BIOCHEMICAL AND GENETIC PROPERTIES OF $HPRT_{Cape\ Town}$

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ABSTRACT

An unusual partial HPRT deficient mutant, $HPRT_{Cape\ Town}$, was observed to have a low activity in erythrocyte lysates at high concentrations of the purine substrates, hypoxanthine and guanine. This substrate inhibition was not observed with the substrate PPRP. The low activity was not associated with changes in the K_m or V_{max} for any of the substrates (Steyn and Harley, 1984).

The kinetics of the proband's enzyme was studied in lymphoblast extracts. The characteristic substrate inhibition was observed which showed that this phenomenon was not confined to erythrocytes but was a more generalized phenomenon. This result implies that the decreased HPRT activity observed in the proband is due to substrate inhibition by the purine bases.

The HPRT enzyme is coded for by a gene which is located on the X chromosome (Pai et al., 1980). The proband's daughter was therefore studied in order to determine the cause of the mutation. It was not known whether the substrate inhibiton was the result of a mutation in the gene coding for the enzyme, a mutation which results in altered post-translational modification or the absence or alteration of factors influencing normal HPRT kinetics.

The daughter's transformed lymphoblasts exhibited growth patterns in selective media that resembled those of her father. The daughter's enzyme prepared from lymphoblast extracts exhibited the characteristic substrate inhibition. These results suggest that this cell line

results from the selection of a clone or clones which have suppressed the function of the X chromosome carrying the maternal and presumably normal HPRT allele. The daughter's enzyme prepared from erythrocyte lysates exhibited intermediate enzyme activity between that of the proband and a normal control. This result suggests that the daughter is an obligate heterozygote and that the defect is due to a mutation in the HPRT gene itself.

The defect was studied at the gene level. No difference was observed in the banding patterns of the proband's DNA and control DNA which were digested with various restriction enzymes and hybridized to ^{32}P-labelled HPRT cDNA. The size of the HPRT mRNA of the proband was the same as the control. These results imply that there is no major gene alteration; this is expected since the proband only has a partial deficiency of the enzyme.

The HPRT cDNA was subcloned into a riboprobe vector, pGEM-3. The T7 promoter was used to transcribe antisense RNA strands which were then hybridized to the proband's RNA and control RNA. No difference was observed in the size of the protected fragment. This result does not exclude the possibility of a point mutation as the cause of the defect in $HPRT_{Cape\ Town}$.

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LIST OF ABBREVIATIONS

ADA: adenosine deaminase

ADP: adenosine diphosphate

AMP: adenosine monophosphate

APRT: adenine phosphoribosyltransferase

ATP: adenosine triphosphate

dATP: deoxyadenosine triphosphate

B.K.: the initials of the proband's daughter

BLOTTO: bovine lacto transfer technique optimizer

BSA: bovine serum albumin

CDP: cytidine diphosphate

cpm: counts per minute

CTP: cytidine triphosphate

dCTP: deoxycytidine triphosphate

DMEM: Dulbecco's Modified Eagle's Media

DNA: deoxyribonucleic acid

cDNA: complementary deoxyribonucleic acid

DEAE: diethylaminoethylcellulose

DNase: deoxyribonuclease

DTT: dithiothreitol

EBV: Epstein-Barr virus

EDTA: ethylenediaminetetra-acetic acid

FAD: flavin adenine nucleotide

GDP: guanosine diphosphate

GMP: guanosine monophosphate

GTP: guanosine triphosphate

dGTP: deoxyguanosine triphosphate

HAT medium: hypoxanthine, aminopterin, thymidine

HPRT: hypoxanthine-guanine phosphoribosyltransferase

IMP: inosine monophosphate

K_i: dissociation constant for the second substrate molecule

K_m: Michaelis constant

MAGAT: mycophenolic acid, adenine, guanine, amethopterin and thymidine

MOPS: morpholinopropanesulfonic acid

NAD: nicotinamide adenine dinucleotide

NADP: nicotinamide adenine dinucleotide phosphate

OD: optical density

PEG: polyethylene glycol

PEI: polyethylene imine

pfu: plaque forming units

PNP: purine nucleoside phosphorylase

PP_i: inorganic pyrophosphate

PPRP: 5-phospho-D-ribosyl-1-pyrophosphate

RNA: ribonucleic acid

mRNA: messenger ribonucleic acid

rRNA: ribosomal ribonucleic acid

RNase: ribonuclease

RNasin: ribonuclease inhibitor

S: substrate concentration

18S, 28S: rRNA components from the mammalian ribosome. The rRNA is characterized by its sedimentation coefficient expressed in Svedberg units (S).

SDS: sodium dodecyl sulphate

T.K.: the initials of the proband

Tris: Tris(hydroxymethyl)-methylamine

dTTP: thymidine triphosphate

UDP: uridine diphosphate

UTP: uridine triphosphate

UV: ultraviolet

V: initial velocity

V_{max}: maximum velocity

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1.0 INTRODUCTION

1.1 PURINE METABOLISM

Man is able to synthesize purine and pyrimidine nucleotides de novo. These nucleotides have many roles which include serving as precursors for RNA and DNA. The purines also act as a high energy source (ATP), as regulatory signals (cyclic AMP and cyclic GMP) and as components of the coenzymes FAD, NAD, NADP and as a component of the methyl donor, S-adenosylmethionine. The pyrimidines act as high energy intermediates in carbohydrate metabolism (UDP-glucose and UDP-galactose) and in lipid synthesis (CDP-acylglycerol).

The de novo synthesis of IMP from ribose-5-phosphate and ATP is a complex process which requires the expenditure of 6 high energy phosphodiester bonds (by ATP hydrolysis) as well as utilizing glycine, glutamine, aspartic acid and one carbon unit from methenyl-tetrahydrofolate (Figure 1). IMP is an intermediate in the formation of AMP and GMP (Figure 2), both pathways requiring further expenditure of energy.

Two pathways exist for the salvage of purines which, either result from the catabolism of endogenous ribonucleotides, or from the ingestion of purine-containing foods. The first and most important pathway involves the phosphorylation of the purine base by a specific enzyme which requires 5-phosphoribosyl-1-pyrophosphate (PPRP) as a cosubstrate (Figure 3). The second pathway involves phosphorylation of the purine nucleosides and then a kinase reaction to form the ribonucleotide. Both these pathways only require the expenditure of one high energy bond (either PPRP or ATP). Thus the salvage pathways have an

