

1.300 MOLECULAR ANALYSIS:

The HPRT locus has been mapped to Xq26-Xq27 using human/mouse somatic cell hybrids containing various portions of the human X chromosome (Becker *et al.*, 1979). This locus is also X-linked in other mammals such as mouse and hamster (Pearson *et al.*,1979).

Isolation of HPRT specific mRNA was hindered due to low levels of expression in somatic tissues (0.005-0.04% of total mRNA). A mouse neuroblastoma cell line NBR4 that overproduced a mutant HPRT, facilitated the isolation of an HPRT cDNA (Melton, 1981). Eventually a cDNA clone pHPT5, was produced that contained an open reading frame of

654 nucleotides, along with 100 and 550 nucleotides of 5' and 3' untranslated sequence (Konecki et al., 1982). This same group used this probe to identify cDNA equivalents in human and hamster cDNA libraries. Transfection of human DNA into HPRT-ve mouse cells, allowed the isolation of a partial genomic human HPRT clone and eventually a full length expressible HPRT cDNA was isolated (Jolly et al.,1982 and 1983).

The availability cDNA probes has facilitated detailed analysis of the mouse and human structural genes. Mouse and human HPRT genes were remarkably similar with the mouse gene spanning roughly 34 kilobase pairs (Kbp), while the human counterpart spanned 44 Kbp (Melton et al., 1984, and Stout and Caskey, 1985). Eight intervening sequences account for more than 95% of this length, while the nine exons make up the remainder of the gene (FIG.2).

This relatively complex gene locus has no recognizable CAAT box in the promoter, and the nearest sequence resembling a TATAA box is located more than 700 bp 5' to the cap site instead of 20-30 bp as in the case of most other eukaryotic genes. Transfection of HPRT minigene constructs contaning various portions of the 5' flanking sequence into HPRT-ve cell lines has revealed at least three transcription initiation sites in the mouse HPRT promoter (Melton et al., 1986). Two of these sites occur 91 and 85 bases upstream of the the initiation codon, with the main transcription start site being located at 118 bases 5' to the ATG start codon. In the human HPRT promoter, transcription initiation

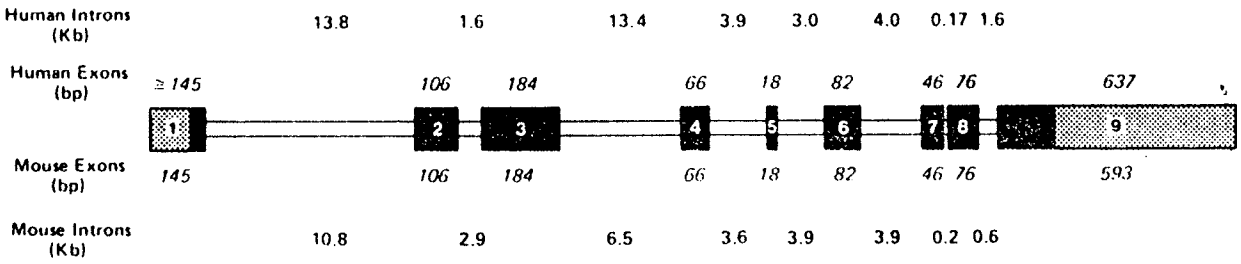


FIG. 2 Structural Organization of Human and Mouse HPRT Genes (Stout and Caskey, 1985)

Stippled boxes represent 5' and 3' - untranslated regions.

is also heterogeneous, with many sites located 104-169 bases upstream to the start codon (Patel et al., 1986). Full functional promoter activity is contained within a 234 bp region 5' to the initiation codon.

The HPRT promoter resembles promoters of other housekeeping genes such as (PGK and G6PD) in that it is very GC rich and contains several copies of the sequence GGGCGG (Patel et al., 1986) which may serve as binding sites for promoter specific transcription factors. Non-housekeeping X-linked genes such as those for clotting factors VIII and IX, have no such features in their promoters (Patel et al., 1986). There are 46 and 35 potential methylation sites which take the form of CpG clusters located in 400 bases 5' to the start ATG codon in human and mouse HPRT genes respectively. It has been suggested that differential methylation in this region may provide a means of conferring functional hemizygosity at this locus in females (Wolf and Migeon, 1985). Hypomethylation is observed in the 5' region of the active HPRT allele, while nonuniform methylation is seen in the inactive allele.

More importantly, the availability of cloned HPRT sequences have allowed the precise elucidation of molecular lesions in HPRT-ve diseases such as Lesch-Nyhan syndrome and gouty arthritis. Molecular analysis of this locus is difficult because of the complex nature of the gene and the existence of crosshybridizing pseudogenes located on

human chromosomes three, five, and two sequences on chromosome 11 (Patel et al., 1984).

Three common HPRT alleles have been demonstrated after molecular hybridization of genomic DNA digested with Bam HI (Nussbaum et al., 1983b). These alleles occur as pairs of restriction fragments that hybridize to HPRT cDNA (22/25, 12/25 and 22/18 Kbp) with respective frequencies of 0.77, 0.16, and 0.07 in Caucasian populations). A recombinant probe (p6-A1) which recognizes the locus DSX10, an anonymous DNA marker tightly linked to HPRT, detects two common Taq I alleles, of 5.0 and 7.0 Kpb which have frequencies of 0.67 and 0.33 respectively (Boggs et al., 1984). The DSX10 locus is outside the HPRT locus, but within Xq26 band, as some Lesch-Nyhan patients have a complete HPRT gene deletion, yet are positive for DXS10 (Boggs et al., 1984). These restriction fragment length polymorphisms (RFLP's) show no association with HPRT deficiency, but may be useful for carrier detection and prenatal diagnosis in informative families.

Haldane's postulation that X-linked lethal disorders conferring no heterozygote advantage were often the result of new mutations (Haldane, 1935), was the basis for the theory that HPRT deficiency disorders were heterogeneous in nature. Protein sequencing work has confirmed ideas from enzyme kinetic studies that HPRT-ve conditions were heterogeneous at the amino acid level. Molecular studies at the DNA and RNA level contribute more evidence for this case.

Recently Yang et al.(1984) found that DNA from five of 28 unrelated L-N patients exhibited abnormal molecular hybridization patterns when probed with HPRT cDNA. In this study, 83% of the mutations in HPRT were most likely due to single base substitutions, while seventeen percent of the L-N patients had major alterations of the HPRT gene, including complete or partial deletions, or partial duplications of HPRT. Not surprisingly, patients with deletions of HPRT produced no detectable HPRT mRNA.

Analysis of 19 Chinese hamster cell HPRT-ve mutants (ten spontaneous, nine U.V. induced) revealed that only two had mutations detectable by molecular hybridization (Fuscoe et al., 1983). In a study of 20 HPRT-ve, CRM-ve (cross-reacting material negative) Chinese hamster cell lines which gave normal molecular hybridization patterns, 18 of these produced normal levels of HPRT specific mRNA (Nussbaum et al.,1983a).

A study involving selection of HPRT-ve human T-lymphocyte clones by culture in 6-TG revealed major alterations at the HPRT locus in 57% of the clones (Turner et al., 1985). Twelve of 21 independently derived 6-TG resistant T-cell clones exhibited abnormal molecular hybridization patterns when compared to unselected T-cell clones. In contrast, Albertini et al.(1985a and b) have demonstrated that in vivo derived 6-TG resistant T-lymphocytes show gross alterations at HPRT in only 27% of the cases. Although these 6-TG resistant T-cells are the somatic

equivalents to L-N cells in normal individuals because they lack HPRT activity, a lower incidence of point mutations as the cause of HPRT disfunction is observed. Stout and Caskey (1985) argue that the stringent selection conditions employed in these experiments favour mutations causing complete HPRT deficiency. Consequently, cells possessing major alterations at this locus may be represented at a disproportionately high frequency in populations of HPRT-ve T-cells.

HPRT deficent disorders are, molecularly a heterogeneous group, of which many are due to new mutations. In a composite survey of 15 unrelated L-N patients and nine patients with gout or nephroliathis, Wilson et al (1986) showed that these patients could be placed into at least 16 distinct mutational groups based on biochemical and molecular parameters. Cells from most L-N patients do not produce immunologically detectable amounts of HPRT protein, yet are mRNA+ve, and have normal molecular hybridization patterns when probed with HPRT cDNA. These observations suggest that most HPRT lesions are probably point mutations effective at the post-transcriptional level resulting in inefficient translation or reduced enzyme activity and/or stability.

1.400 Research Aims

Richardson et al (1973), using conventional biochemical techniques, provided preliminary evidence for the existence of different HPRT mutations in the Y and D-families. The purpose of this study was to characterize the nature of these same lesions at the molecular level using the following techniques.

1) Molecular hybridization analysis a) to determine if any rearrangements have taken place at the DNA level, b) to identify any unique familial RFLP's, c) to confirm carrier or non-carrier status for females at risk for having a L-N fetus.

2) Molecular hybridization of RNA to determine if HPRT specific mRNA is being expressed in L-N fibroblasts from the affected members of the two families, along with size of mRNA.

3) Measurement of hypoxanthine utilization by L-N fibroblasts under aminopterin block by assessing the incorporation of hypoxanthine into acid insoluble material. Results from this indirect HPRT assay were compared to the values obtained by Richardson et al.(1973) using red blood cell (RBC) lysates.

2.000 MATERIALS AND METHODS;

2.100 Growth of Human Fibroblasts:

Fibroblasts initially obtained from skin biopsies in 1970 and 1971 and stored in liquid nitrogen, were recovered by rapid thawing and maintained in Modified McCoy 5A medium supplemented with 10% fetal bovine serum (FBS), and 0.026M sodium bicarbonate. Penicillin at 100 units/ml, and streptomycin at 10 µg/ml were included in the medium to minimize bacterial contamination. Cells were grown at 37°C under a 5% carbon dioxide atmosphere at 100% relative humidity. Monolayers were allowed to reach confluency and were then harvested for DNA or RNA extractions.

2.200 Extractions:

2.210 DNA Extraction:

Fibroblast monolayers were washed twice in ice cold PBS and removed by trypsinization (5 minutes in 0.05% trpysin at 37°C) or treating with 0.1M EDTA pH 8.0 (ethylene diamine tetraacetate, sodium salt) followed by scraping with a rubber policeman. Cells were pelleted by centrifuging at 4000 rpm for ten minutes in a Beckman JA 7.5 rotor at 4°C, washed with PBS and centrifuged once again.

Cells were suspended in 10mM Tris-HCl (pH 8.0), 500mM NaCl, and 2mM EDTA at a density of 10^7 cells/ml. Cell lysis was accomplished by addition of SDS (sodium dodecyl sulfate) to 0.5%, followed by digestion with Proteinase K (250 µg/ml) at 65°C for three hours. The cell lysate was extracted once with TNE_2 saturated phenol followed by centrifugation at 5000 rpm for ten minutes in a JA 7.5 rotor at 20°C to separate the phases. The aqueous phase was extracted with phenol:chloroform (1:1) and finally with chloroform.

DNA was precipitated by addition of an equal volume of cold isopropanol followed by gentle mixing. The pellet was washed once in cold 70% isopropanol and once with pure isopropanol,and dried under vacuum. The DNA was dissolved in 50 mM Tris-HCl (pH 8.0), 100mM NaCl, 10 mM EDTA (2 ml/original 10^7 cells) and heat-treated RNAse was added to a final concentration of 100µg/ml. Digestion proceeded for three hours at 37°C. This mixture was then subjected to three organic solvent extractions as above. Sodium acetate was added to a final concentration of 0.2M, and DNA was reprecipitated and washed as before with isopropanol. After drying, the nucleic acid pellet was dissolved at a concentration of 500µg/ml in TNE_2 and stored at 4°C.

2.220 RNA Extraction:

Fibroblast monolayers were washed twice with ice cold PBS and harvested by adding 0.1M EDTA (pH 8.0) and scraping with a rubber

policeman. Cells were pelleted at 4000 rpm for ten minutes in a JA 7.5 rotor at 4°C and lysed with 4M guanidinium isothiocyanate, 0.5% sodium N-lauryl sarcosine, 25mM sodium citrate, and 0.1M 2-mercaptoethanol (15ml/10^9 cells). DNA was sheared by forcing the suspension through an 18-gauge needle until viscosity was lost.

One gram of CsCl was added per 7.0 ml of homogenate, and the resulting solution was layered onto 3.0 ml of 5.7M CsCl, 100mM EDTA (pH 8.0) in a Beckman Quick-Seal 5/8 X 3 inch polyallomer ultracentrifuge tube. Paraffin oil was used to top up the tube, and RNA was pelleted by ultracentrifugation in an Beckman 80Ti rotor at 36,000 rpm for 16 hours at 20°C.

Supernatants were carefully decanted and the tube inverted to drain excess fluid from the pellet. The RNA was dissolved in a small volume of sterile 10mM Tris-HCl (pH 7.5), 5mM EDTA, followed by extraction with a 4:1 mixture of chloroform:1-butanol. The organic phase was back-extracted with the Tris-EDTA solution, and the two aqueous phases combined. 4M sodium acetate (pH 5.5) was added to a final concentration of 0.2M, and three volumes of absolute ethanol were added. After allowing precipitation to occur for at least three hours at -20°C, the RNA was pelleted by centrifuging at 8000 rpm for 20 minutes in a Sorval J.A. 20 rotor at 0°C. The RNA was washed in 70%, then 100% ethanol, and dissolved in sterile water at a concentration of 0.5-1 mg/ml. RNA stock solutions were stored at -70°C in 1.5 ml microfuge tubes.

2.230 Plasmid Extraction:

Plasmid extraction was performed according to the method of Garger et al.(1983). The DNA was dissolved in TNE_2 at 4°C.

2.240 Insert Extraction:

Plasmid pPR1, containing a full length human HPRT cDNA (Jolly et al., 1984), was digested with Pst I to excise the cloned insert from the vector. Two cycles of electrophoresis through a 1% agarose gel were used to separate the HPRT insert from the vector. A gel slice containing the band of interest was electro eluted to recover the insert (Maniatis et al., 1982). The HPRT insert was purified and concentrated by using a BioRad Rapid DNA purification mini-column, followed by ethanol precipitation. The DNA recovered was dissolved at a concentration of 100 ng/ml in TNE_2 and stored at 4°C.