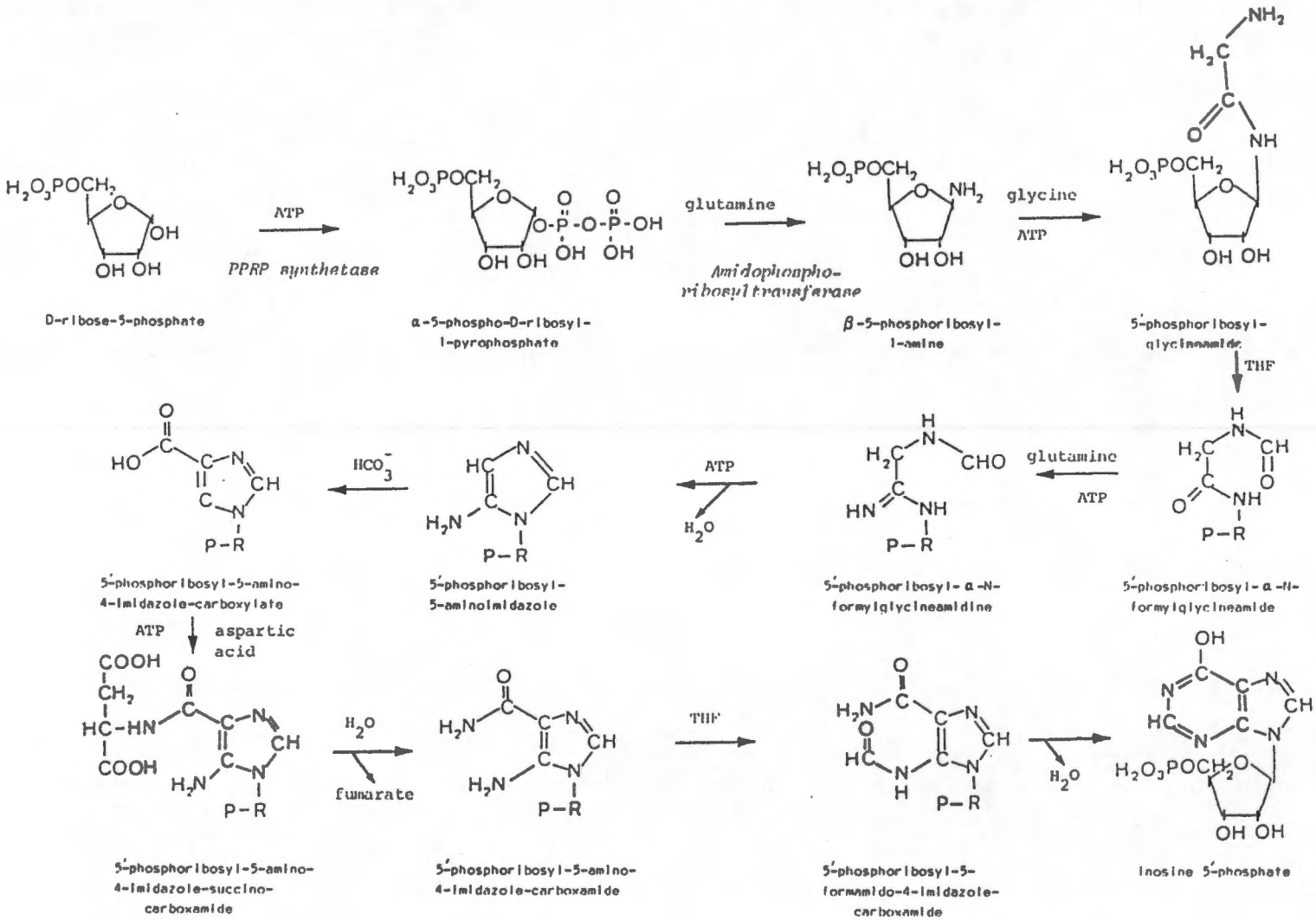


Figure 1 De novo purine biosynthesis

P-R: ribose-5-phosphate; THF: tetrahydrofolate

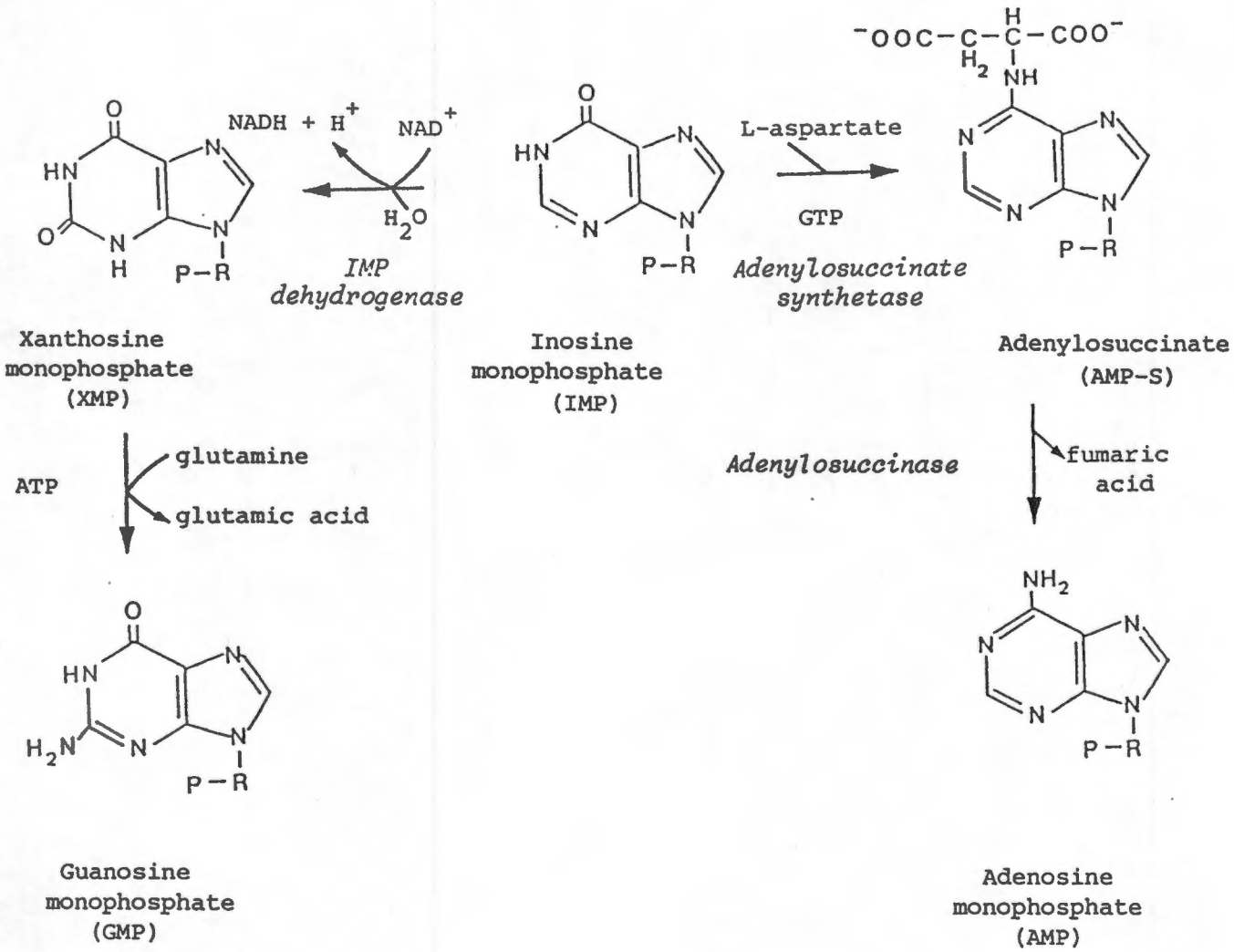


Figure 2 Biosynthesis of AMP and GMP

P-R: ribose-5-phosphate

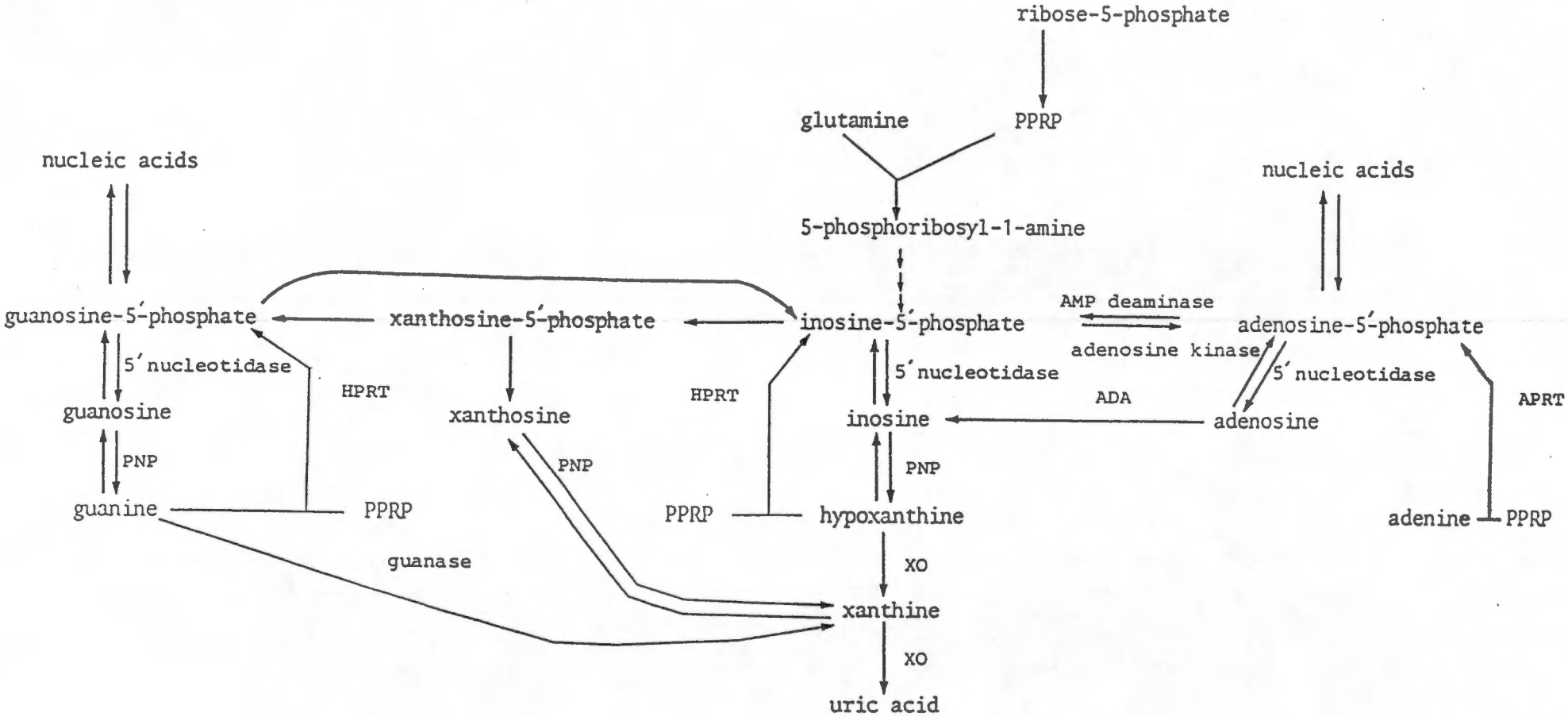


Figure 3 Purine salvage pathways

ADA: adenosine deaminase
APRT: adenine phosphoribosyltransferase
HPRT: hypoxanthine phosphoribosyltransferase
PNP: purine nucleoside phosphorylase
XO: xanthine oxidase

important role to play in the conservation of energy and purine metabolites in the cell, especially in those cells that have a decreased ability for de novo purine biosynthesis e.g. erythrocytes (Fontenelle and Henderson, 1969).

1.1.1 Regulation of purine metabolism

Purine metabolism is regulated in a number of ways. It is important to regulate de novo purine biosynthesis because of its high energy requirements. An important regulator of the de novo pathway is the PPRP concentration. This is determined by the balance between its rate of synthesis and its rate of utilization / degradation. The rate of utilization is largely dependent on its consumption by the salvage pathway where it acts as a cosubstrate for the phosphoribosyl-transferases. The rate of synthesis of PPRP depends, firstly on the availability of its precursors, especially ribose-5-phosphate, and secondly on the catalytic activity of PPRP synthetase. This enzyme has a requirement for inorganic phosphate which acts as an allosteric activator. PPRP synthetase activity also depends on the concentration of purine and pyrimidine nucleotides which inhibit its activity (Figure 4) (Wyngaarden and Kelley, 1983).

Further control of de novo purine biosynthesis is exerted on the enzyme responsible for the committed step, amidophosphoribosyltransferase. This enzyme is allosterically inhibited by the end products of the reaction, AMP and GMP. These nucleotides promote aggregation of the enzyme into a metabolically, inactive form while PPRP causes disaggregation to the active, monomeric form (Holmes et al., 1973).

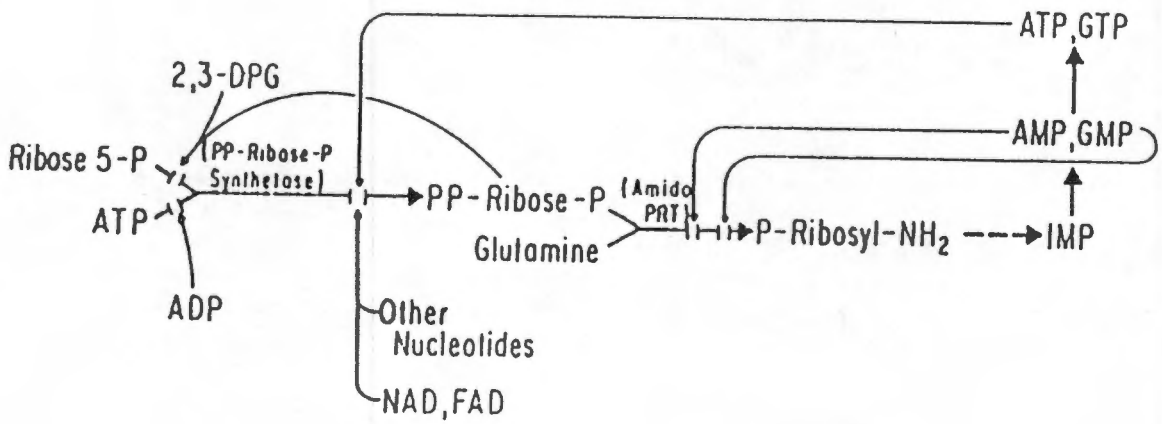


Figure 4 Diagrammatic representation of the feedback inhibitors of purine biosynthesis reactions catalyzed by PPRP synthetase and amidophosphoribosyltransferase (amido PRT)

2,3-DPG: 2,3-diphosphoglycerate