The recombinant plasmid p36B-2 (Nussbaum, 1986) was digested with Hind III to remove the insert, which was then purified in the same manner as the HPRT insert.

2.300 Restriction Endonuclease Digestions:

Reactions were typically performed in 25µl volumes following assay conditions specified by the suppliers (Boehringer-Manheim Company and Pharmacia). DNA was digested at a concentration of 300ng/µl using a three fold excess of enzyme. Spermidine was added to a concentration of 2mM to aid digestion of DNA. Restriction of DNA usually proceeded for 12-16 hours, and was terminated by adjusting to 0.05% orange G, 2% Ficoll, and 10mM EDTA.

2.400 Electrophoresis:

2.410 DNA Electrophoresis:

DNA was electrophoresed through agarose gels containing 40mM Tris-Acetate (pH 8.0), 5mM sodium acetate, 1mM EDTA, and 1 µg/ml of ethidium bromide. Gel concentrations ranged from 0.8-1.0%, and running times were typically 17-20 hours at 20 volts/45 milliamps in a submarine gel system. DNA was visualized using a short wave U.V. transilluminator, and photographed through red and yellow filters using Kodak Tripan-X film.

The molecular weight marker used on each gel was 200 ngm of lambda phage DNA digested with Hind III and Eco RI.

2.420 RNA Electrophoresis:

Fifty µg of total cellular RNA was dissolved in a solution of 50% formamide, 20 mM MOPS (morpholinopropanesulfonic acid), 5mM sodium acetate, 1mM EDTA (pH 7.0), and 6.5% formaldehyde in a volume of 40 µl or less. The RNA was denatured by heating the mixture at 65°C for 15 minutes and cooling on ice for ten minutes. This solution was adjusted to 5% glycerol, 0.1M EDTA, and 0.04% bromophenol blue prior to gel loading.

RNA was electrophoresed through 1.3% agarose gels containing 6.5% formaldehyde, 20mM MOPS pH 7.0, 5mM sodium acetate, 1mM EDTA, and 1 µg/ml of ethidium bromide . Gels were run in a submarine system in the same buffer (without formaldehyde and ethidium bromide) for 15-20 hours at 25-30 volts. RNA was visualized and photographed using the same method as for DNA gels.

2.500 Transfer of Nucleic Acids to Nitrocellulose:

2.510 Transfer of DNA to Nitrocellulose:

DNA was transferred from agarose gels (containing 7.5 µg DNA/lane) to nitrocellulose membranes according to Southern (1975) with

some modifications. Gels were treated with 0.25M HCl for 15 minutes if fragments larger than ten Kbp were to be transferred, followed by denaturation of the DNA by shaking the gel in two changes of 0.5N NaOH, 1.5M NaCl for 30 minutes each. Gels were neutralized by shaking in three changes of 1M Tris-HCl (pH 8.0), 1.5M NaCl for 20 minute periods. All procedures were carried out at room temperature unless otherwise specified.

DNA was transferred to boiled nitrocellulose using 10XSSC essentially according to Southern (1975). Transfer continued for at least 20 hours. Nitrocellulose was rinsed in 6XSSC to remove unbound DNA, dried at RT, and then baked at 80°C under vacuum for two hours. Blots were prehybridized immediately or stored dry at 4°C for future use.

2.520 Transfer of RNA to Nitrocellulose:

RNA was transferred to nitrocellulose in the same manner as DNA except the acid hydrolysis, denaturation, and neutralization steps were omitted, and transfer was accomplished with 20X SSC.

2.600 DNA Labelling:

Probes for molecular hybridization were labelled with alpha-^{32}P dCTP and dATP using a random hexanucleotide priming and Klenow fragment extension system (Feinberg et al, 1983).

Probe DNA, (100-400 ng in 2 µl or less of TNE_2) was denatured by boiling for two minutes in a 500 µl microfuge tube, followed by quick cooling on ice. 1.25 µl of a random mixture of hexameric oligonucleotides (0.1 O.D. units/ml, Pharmacia Ltd.) was annealed at room temperature to the denatured probe in the presence of 0.2M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 6.6),5 mM MgCl, 10mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 8.0), 50 µM dTTP, 50 µM dGTP, and 400 ng/ml of bovine serum albumin. Fifty µCi each of alpha-^{32}P-dCTP and -dATP were added, and the random hexamer primers extended by incubating with 4.5 units of Klenow fragment two to 16 hours at room temperature. Reaction volumes were usually 25 to 30 µl.

The reaction was stopped by adjusting the mixture to 0.2 % SDS, 10 mM EDTA, and 5% glycerol. Labelled probe was separated from free nucleotides by passing the reaction through a sephadex G-75 column equilibriated with TNE_2.

2.700 Hybridizations:

2.710 DNA/DNA Hybridizations:

Nitrocellulose filters were prehybridized at least 4 hours at 42°C in 20 ml of 50% formamide (recrystallized twice), 5XSSC, 10X

Denhardt's solution, 0.5% SDS, 50mM sodium phosphate (pH 6.4), 10 mM EDTA, and 100 µg/ml herring sperm DNA (denatured by boiling at 100°C for 5 minutes, and quenching on ice) at 42°C. Hybridizations were carried out in 10 ml of 50% formamide, 5XSSC, 5X Denhardt's solution, 0.5% SDS, 50mM sodium phosphate (pH 6.4), 10mM EDTA, 10% dextran sulfate, 100 µg/ml denatured herring sperm DNA along with 2 X 10^8 cpm/ml of denatured probe and 10^5 cpm ^{32}P-labelled Lambda phage DNA.

After at least 30 hours of hybridization at 42°C, the filters were washed two times in 2X SSC, 0.1% SDS for 10 minutes at RT, and then once or twice (depending upon radioactivity of filter) in 0.5X SSC, 0.1% SDS at 50-55°C for 30 minutes. The filters were then wrapped in plastic wrap and exposed at -70°C to preflashed Kodak X-OMAT AR-5 X-ray film using a Dupont Lightning Plus intensifying screen for 12-72 hours.

2.720 DNA/RNA Hybridizations:

Nitrocellulose filters were prehybridized at least 4 hours at 42°C in 10 ml of 50% formamide, 5X SSC, 5X Denhardt's solution, 0.1% SDS, 10mM EDTA, and 100 µg/ml denatured salmon sperm DNA. Hybridization was carried out in 5 ml of the same solution containing ^{32}P-labelled HPRT cDNA for 40-48 hours at 42°C.

Filters were washed using the same conditions as for DNA blots,

followed by exposure to Kodak X-OMAT AR-5 film as before.

2.800 Measurement of Hypoxanthine utilization:

The ability of L-N fibroblasts to incorporate hypoxanthine into acid insoluble material was measured using the method of Fenwick and Caskey (1975) with modifications.

2 - 3 X 10^6 cells were seeded in 100mm petri dishes containing ten ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. The cells were incubated for 4 days, after which time the medium was aspirated off and replaced with 4.0 ml of DMEM + 10% FBS containing 10 µM aminopterin and 2 µCi of 8-^{14}C-hypoxanthine. After six hours, this medium was removed, and the cells washed twice with ice cold PBS. Two ml of sterile water were added to each plate, and the cells removed with a rubber policeman.

Lysis of the cells was accomplished by three cycles of rapid freeze/thaw between liquid nitrogen (-180°C) and a 37°C water bath. 10% of this lysate was removed for protein assay according to the method of Bradford (1976) and 3.0 ml of cold 20% TCA was added to the remainder to precipitate nucleic acids. Precipitates were recovered on glass filters (Whatman GF/A) by vacuum filtration. The filters were washed with cold 5% TCA twice, with cold ethanol twice, dried, and radioactivity was then determined by liquid scintilation counting.

FIG. 3 D-family pedigree

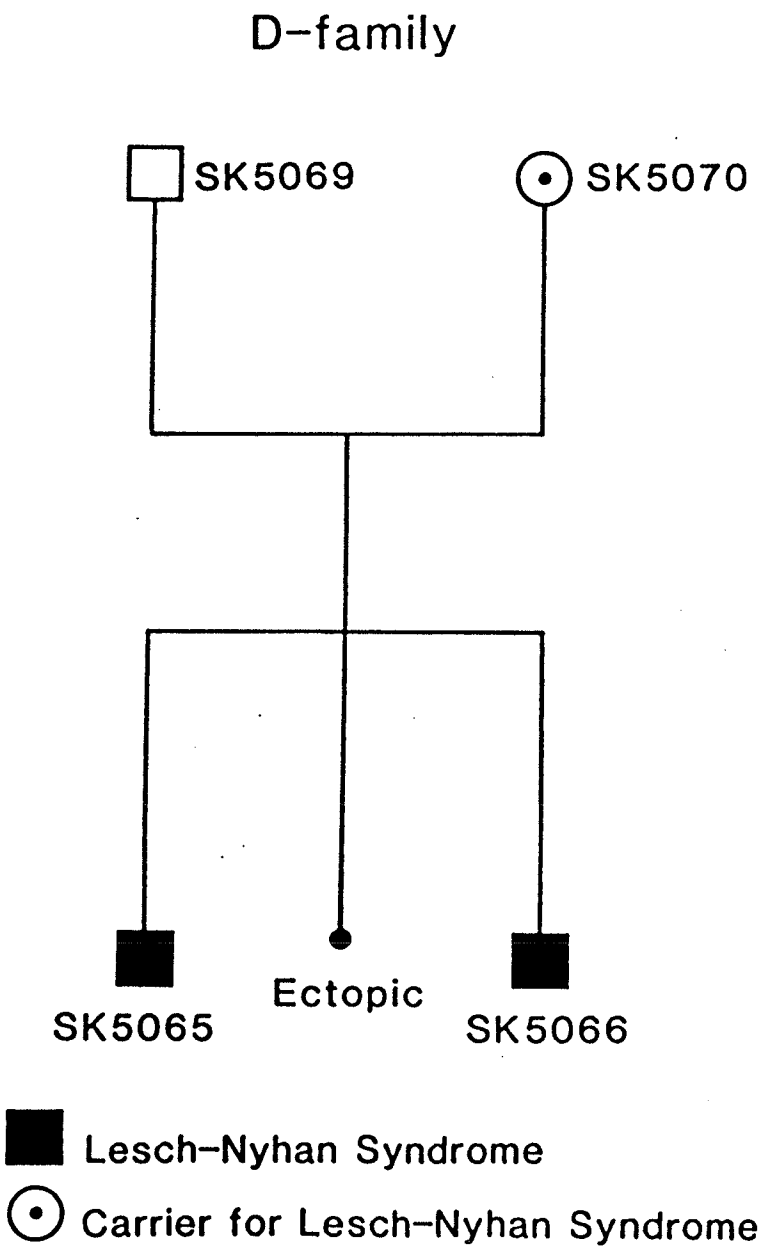
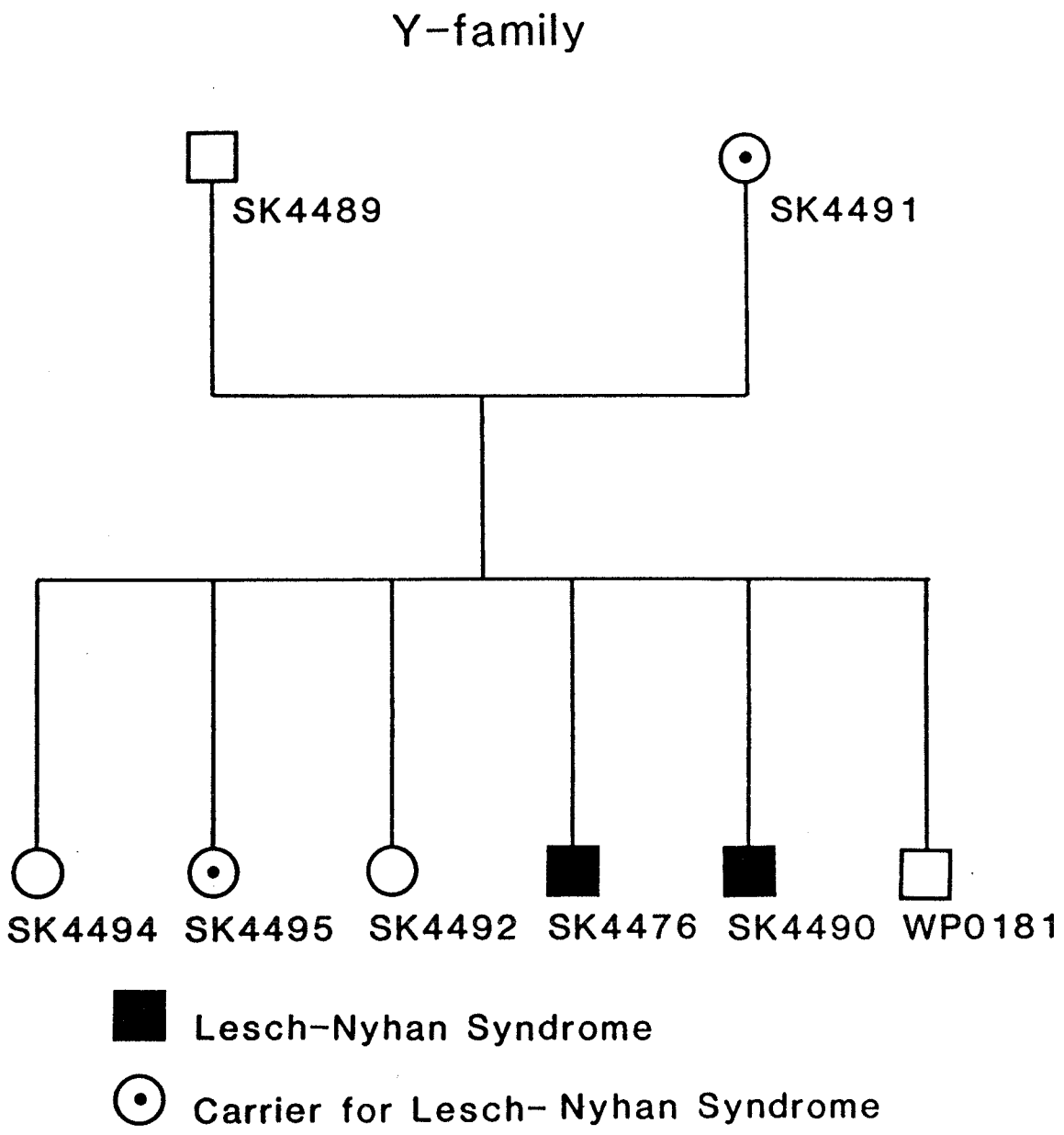