P-ribosyl-NH_2 : β-5-phosphoribosyl-1-amine

(Wyngaarden and Kelley, 1983)

The purine ribonucleotide interconversions (figure 2) are also regulated. GMP inhibits IMP dehydrogenase, the enzyme which catalyzes the formation of xanthosine monophosphate which is then converted to GMP. This second step in the conversion of IMP to GMP requires ATP as an energy source. AMP and GDP inhibit adenylosuccinate synthetase which catalyzes the conversion of IMP to adenylosuccinate. Adenylosuccinate is then converted to AMP by adenylosuccinase. The first reaction in this two step conversion requires GTP as an energy source. The end products AMP and GMP thus control their own biosynthesis as well as controlling the synthesis of the other nucleotide by providing an energy source. Thus the synthesis of GMP can be balanced by the corresponding AMP production and vice versa (Wyngaarden and Kelley, 1983).

AMP and GMP also inhibit their respective phosphoribosyltransferases, thus regulating their synthesis from free bases (Kelley et al., 1969).

Ribonucleoside diphosphates are converted to deoxyribonucleoside diphosphates by the enzyme ribonucleoside diphosphate reductase. The deoxyribonucleoside diphosphates are then phosphorylated to form the deoxynucleoside triphosphates. Deoxynucleotide synthesis is depicted in Figure 5. This process is regulated. Accumulation of dATP inhibits the reductase enzyme, while the accumulation of dTTP inhibits the reduction of the pyrimidines, UDP and CDP. Accumulation of dGTP inhibits the reduction of GDP, UDP and CDP (Thelander and Reichard, 1979).

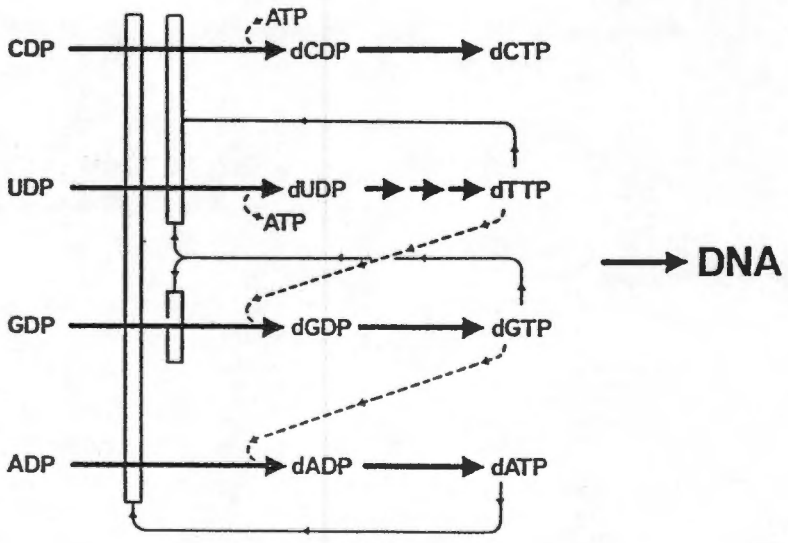


Figure 5 Regulation of deoxynucleotide synthesis
Open bars represent inhibition and broken arrows represent activation
(Thelander and Reichard, 1979)

1.1.2 Inborn errors of purine metabolism in man

1.1.2.1 Adenine phosphoribosyltransferase

Adenine phosphoribosyltransferase (APRT EC 2.4.2.7) phosphorylates adenine to produce AMP. PPRP is a cosubstrate of the enzyme. A deficiency of APRT, which is autosomally recessively inherited, presents clinically as urolithiasis, the stones consisting predominantly of 2,8 dihydroxyadenine. Besides the excretion of adenine and its metabolites, there are no other biochemical abnormalities, thus APRT is not vital for the overall control of purine metabolism (Simmonds and Van Acker, 1983).