FIG. 4 Y-family pedigree



3.000 RESULTS;

3.100 Molecular Hybridization Analysis using HPRT cDNA;

Genomic DNA from Lesch-Nyhan and control males in the Y- and D-families was digested with 23 and 24 restriction enzymes respectively, and probed with a full length human HPRT cDNA. Molecular hybridization patterns for Y- and D-family males are illustrated in figures 5 and 6 respectively. For the Y-family (Fig.4), cell line SK4476 was used as the source of L-N DNA, and WP0181 was used as the control. In the D-family, SK5066 and SK5065 were used as the sources of L-N DNA, and SK5069 as the control (Fig. 3).

Figure 5 shows Bgl I and Bgl II digests of DNA from Y-family males probed with HPRT cDNA, while Fig. 6 illustrates DNA from D-family males digested with Eco RI, Bam HI, Pst I, and Hind III, and Taq I after molecular hybridization.

DNA from Y-family males was digested with Eco RI, Bam HI, Hind III, Msp I, Taq I, Bgl I, Bgl II, Pst I, Xmn I, Xba I, Ban II, Apa I, Eco RV, Bxt XI, Ava II, Kpn I, Rsa I, Mbo I, Alu I, Hae III, Dde I, Hinf I, and Bst I, followed by probing with HPRT cDNA. DNA from D-family males was analyzed in the same manner but restriction enzyme Pvu II was used in addition to the other 23 enzymes.

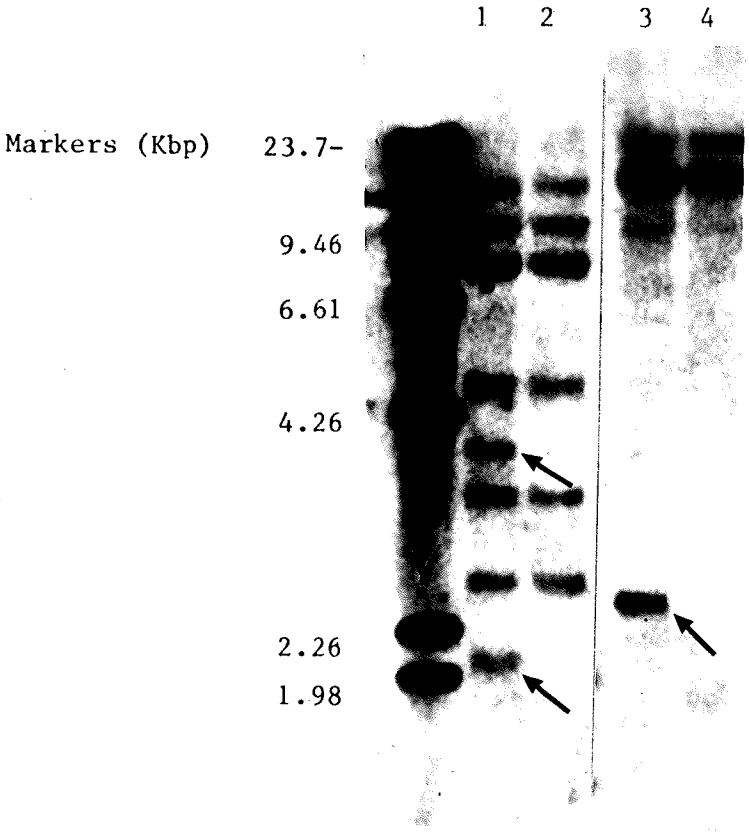


FIG. 5 Southern Blot Analysis of Y-Family Male DNA probed with HPRT cDNA.

Control male-WP0181
L-N male-SK4476

Lanes 1 and 2 - Bgl I digest of SK4476 and WP0181 DNA respectively.

3 and 4 - Bgl II digest of SK4476 and WP0181 DNA respectively.

Note- Bands denoted by arrows result from plasmid contamination in SK4476 DNA.

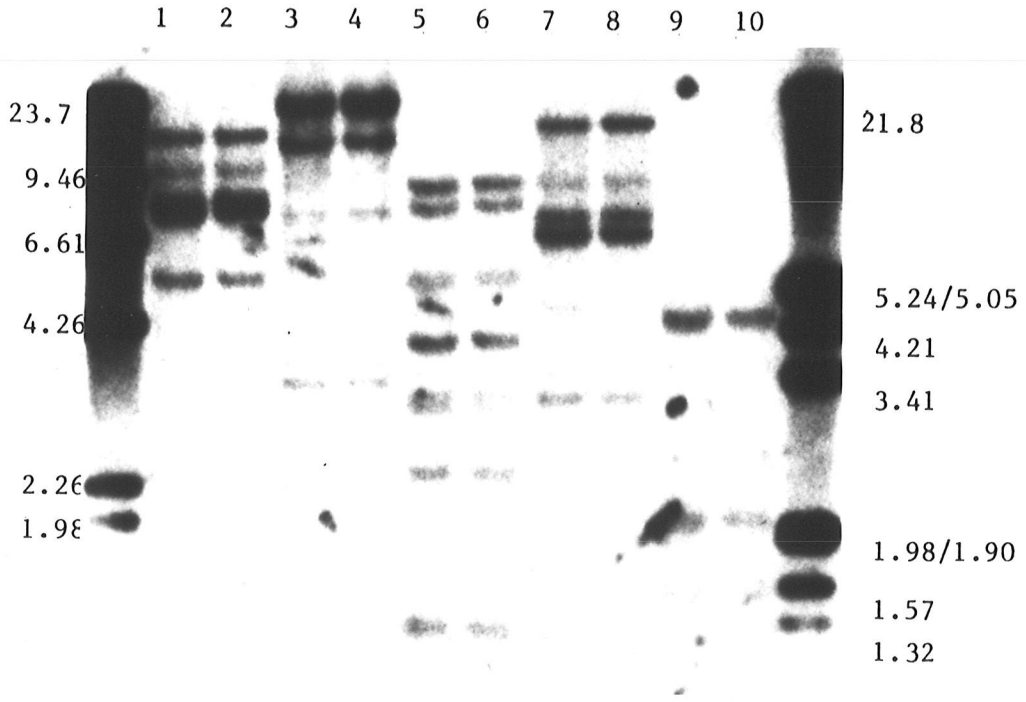


FIG. 6 Southern Blot Analysis of D-Family DNA probed with HPRT cDNA

Control male-SK5069
L-N male-SK5066

Lanes 1 and 2 - Eco RI digest of SK5066 and SK5069 DNA respectively.

3 and 4 - Bam HI digest of SK5066 and SK6069 DNA respectively.

5 and 6 - Pst I digest of SK5066 and SK6069 DNA respectively.

7 and 8 - Hind III digest of SK5066 and SK5069 DNA respectively.

9 and 10 - Taq I digest of SK5066 and SK5069 DNA respectively.

No real differences between Southern blot patterns of control and L-N DNA were observed when HPRT cDNA was used as the probe. In some digests, hybridizing bands present in SK4476 DNA were not observed in control DNA WP0181 (Fig. 5). To further investigate these differences, the DNA blots were stripped of the HPRT probe,and rehybridized with radiolabelled plasmid (pAT 153). The anomalous bands present in SK4476 DNA, but not in WP0181 DNA, were due to low level contamination of homologous plasmid sequences in the SK4476 DNA sample. DNA from L-N and control males from both families exhibited the 25/22 Kbp Bam HI allele of HPRT.

3.200 Molecular Hybridization Analysis of Fibroblast RNA;

Total cellular RNA from Lesch-Nyhan cell lines SK4476 and SK5066, and from control male line WP0181 was probed with HPRT cDNA. HPRT specific mRNA is approximately 1600 bases, and comprises 0.005-0.01 % of mRNA in somatic cells (Stout and Caskey, 1985).

Fig. 7 shows the results of probing total fibroblast RNA with radiolabelled HPRT cDNA after fractionation on an agarose gel and transfer to nitrocellulose. A band of equal intensity slightly below the 18s rRNA band is observed in all three RNA samples. This would appear to be the HPRT mRNA band, as no other distinct hybridizing bands, other than rRNA, are observed on the autoradiogram. Therefore,

at this level of detection, no differences at the RNA level are observed in the Y and D-families.

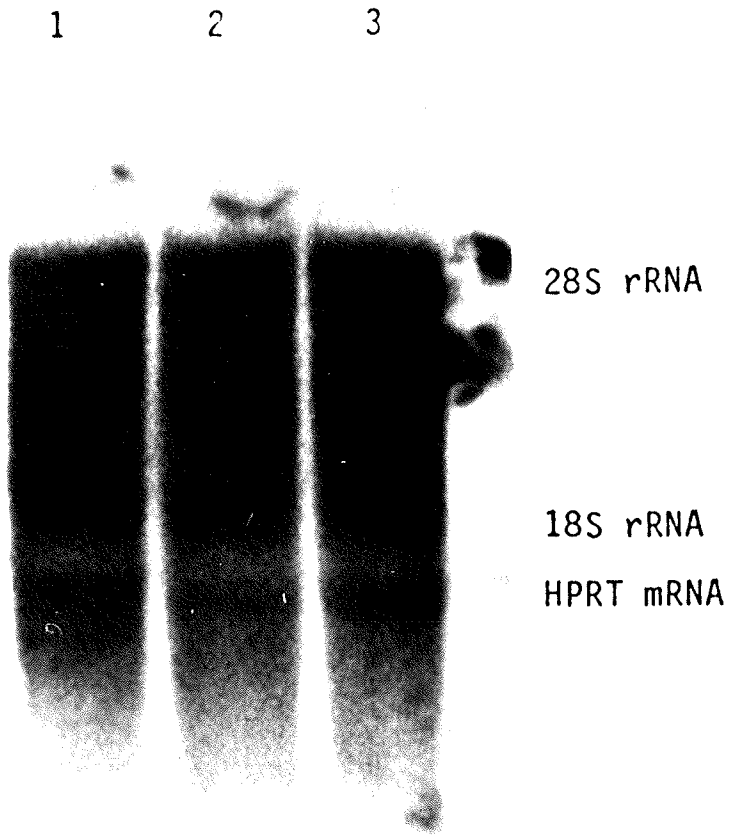


FIG. 7 Northern Blot Analysis of Fibroblast RNA

Lane 1 - WP0181 RNA (control male)

2 - SK5066 RNA (L-N male, D-Family)

3 - SK4476 RNA (L-N male, Y-Family)

3.300 Incorporation of 8-^{14}C-Hypoxanthine into Acid Insoluble Material;

The relative abilities of L-N fibroblasts from members of the Y- and D-families to incorporate ^{14}C-labelled hypoxanthine to TCA insoluble material are listed in Table 1. Values from this whole cell HPRT assay were compared with relative HPRT levels obtained by Richardson et al. (1973) using erythrocytes from the same family members.

Table 1. Comparison of relative levels of HPRT as measured by ability to incorporate ^{14}C-Hypoxanthine in intact fibroblasts compared to red blood cell lysates.

Family member	SK4476	SK5066	Control
*Relative ability to incorporate 8-^{14}C-hypoxanthine (fibroblasts)%	3.1	0.5	100
°Relative HPRT level (using RBC lysates) %	0.1	0.005	100

* Results from present study (WP0181 used as control)

° Results from Richardson et al., 1973.

It can be seen from Table 1 that values for indirect whole cell HPRT assays differ significantly from those using a cell lysate method, in that the values from the latter study are lower by factors of 31 and 100 in the Y- and D-families respectively. These results are in agreement with those of Page et al. (1982), in that higher relative HPRT levels are found when the assays are performed on whole cells as opposed to cell lysates.

3.400 Molecular Hybridization Analysis using Probe 36B-2:

Probe 36B-2 is an X-chromosome derived HindIII probe, 2.26 Kbp in length, which has been mapped to the anonymous locus DXS10 in Xq26. This probe detects the same 5 and 7 Kbp Taq I alleles of DXS10 as probe 6A-1, however the constant 3.5 Kbp band which is present in Taq I digests is not present when using 36B-2 (Nussbaum, 1986., and Boggs et al., 1984).

3.410 D-Family;

Figure 8 shows DNA from D-family members digested with Taq I, and probed with 36B-2. The mother (SK5070) is heterozygous at DXS10, displaying the 7.0 and 5.0 Kbp alleles,as well as being an obligate carrier for L-N. Both affected L-N males are hemizygous for the 7.0 Kbp allele, and thus the L-N phenotype is segregating with this allele, the father (SK5069) is fortuitously also displaying the same band.