1.1.2.2 Adenosine deaminase and purine nucleoside phosphorylase

Adenosine deaminase (ADA EC 3.5.4.4) and purine nucleoside phosphorylase (PNP EC 2.4.2.1) both catalyze reactions which are involved in purine degradation (figure 3). ADA deaminates adenosine to inosine and PNP catalyzes the conversion of inosine or guanosine to hypoxanthine and guanine respectively. PNP has no activity with adenosine as a substrate (Kredich and Hershfield, 1983). A deficiency of either enzyme causes abnormalities in purine nucleoside metabolism that are toxic to lymphocytes. In ADA deficiency, deoxyadenosine accumulates and is converted to dATP which inhibits ribonucleotide diphosphate reductase (figure 5). In PNP deficiency, deoxyguanosine accumulates and is converted to dGTP which inhibits the synthesis of dCTP and dTTP, therefore DNA synthesis in response to an immunogenic challenge fails because of a lack of pyrimidines (Watts, 1983). Most

patients with ADA deficiency lack cell-mediated and humoral immunity and have severe combined immunodeficiency disease. Patients with a deficiency of PNP only have a defective cell-mediated immunity (Kredich and Hershfield, 1983).

1.1.2.3. Xanthine oxidase

Xanthine oxidase (EC 1.2.3.2) catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid (figure 3). A deficiency of this enzyme has been found to be asymptomatic in some cases, while in others xanthine calculi of the urinary tract developed. The mode of inheritance is autosomal recessive (Holmes and Wyngaarden, 1983).

1.1.2.4 5-Phosphoribosyl-1-pyrophosphate synthetase

5-phosphoribosyl-1-pyrophosphate (PPRP) synthetase (EC 2.7.6.1) catalyzes the formation of PPRP from ATP and ribose-5-phosphate. A number of variants of this enzyme have been identified. These variants result in an excessive rate of generation of PPRP which causes an acceleration of de novo purine biosynthesis. The patients exhibit hyperuricemia, uricosuria and gout. The mode of inheritance is X-linked (Wyngaarden and Kelley, 1983).

1.1.2.5 Hypoxanthine-guanine phosphoribosyltransferase

The remainder of this introduction will be devoted to the structure and function of hypoxanthine-guanine phosphoribosyltransferase (HPRT, EC 2.4.2.8) as well as the consequences of a deficiency of it.

1.2 HPRT STRUCTURE AND FUNCTION

HPRT catalyzes the transfer of phosphoribose from PPRP to the 9 position of hypoxanthine or guanine to form IMP or GMP respectively (Figure 6) (Kornberg et al., 1955). In man, unlike in bacterial cells, a single enzyme catalyzes the reaction for both substrates. This has been suggested by the fact that the two substrates have identical rates of heat inactivation, identical electrophoretic mobilities under several conditions and the genetic loss of one reaction is accompanied by loss of the other (Henderson, 1968).

HPRT is a soluble, cytoplasmic protein which is widely distributed in tissues. It accounts for 0.005-0.04% of total proteins (Stout and Caskey, 1985). In man, the highest activity is found in the brain, within the basal ganglia. Relatively high levels have also been found in leukocytes, fibroblasts and gonadal tissue. Generally, HPRT activity is highest in rapidly dividing tissue (Kelley and Wyngaarden, 1983).

The HPRT protein is 217 amino acids in length with a subunit molecular weight of 24 470. The initial methionine has been cleaved. The amino terminal alanine is acetylated (Wilson et al., 1982a).

There has been a controversy over the number of subunits in the native enzyme molecule. Certain investigators have compared the apparent molecular weights of the native enzyme molecule and the subunits and proposed that the enzyme exists as a trimer in Chinese hamster brain (Olsen and Milman, 1974) and in mouse liver (Hughes et al., 1975).

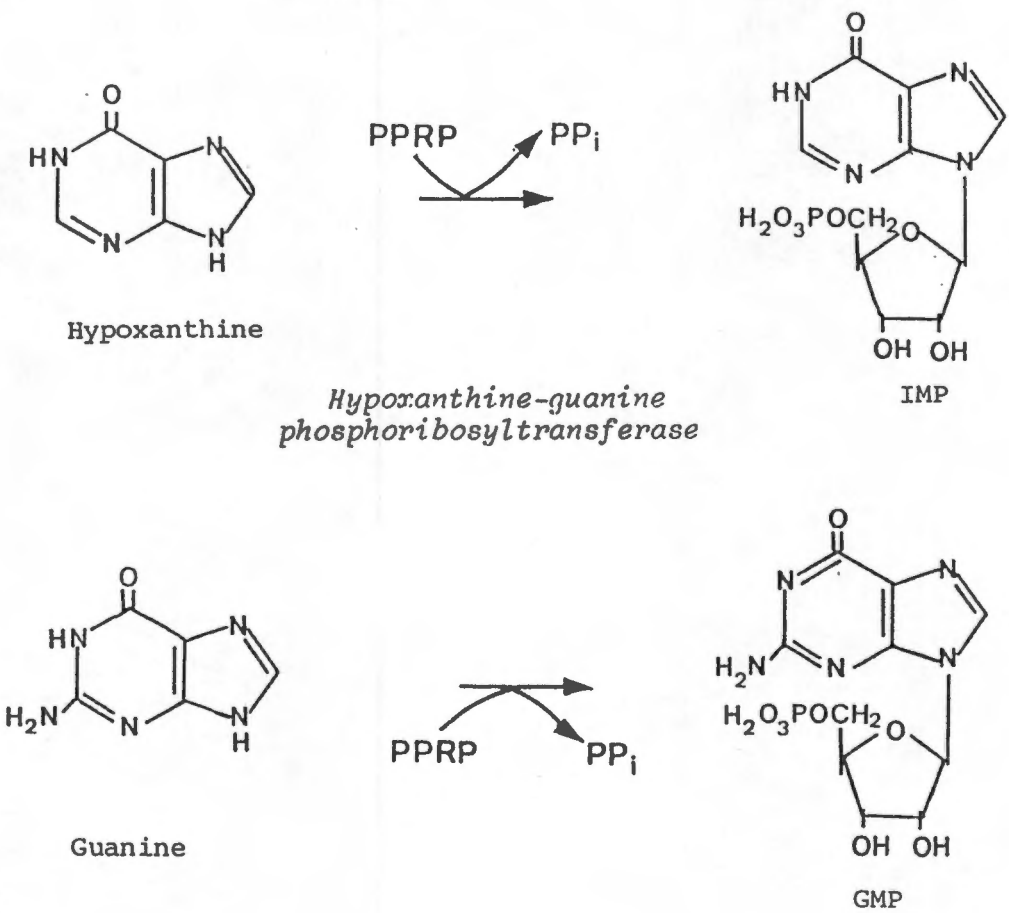


Figure 6 The reactions catalyzed by HPRT

P-R: ribose-5-phosphate
PPRP: 5-phosphoribosyl-1-pyrophosphate
PP_i : inorganic pyrophosphate

Muensch and Yoshida (1977) obtained different results for the apparent size of the subunits of human erythrocyte HPRT using different methods. With sedimentation equilibrium methods, they obtained a subunit size of 41 000-45 000 and the size of the native molecule was estimated to be 84 000, thus suggesting a dimer. However, with SDS polyacrylamide gel electrophoresis, they estimated the subunit size to be 26 000, thus suggesting a trimeric structure. Arnold and Kelley (1971) also compared apparent molecular weights of the native molecule and the subunits and proposed a dimeric structure. Johnson et al. (1979) examined the interaction between mouse and human enzyme subunits in hybrid cells under conditions of isoelectric focusing (low ionic strength) and observed the formation of dimers. However, in high ionic strength, the dimers associate to form tetramers. Holden and Kelley (1978) covalently crosslinked the purified enzyme with dimethyl-suberimidate, dimethyladipimidate and glutaraldehyde and analyzed the products on SDS gels. They observed 4 bands indicating the presence of a tetramer, which is most likely to consist of a dimer of dimers.