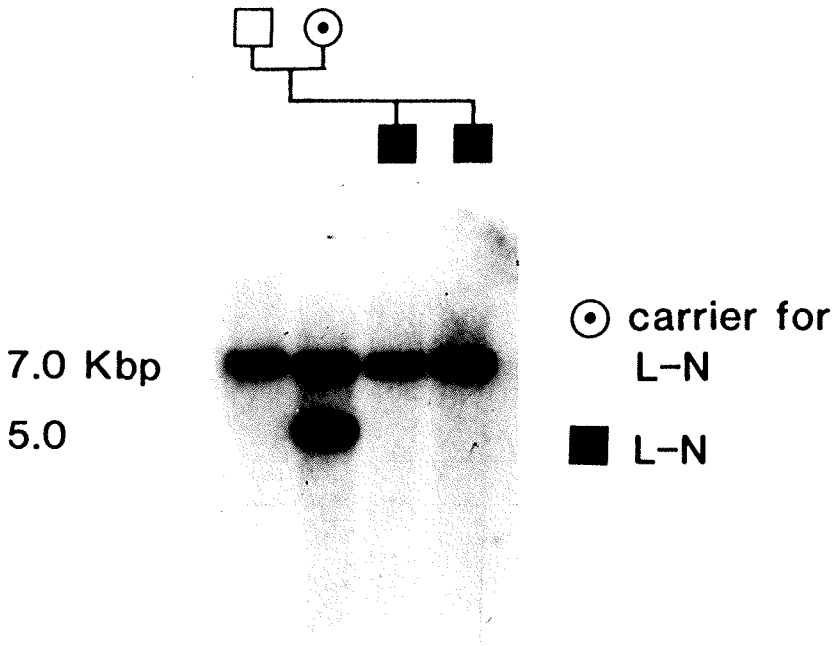


FIG. 8 Taq I Digest of D-Family DNA Probed with p36B-2.

In this family, the L-N gene is segregating with the 7.0 Kbp allele.

3.420 Y-Family:

As in the D-family, the obligate carrier mother (SK4491) is heterozygous for the Taq I alleles of DXS10. However, in this kindred, both L-N males carry the 5.0 Kbp band, while the non-affected male has the 7.0 Kbp allele. The youngest and eldest daughters (SK4492 and SK4495) are also heterozygous at DXS10, and the middle daughter (SK4494) is homozygous for the smaller DXS10 allele. The father, coincidentally is hemizygous for the same DXS10 allele as the two L-N males. In this family, the mutant HPRT is segregating with the 5.0 Kbp allele of DXS10, and the carrier/non-carrier status of the three daughters are in agreement with those assigned by Migeon (1976).

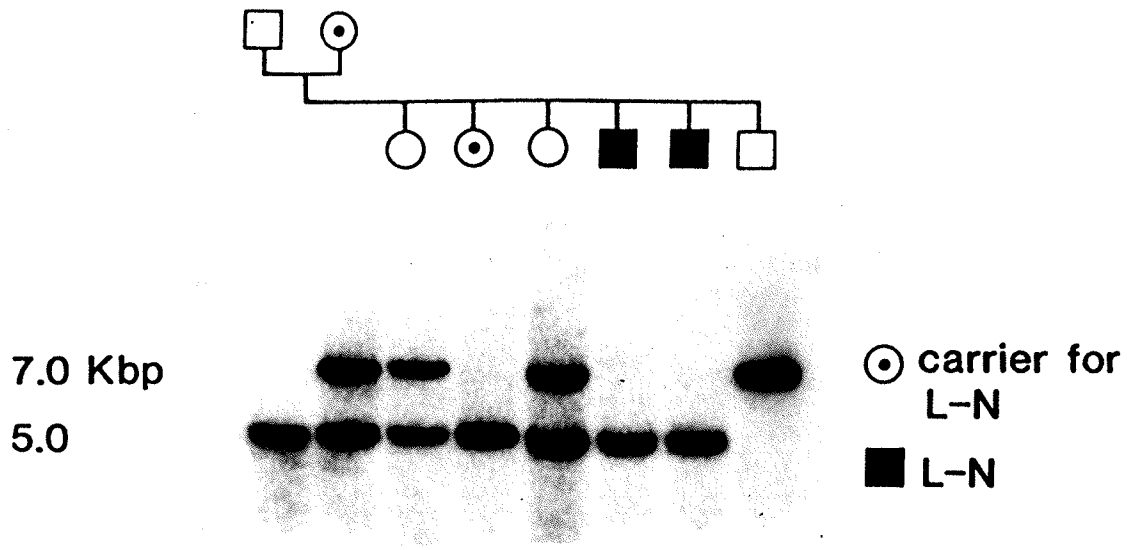


FIG. 9 Taq I Digest of Y-Family DNA Probed with p36B-2.

In this family, the L-N gene is segregating with the 5.0 Kbp allele.

4.000 DISCUSSION:

4.100 DNA Studies:

Molecular hybridization analysis of the HPRT locus in these two families has provided some insight into the nature of the mutations at this locus. In informative families, carrier detection and/or prenatal diagnosis may be accomplished in informative families by using the Bam HI polymorphisms of HPRT (Nussbaum et al., 1983b). In this study, the strategy was to look for differences in banding on Southern blots between L-N and control male DNA's from both families. Ultimately, any differences found would have been verified by examining DNA blots using DNA from all the family members.

L-N and control male DNA from Y- and D-families were examined using 23 and 24 restriction enzymes respectively. No differences that could be attributed to HPRT deficiency were found between control and L-N DNA from the two families when HPRT cDNA was used as a probe. Males from the Y-family both carried the 25/22 Kbp Bam H1 allele, which is the most common. It can be inferred from this that the mother (SK4491) is homozygous for the 25/22 Kbp Bam HI allele of HPRT . Heterozygosity for this locus with regard to Bam HI can not be inferred in this manner for the D-Family mother because DNA from a normal son was not availble.

If the HPRT mutations in the Y- and D-families involved major

alterations such as partial deletions or duplications of one or more exons, changes in multiple molecular hybridization patterns would have been apparent. When no differences between L-N and control male DNA's were observed after the first few enzymes used, it became apparent that the familial lesions at HPRT were likely to be point mutations, or very small deletions or duplications. At this point, the chances of identifying the specific mutations as new RFLP's decreased, as only 17% of L-N patients show abnormal bands on Southern blots (Yang et al., 1984). To date, only one mutant HPRT protein (HPRT Toronto) that has been sequenced, has an altered restriction site detectable by molecular analysis. The single base substitution in exon 3 results in abolition of a Taq I restrition site (Wilson et al., 1983c)

Screening control and L-N DNA from the Y- and D-families with more restriction enzymes might possibly reveal unique familial RFLP's due to the HPRT lesions. However, this process would be very expensive, and prenatal diagnosis and/or carrier detection is not necessary in the families now, with the exception of SK4495, the only individual now at risk for having a L-N son and/or carrier daughter.

One method to identify the specific mutation in each family would require cloning and and sequencing of the mutant HPRT genes. Due to the large size of the HPRT gene, isolating a cDNA to the mutant alleles would be the more feasible approach.

Alternatively, methods are now available that allow identification of point mutations in total genomic DNA without cloning and sequencing. Much of this type of work has been done using the well characterized beta-globin mutations. One such method requires synthesis of a labelled RNA probe complementary to wild-type DNA which is hybridized to globin DNA containing a single base substitution. Following digestion with Ribonuclease A, the mismatch is localized by examining cleavage product sizes determined through electrophoresis (Meyers et al., 1985). Greater than 50% of all single base mutations in beta-thalassemia can be detected in this fashion. The complex nature of the HPRT locus raises the question as to whether L-N Syndrome DNA's would be amenable to this type of analysis. However, by making cDNA from mutant HPRT mRNA, identification of single base substitutions without sequencing, should be possible by this method.