Isoelectric focusing of the human erythrocyte enzyme has revealed that there are multiple forms of the enzyme (Arnold and Kelley, 1971; Davies and Dean, 1971; Gulumian and Wakid, 1975; Johnson et al., 1982). Rubin et al. (1971) identified two peaks of activity in human erythrocyte HPRT after DEAE-cellulose chromatography. Bakay and Nyhan (1975) separated four isoenzymes by polyacrylamide gel electrophoresis. This electrophoretic heterogeneity cannot be explained by multiple genes because a single genetic event leads to a complete deficiency of the enzyme in man nor can it be explained by multiple alleles because some of the studies were performed on erythrocytes from males who, since the

enzyme is X-linked, would only have one allele (Arnold and Kelley, 1971). The fibroblast enzyme does not exhibit electrophoretic heterogeneity (Zannis et al., 1980) and neither does the enzyme prepared from cultured lymphoblasts and white blood cells (Johnson et al., 1982). Wilson et al. (1982a) have isolated and characterized tryptic peptides of human HPRT and identified asparagine/aspartic acid heterogeneity at amino acid 106. Studies have indicated that this is due to deamidation of the protein in vivo. This post translational modification partly accounts for the observed electrophoretic heterogeneity.

HPRT can bind and ribosylate a variety of different substrates besides hypoxanthine and guanine. The enzyme binds 6-oxo or 6-thiopurines but not 6-amino compounds. A 2-amino group enhances binding whereas a 2-hydroxyl group diminishes it. Methylation of any of the ring nitrogens except N-1 prevents binding by the enzyme (Krenitsky et al., 1969). Included in these substrates are the toxic purine analogues, 6-thioguanine, 8-azaguanine, 6-mercaptopurine and 6-aza-hypoxanthine which are used as a means to select $HPRT^-$ cells. HPRT can also bind allopurinol. Xanthine is also a substrate, although the reaction proceeds at 0.3% of the rate of the reaction with hypoxanthine or guanine (Seegmiller, 1976).

Magnesium activates the enzyme by complexing with PPRP. PPRP in the dimagnesium salt form, is the true substrate for HPRT. The HPRT catalyzed reaction is inhibited at high $MgCl_2$ concentrations due to a competitive inhibition of Mg^{2+} with respect to the dimagnesium salt of PPRP. This suggests that these ionic species bind to the same enzyme

form (Salerno and Giacomello, 1981). The enzyme is most active at magnesium concentrations ranging from 5-20 mM (Kelley and Wyngaarden, 1983). An activity versus substrate concentration plot exhibits hyperbolic kinetics with increasing PPRP concentration. However when the magnesium to PPRP ratio is very low, the curve is sigmoidal in character (Krenitsky et al., 1969).

The substrates bind in an orderly sequence, PPRP first and then the purine base, with the formation of a ternary complex, E:PPRP:purine. The products are released in a random order (Salerno and Giacomello, 1979). However, when the magnesium concentrations are relatively high (in large excess of the PPRP concentration), the predominant reaction sequence is a ping-pong mechanism involving an enzyme-phosphoribosyl intermediate (Krenitsky and Papaioannou, 1969).

The apparent K_m in human erythrocytes for guanine is 5×10^{-6} M, for hypoxanthine it is 1.7×10^{-5} M and for PPRP it is 2.5×10^{-4} M (McDonald and Kelley, 1971).

1.3 GENE STRUCTURE

The human HPRT enzyme is coded for by a single gene which resides on the X chromosome in the region Xq26-27 (Figure 7) (Pai et al., 1980).

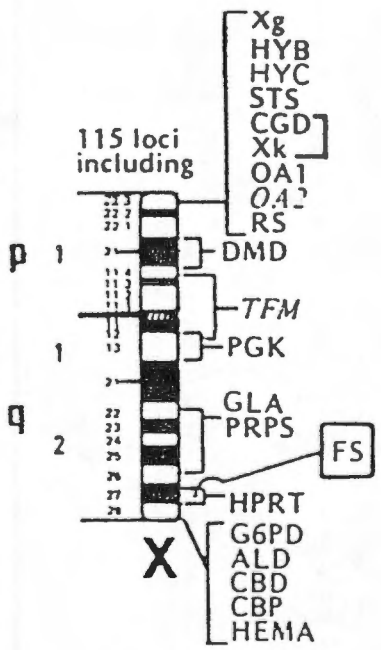


Figure 7 Gene map of the human X chromosome (McKusick, 1983)

The cDNA has been cloned and has been used to study the gene structure. Brennand et al. (1982) identified a mouse neuroblastoma cell line which produces 20-50 fold increased levels of an altered HPRT protein. They purified HPRT mRNA from this cell line and used it to synthesize HPRT cDNA. This cloned mouse cDNA was used to identify human and mouse HPRT cDNA recombinants (Patel and Caskey, 1985). Jolly et al. (1982) transfected HPRT$^-$ mouse cells with total human DNA and then isolated a genomic clone containing a portion of the HPRT gene. This clone was then used as a probe to isolate a full length cDNA clone encoding the human HPRT enzyme from a human cDNA library (Jolly et al., 1983). The inferred amino acid sequence was determined from the nucleic acid sequence of this cDNA and it was in complete agreement with the amino acid sequence of the protein determined by Wilson et al. (1982a).

The gene codes for a mRNA which is 1 600 bases in length (Lin et al., 1982) and has an open reading frame of 654 nucleotides. The 3' noncoding region terminates with a polyadenylated sequence. HPRT mRNA accounts for 0.01% of total mRNA (Melton et al., 1981). The human gene is approximately 42-44 kilobases in length and is composed of 9 exons and 8 introns (Kim et al., 1986 and Patel et al., 1986). The coding region thus accounts for less than 5% of the gene. The exons range in size from 18 to 637 nucleotides and the introns from 0.17 to 13.3 kilobases in length (Figure 8).

The splice junctions at the boundaries of each exon conform to the consensus sequences from other eukaryotic genes (Kim et al., 1986; Patel et al., 1986). Most eukaryotic genes which code for mRNAs

contain two regulatory sequences in their 5' flanking region, the TATA box and the CAAT sequence. The HPRT gene lacks both these sequences, but the 5' noncoding region is rich in guanine and cytosine residues (G+C rich) (approximately 80% in contrast to approximately 46% in the coding region and approximately 32% in the 3' untranslated region (Patel et al., 1986)) and contains several copies of the sequence 5'-CCGCCC-3'. The HPRT mRNAs have multiple initiation sites which are located about 104 to 169 base pairs upstream from the translation initiation codon. These features have also been found in other housekeeping genes (Patel et al., 1986). There are also two regions in the 5' flanking region that are homologous to the enhancer core sequence in the SV40 72 base pair repeats (Kim et al., 1986).
The HPRT gene is highly conserved with 95% homology between human and mouse genes in the 5' coding region. There are only 7 amino acids which differ and nearly all are the result of single nucleotide differences in the corresponding DNA sequences (Chinault and Caskey, 1984). The exon-intron junctions of the human gene are identical to those of the mouse gene, though the sizes of the introns differ (Figure 8), which results in the mouse gene being smaller than the human gene. The mouse gene is 34 kilobases in length (Melton et al., 1984a). However the relative sizes of the introns in these two genes are the same (Kim et al., 1986; Patel et al., 1986). There is 51% homology between the two genes in the 5' flanking region up to approximately 299 base pairs upstream from the translation initiation codon. There is 74% homology in the 3' untranslated region (Kim et al., 1986). The mouse gene also lacks the CAAT sequence and TATA box and like the human gene is G+C rich in the 5' flanking region (Melton et al., 1984a).

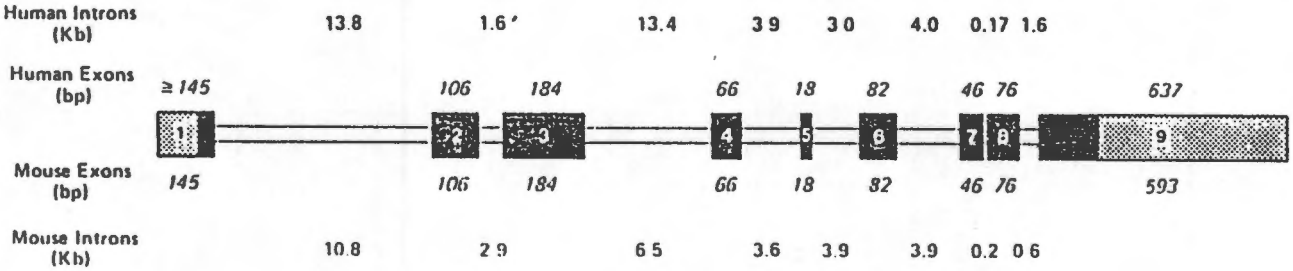


Figure 8 Comparison of the human and mouse HPRT genes

The black boxes represent the exons and the stippled boxes represent 5'- and 3'-untranslated regions of the gene

Kb: kilobases; bp: base pairs

(Stout and Caskey, 1985)

Four HPRT-like autosomal sequences have been identified in the human genome. One is located on chromosome 3, one on chromosome 5 in the region between 5p13 and 5q11 and two on chromosome 11. These sequences probably represent intronless "pseudogenes" which are not expressed (Patel et al., 1984).

At an early stage of embryonic development, one of the X chromosomes in female somatic cells is inactivated and forms a Barr body (Lyon, 1961). This inactivation event is random and either the paternally or maternally derived X chromosome can be inactivated. However once it occurs it is fixed for that cell and its progeny. There is evidence for the role of methylation of cytosine residues in DNA in the maintenance of X-chromosome inactivation (Gartler and Riggs, 1983). The methylation pattern of the HPRT gene has been studied. There is hypomethylation of 5' clustered regions of the gene on the active X chromosome relative to the inactive X chromosome (Wolf et al., 1984; Yen et al., 1984) and extensive methylation of the 3' sequence of the gene on the active X chromosome. This pattern is the same in all tissues, however, the inactive alleles have a variety of patterns (Wolf et al., 1984). Yen et al., 1984 thus suggested that the overall pattern of methylation is important in maintaining X inactivation.

1.4 HPRT DEFICIENCY

1.4.1 Clinical Features

A virtually complete deficiency of HPRT results in the Lesch-Nyhan syndrome (Seegmiller et al., 1967) which was first reported in two brothers who were 5 and 8 years old. They presented with mental retardation, cerebral palsy, choreoathetosis, self-mutilation and hyperuricemia (Lesch and Nyhan, 1964). The self-mutilation is a compulsive behaviour as the pain sensation is intact. The children are also aggressive towards other people (Nyhan, 1978). Patients with a partial enzyme defect have a severe form of gout, hyperuricemia and excessive purine synthesis (Kelley et al., 1967), but generally lack the neurological and behavioural features of the complete deficiency. Generally, there are less neurological symptoms as the residual HPRT activity increases. However, this correlation between enzyme activity and clinical characteristics does not always apply. Dancis et al. (1973); Emmerson et al. (1972) and Geerdink et al. (1973) all reported patients lacking neurological abnormalities, yet no or very low HPRT activities were detected in erythrocytes. Rijksen et al. (1981) described a Lesch-Nyhan patient with 5-10% activity in erythrocytes. Page et al.(1981) demonstrated a correlation between the severity of clinical symptoms and the amount of residual activity in intact fibroblasts. This could be due to the fact that the HPRT activity in intact cells may vary from the activity in erythrocyte lysates.

Watts (1985) proposed that the neurological symptoms of Lesch-Nyhan syndrome are due to an imbalance of the neurotransmitters. GTP is the

substrate for the rate limiting step of de novo pterin synthesis. Tetrahydrobiopterin acts as a cofactor for the mono-oxygenases involved in the production of norepinephrine, dopamine and serotonin. Thus a deficient HPRT would result in decreased GMP and thus GTP levels and thus would affect the production of the mentioned neurotransmitters. Low GTP levels were found only in patients with neurological involvement and not in patients with gout (Simmonds et al., in press) which thus provides support for the theory of Watts (1985).

1.4.2 Associated biochemical abnormalities

Children with the Lesch-Nyhan syndrome excrete 4 to 8 times the normal amount of uric acid based on body weight, in their urine. They also excrete an increased amount of 5-amino-4-imidazole carboxamide, a purine precursor. There is also an increase in the excretion of hypoxanthine relative to xanthine (Seegmiller, 1976).

APRT is increased in erythrocytes of patients with a complete deficiency of HPRT as well as in many patients with a partial deficiency. This is probably due to a stabilization of the APRT by the high concentration of PPRP, which may cause a decreased degradation rate and thus an increased APRT activity (Kelley and Wyngaarden, 1983).

Lommen et al. (1971) observed reduced concentrations of ATP, ADP and AMP in erythrocytes of Lesch-Nyhan patients, but the GTP concentration was observed to be normal. Because of this finding, the enzyme IMP dehydrogenase was studied. HPRT deficient patients have increased IMP dehydrogenase activities in erythrocytes only. This might be due to

insensitivity of this enzyme in HPRT deficient erythrocytes to inhibition by 2,3-diphosphoglycerate (Lommen et al., 1974).

Orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase catalyze the conversion of orotate to uridine 5'-phosphate. Both these enzyme activities are increased only in erythrocytes. There is no evidence for stabilization by PPRP or other dialysable factors (Beardmore et al., 1973).

There is an increased intracellular concentration of PPRP. This is probably due to decreased utilization of PPRP because of a defective HPRT. Because of this increased concentration of PPRP, there is more available as a substrate for PPRP amidotransferase, the rate limiting enzyme for de novo purine biosynthesis. This would cause an increase in synthesis of purines and, ultimately, in their breakdown to uric acid. A diminished salvage pathway and thus diminished replacement of nucleotides, which are feedback inhibitors of the de novo pathway, also cause an increased purine synthesis and ultimately an increased uric acid concentration (Figure 9) (Kredich and Hershfield, 1983).

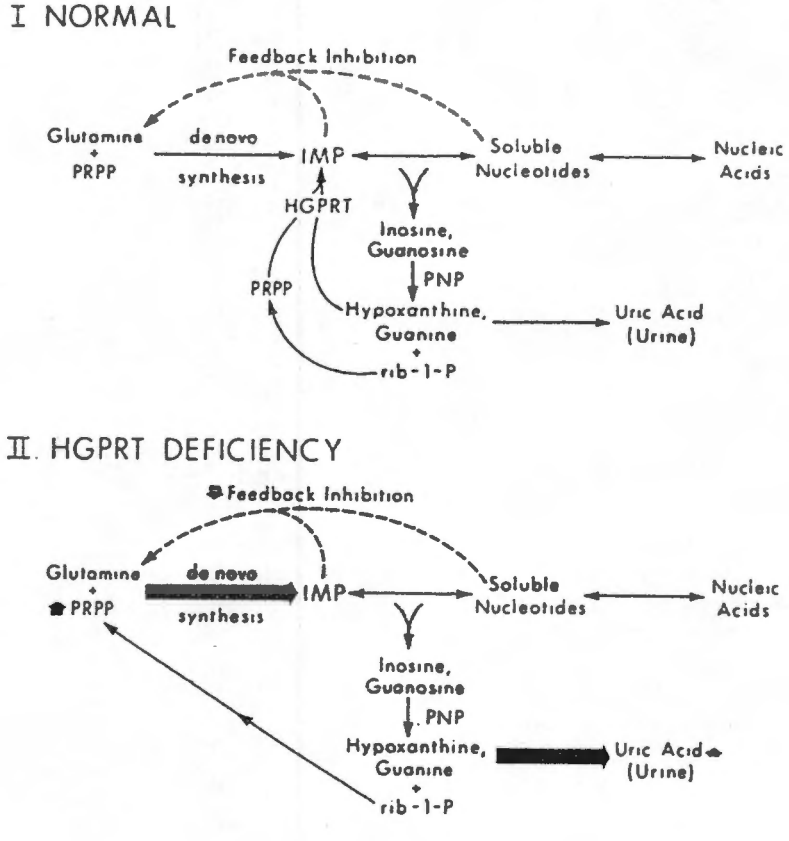


Figure 9 Diagrammatic representation of the factors influencing the cellular content of PPRP and uric acid in HPRT deficient patients compared with normal individuals
(Kredich and Hershfield, 1983)