The described polymorphism of HPRT at the DNA level, is the three allele Bam HI RFLP (Nussbaum et al., 1983b). However, the low frequency of the 22/12 and 22/18 Kbp alleles (0.16 and 0.07 respectively) severely limits the usefulness of the system. Consequently the use of random polymorphic X-chromosome probes may often be more helpful in carrier detection/prenatal diagnosis in certain diseases than cloned DNA to the disease locus itself. DXS10 is an anonymous locus proximal to HPRT in Xq26. One L-N patient with a complete deletion of HPRT still retains DXS10, while a cell line with an alpha-particle induced HPRT deletion displays no DXS10 signal,

although Xq26 is cytogenetically normal (Boggs et al, 1984). Therefore, DXS10 is outside the HPRT locus, but within Xq26 at an unknown distance from HPRT. There has not been a documented recombination between these two loci in 26 informative meiotic events (Nussbaum, 1986).

Both Y and D-family mothers were heterozygous for the DXS10 alleles, and thus probe 36B-2 could be used to confirm carrier status of daughters in the Y family. Results from the analysis with 36B-2 regarding the carrier status of Y family daughters were consistent with those of Migeon (1976). Therefore this probe is useful for carrier detection in both families. Up to this point, DXS10 is the closest polymorphic DNA locus to HPRT. Use of other DNA probes such as 52A, for loci flanking HPRT would also be necessary in the event of an attempted prenatal diagnosis/carrier detection. Probe 52A detects a Taq I polymorphism of DXS51, a locus distal to HPRT (Max.lod=4.3 at O=0.05) (Drayna et al, 1984).

4.200 RNA STUDIES:

HPRT is a housekeeping enzyme expressed at low levels in all mammalian somatic cells, with processed HPRT specific mRNA (1600 bases) comprising 0.005-0.04% of total mRNA. Total RNA from L-N and control fibroblast lines was probed with labelled HPRT cDNA to determine if the mutations in the Y and D families were effective at the pre- or posttranscriptional level.

Molecular hybridization analysis of total RNA from L-N fibroblasts revealed apparently normal levels of HPRT mRNA in SK5066 and SK4476. The band indicated as the HPRT mRNA (Fig. 7) was much more intense with regard to amount of RNA in this area of the blot. There was significant background on the blot due to non-specific binding of the probe, presumably due to the large amounts of 18S and 28S rRNA in relation to the mRNA. The majority of nucleic acid in a total RNA preparation is ribosomal and transfer RNA, with mRNA making up only about 1%. Caskey also reports the appearance of high molecular weight bands on HPRT Northern blots that can not be precisely explained (Caskey, 1986). Ideally, lymphoblastoid cell lines should have been used in this study for RNA analysis due to the much higher HPRT mRNA levels. However, such lines were not available, so fibroblasts were used.

RNA studies show that apparently normal amounts of HPRT mRNA were being produced by affected individuals in both families. The mRNA is also of the same size as in the control, and the HPRT mutations in these families must therefore be acting at the posttranscriptional level.

4.300 ENZYME STUDIES:

In mammalian cells, purines can be introduced into the nucleotide pool through de novo synthesis from glutamine and PRPP, or

by recycling free purines by salvage enzymes HPRT and APRT. De novo purine synthesis is blocked by the action of aminopterin, and as a result, L-N cells do not survive in the selective medium HAT (hypoxanthine, aminopterin, and thymidine).

Apparent enzyme kinetics for the mutant HPRT's in both families were previously reported (Richardson et al., 1973). That study Showed relative HPRT enzyme levels of 0.1% and 0.005% in patient erythrocytes from the Y- and D-families respectively. Recent studies suggest in vitro analysis of this type may give artificially low activities due to instability of mutant enzymes upon cell lysis (Page et al., 1982). Although the HPRT activities in the L-N fibroblasts from both families were similar to each other, they were much higher relative to the control (WP0181) in the present study. Y family L-N fibroblasts (SK4476) had 3.1% of normal ability to utilize hypoxanthine via purine salvage, while the D family fibroblasts (SK5066) had only 0.5% activity. These values are higher by factors of 31 and 100 respectively, and are consistent with the work of Page et al (1982).

There is definitely residual HPRT activity in L-N fibroblasts from both families. Assays for determining the relative enzyme level measure HPRT activity, and not amount of HPRT protein. If antibodies to HPRT were available, immumoprecipitation and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) would allow quantitation of HPRT cross-reacting material (CRM) in the L-N fibroblasts. Following

immunological studies the L-N would be classified as CRM+ve or CRM-ve. If they were CRM+ve, the mutant HPRT proteins could theoretically be purified, and the electrophoretic and enzyme kinetic properties determined.

5.000 SUMMARY:

Two unrelated L-N families have been studied using molecular and biochemical analysis. Results from extensive molecular hybridization analysis using HPRT cDNA as a probe, suggest no major alterations at the HPRT loci have taken place in the two kindreds. Probing Taq I digested DNA with p36B-2, revealed that the mutant HPRT alleles were segregating with different DXS10 alleles in the Y and D families.

Molecular hybridization analysis of L-N fibroblast RNA indicated that the HPRT mRNA was being produced in similar amounts as in the controls. From the DNA and RNA studies described here, it seems the HPRT genes are unaltered structurally, and the mutations are functioning at the posttranscriptional level.

Biochemical evidence for heterogeneity in the L-N syndrome was presented earlier (Richardson et al., 1973). Measuring the HPRT activity in intact cells (present study) simply corroborated the work of Richardson et al.

The mutations at the HPRT in the Y and D families are likely point mutations in different parts of the gene coding region. Both lesions however, seem to cause HPRT-ve condition by reducing the stability or catalytic activity of the HPRT protein itself.

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7.000 APPENDIX

7.100 List of Reagents:

PBS
0.137 M NaCl
0.003 M KCl
0.008 M Na_2PO_4
0.0015 M KH_2PO_4 pH 7.0

TNE_2
0.01 M Tris-HCl (pH 8.0)
0.01 M NaCl
0.002 M EDTA

SSC
0.15 M NaCl
0.015 M sodium citrate (pH 7.0)

Denhardt's solution
0.02 % Bovine serum albumin
0.02 % polyvinyl pyrolidine
0.02 % Ficoll

7.200 List of Abbreviations:

ml = milliliter
µl = microliter
g = gram
mg = microgram
ng = nanogram
cpm = counts per minute
µCi = microCurie
mM = millimolar
M = molar
O.D. = optical density

7.300 List of Fibroblast Lines Used:

Y-family		D-family	
Cell line	Family member	Cell line	Family member
SK4489	Father	SK5069	Father
SK4491	Mother, obligate carrier	SK5070	Mother
SK4494	Daughter, non-carrier	SK5065	Son, L-N
SK4495	Daughter, carrier	SK5066	Son, L-N
SK4492	Daughter, non-carrier		
SK4476	Son, L-N		
SK4490	Son, L-N		
WP0181	Son, normal		

Note: HPRT genotypes of Y-family female fibroblast lines were determined by Migeon (1976) by selection in 6-thioguanine.