1.4.3 Heterozygotes

Because the HPRT gene is X-linked, only hemizygous males exhibit symptoms of the deficiency, whereas females are carriers of the deficiency. However, because of the inactivation of one of the X chromosomes in females, they are functionally hemizygous. The inactivation is a random process, thus heterozygotes for HPRT deficiency should have a mixture of normal and mutant cells. This mosaicism has been observed in fibroblasts (Migeon et al., 1968) and hair follicles (Gartler et al., 1971) of heterozygotes and is used for diagnosis of heterozygotes for the Lesch-Nyhan syndrome. However, erythrocytes appear to be normal (Nyhan et al., 1970; Emmerson et al., 1972; McDonald and Kelley, 1972; Johnson et al., 1976). In contrast, heterozygotes of the partial deficiency have two populations of cells in erythrocytes (Bakay et al., 1972; Emmerson et al., 1972; Fox et al., 1976). The absence of mutant HPRT cells in erythrocytes of Lesch-Nyhan heterozygotes is due, either to an inactivation of the X chromosome that is not random, or selection against the mutant cells after random inactivation (Nyhan et al., 1970). However, it is not always true that heterozygotes for the partial deficiencies exhibit reduced HPRT activity in erythrocytes. Snyder et al. (1984) reported a family where four related males had partial HPRT activity but lacked severe neurological symptoms, yet the obligate heterozygotes had normal HPRT activity in their erythrocytes. It has also been shown that a heterozygote for the Lesch-Nyhan syndrome exhibited intermediate activity in erythrocytes (Shaltiel et al., 1981).

1.5 HPRT MUTANTS

Lesch-Nyhan patients cannot reproduce and the heterozygotes have no apparent selective advantage (Wilson et al., 1983a). Haldane's (1935) principle predicts that in order for this syndrome to be maintained in the population, new mutations must occur frequently at the HPRT locus.

Many HPRT deficient patients have been described and they all have different mutations. Mutants have been described that differ with respect to isoelectric points (Wilson et al., 1982b; Wilson et al., 1983b; Wilson and Kelley, 1983; Wilson and Kelley, 1984), electrophoretic mobilities (Bakay and Nyhan, 1972; Bakay et al., 1972; Sweetman et al., 1978; Wilson et al., 1982b; Wilson et al., 1983b; Wilson and Kelley, 1983; Wilson and Kelley, 1984; Wilson et al., 1986), thermolability (Kelley and Meade, 1971; Dancis et al., 1973; Rijksen et al., 1981; Wilson et al., 1982b; Snyder et al., 1984), in vivo stability (Arnold et al., 1972), altered sensitivity to inhibition by GMP (Kelley and Meade, 1971; Fox et al., 1975; Gutensohn and Jahn, 1979; Wilson et al., 1983b), decreased enzyme concentration (Wilson et al., 1983b; Wilson et al., 1983c), decreased maximal velocity (Wilson and Kelley, 1984) and altered Michaelis constants (McDonald and Kelley, 1971; Bakay et al., 1972; Benke et al., 1973; Sweetman et al., 1978; Page et al., 1982; Wilson et al., 1983c; Wilson and Kelley, 1984; Wilson et al., 1986).

Many of the variant enzymes have been purified and the structural and catalytic characteristics have been studied. Because each of the variants exhibited a unique set of structural and catalytic

characteristics, Wilson et al. (1983a) have identified each variant with the city in which the patient with the defect lived. Seven variants have so far been characterized (Table 1). Wilson et al., 1986 have identified a patient with an enzyme variant which appears identical to $HPRT_{London}$ even though the two patients share no apparent common heritage. Tryptic peptides of four of these variants have been mapped and sequence analysis performed on aberrant peptides in order to define the precise abnormality in the primary structure of the enzyme. All four variants have single amino acid substitutions which can be explained by a single nucleotide change (Wilson and Kelley, 1983; Wilson et al., 1983b; Wilson et al., 1983c; Wilson and Kelley, 1984) (Table 2).

Yang et al., 1984 studied restriction fragments of DNA from five Lesch-Nyhan patients. They all had different, major gene alterations (Figure 10).

All these mutants are evidence for the genetic heterogeneity in HPRT variants and thus confirm Haldane's principle.

Mutant enzyme	Clinical Presentation	Specific activity (% of control)	Immunoreactive protein (% of control)	Altered kinetic properties	Electrophoretic properties	Isoelectric point	Other altered enzyme properties	mRNA	References
$HPRT_{Toronto}$	Gout	33	52	normal	anodal	acidic	decreased intracellular concentration increased sensitivity to product inhibition by GMP	+	2, 4, 6
$HPRT_{London}$	Gout	59	35	5 fold increase in K_m for hypoxanthine	normal	normal	decreased intracellular concentration	+	4, 7
$HPRT_{Munich}$	Gout	3	79	20 fold decreased V_{max} 108 fold increased K_m for hypoxanthine	cathodal	basic	decreased sensitivity to product inhibition by GMP	+	3, 4, 8
$HPRT_{Ann\ Arbor}$	Gout	10	11	increased K_m for hypoxanthine and PPRP	cathodal	basic	more labile to heat inactivation	+	1, 4, 5
$HPRT_{Kinston}$	Lesch-Nyhan	0.3	72	increased K_m for hypoxanthine, guanine and PPRP	normal	acidic	more labile to heat inactivation	+	4, 9
$HPRT_{Yale}$	Lesch-Nyhan	<0.7	92	not measured	cathodal			+	9
$HPRT_{New\ Haven}$	Lesch-Nyhan	<0.7	50	not measured	anodal			+	9

References

1. McDonald and Kelley, 1971
2. Fox et al., 1975
3. Gutensohn and Jahn, 1979
4. Wilson et al., 1982b
5. Wilson and Kelley, 1983
6. Wilson et al., 1983b
7. Wilson et al., 1983c
8. Wilson and Kelley, 1984
9. Wilson et al., 1986

Table 1 Heterogeneity of HPRT mutants

Mutant enzyme	Clinical presentation	Mutation		Reference
		amino acid change	position	
$HPRT_{Toronto}$	Gout	arginine to glycine	50	Wilson et al., 1983b
$HPRT_{London}$	Gout	serine to leucine	109	Wilson et al., 1983c
$HPRT_{Munich}$	Gout	serine to arginine	103	Wilson and Kelley, 1984
$HPRT_{Kinston}$	Lesch-Nyhan	aspartic acid to asparagine	193	Wilson and Kelley, 1983

Table 2 Amino acid substitutions in HPRT variants

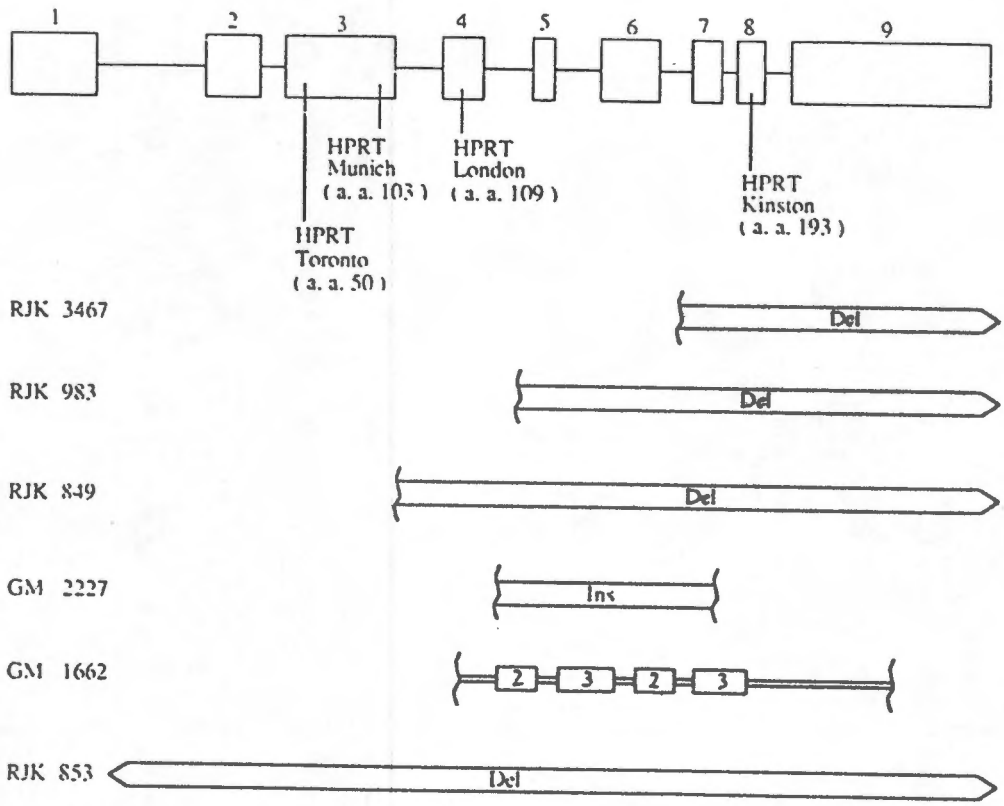


Figure 10 Human HPRT mutants

Point mutations are indicated within the respective exons. Major gene alterations are indicated by del (deletion) and ins (insertion). GM 1662 is an endoduplication mutant.
(Caskey, 1987)

1.6 $\underline{HPRT}_{Cape\ Town}$

The proband (T.K.) presented with ureteric colic, arthritis, mental retardation and very mild neurological abnormalities. Purine salvage was studied in fibroblast cultures and the proband was found to have 10% of control HPRT activity (Cassidy et al., 1980). Detailed studies were performed on the kinetics of the enzyme from erythrocyte haemolysates. The low HPRT activity was not associated with changes in the K_m or V_{max} for any of the enzyme substrates. However, the enzyme exhibited low activity at high concentrations of the purine substrates, hypoxanthine and guanine, but not with PPRP. The type of inhibition was found to be uncompetitive substrate inhibition. The variant enzyme exhibited an ordered sequential reaction mechanism with the inhibitory substrate binding after the non-inhibitory substrate and forming a dead-end complex with an enzyme-pyrophosphate intermediate. This traps the enzyme in an inactive form (Figure 11) (Steyn and Harley, 1984).

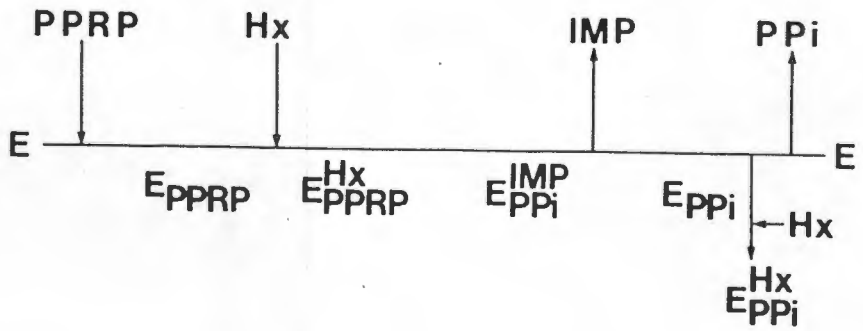


Figure 11 Proposed reaction mechanism for $HPRT_{Cape\ Town}$ at high concentrations of hypoxanthine (Steyn and Harley, 1984)

Because this enzyme represents a different HPRT variant, it was named $HPRT_{Cape\ Town}$, after the city in which the proband lives.

The proband's lymphoblasts exhibited growth patterns similar to those of $HPRT^-$ cells i.e. growth in thioguanine-containing medium, but lack of growth in HAT medium or MAGAT medium (mycophenolic acid, adenine, guanine, amethopterin and thymidine (Steyn and Harley, 1985)) indicating that the cells were deficient in hypoxanthine and guanine salvage. Purine incorporation was measured to be less than 10% of controls, which is in agreement with the proposal that there are less neurological abnormalities as the residual enzyme activity increases. $HPRT_{Cape\ Town}$ cells exhibited 8 fold higher de novo purine synthesis than control cells. The intracellular concentration of guanine (44-150 µmol/l) (Harkness, unpublished results) was in excess of the $K_{i(app)}$ for this substrate (28 µmol/l) which implies that the low intracellular activity is due to substrate inhibition by guanine (Steyn and Harley, 1985).

Wohlhueter (1975) reported enzyme kinetics features which could be interpreted as substrate inhibition in normal rat liver HPRT, whereas the enzyme from rat hepatoma cells exhibits normal hyperbolic kinetics. This implies that the altered HPRT activity in rat liver is a secondary phenomenon. It was not certain whether the substrate inhibition in $HPRT_{Cape\ Town}$ is a consequence of a mutation in the gene coding for the enzyme, a mutation resulting in altered post-translational modification or even the absence or alteration of a factor influencing normal HPRT kinetics.

In order to determine whether the substrate inhibition exhibited by $HPRT_{Cape\ Town}$ is confined to erythrocytes, or is a more generally expressed phenomenon in other cell types, the kinetics of enzyme preparations from cell free extracts of Epstein-Barr virus transformed lymphoblasts were studied.

The kinetics of the enzyme in erythrocyte lysates and cell free extracts of lymphoblasts from the proband's daughter (B.K.) were studied in order to determine the type of mutation.

It is known that the enzyme is labile in the partially purified form (Steyn and Harley, 1984), thus a full characterization of the defect by purification to homogeneity would not be feasible. The defect was thus studied at the gene level. Restriction fragments of the proband's DNA were compared with a normal control. The RNA was studied by Northern blotting and S1 nuclease analysis. Finally, an attempt was made to clone and sequence the mutant cDNA to define the precise nature of the defect.

2.0 BIOCHEMICAL PROPERTIES

2.1 INTRODUCTION

HPRT is coded by a gene that resides on the X chromosome (Pai et al., 1980). The proband's daughter was therefore studied in order to resolve the type of mutation causing the defect in $HPRT_{Cape\ Town}$. If the defect is in the gene coding for the enzyme, the daughter would exhibit the characteristics of a heterozygote. However, if the defect was due to the absence or alteration of a cofactor for HPRT, this would not necessarily be coded for on the X chromosome and therefore, the daughter would not be an obligate heterozygote.

The proband's daughter's enzyme was studied in two ways. Firstly, the kinetics of the enzyme from erythrocyte lysates and cell-free extracts of EBV-transformed lymphoblasts were studied. Secondly, the growth of transformed lymphoblasts in selective media was studied.

The enzyme kinetics were studied using a radiochemical method with [8-^{14}C] hypoxanthine as the enzyme substrate. The product, IMP, was separated from unreacted substrate by PEI-cellulose thin layer chromatography. Aliquots were taken at defined time intervals (within the linear part of the product versus time reaction) and the data fitted to the equation for a straight line. The rate of formation of the product was determined from the slope of the line.

The growth of transformed lymphoblasts was studied in two selective media, HAT and 6-thioguanine. HAT contains hypoxanthine, aminopterin and thymidine. Aminopterin inhibits the tetrahydrofolate requiring steps of de novo purine biosynthesis as well as the addition of the

5-methyl group in thymidine synthesis (De Mars, 1971). The cells are therefore reliant on the salvage of hypoxanthine by HPRT and thymidine by thymidine kinase for their supply of purines and pyrimidines respectively. Cells which are deficient in HPRT activity cannot salvage hypoxanthine and are thus unable to proliferate in HAT medium.

6-thioguanine, a toxic analogue of guanine, is a substrate of HPRT. It is converted to its toxic nucleotide form by HPRT activity, therefore cells which are HPRT deficient will be resistant to its cytotoxic effect (Seegmiller, 1976).

The kinetics of enzyme preparations from cell-free extracts of EBV-transformed lymphoblasts were studied to determine whether the substrate inhibition was confined to erythrocytes or whether the substrate inhibition was a more generally expressed phenomenon.

The proband will be referred to as T.K. and his daughter as B.K.

2.2 RESULTS AND DISCUSSION

2.2.1 Enzyme kinetics in erythrocyte lysates

The effect of hypoxanthine concentration on the HPRT activity in control, T.K. and B.K.'s erythrocyte lysates is shown in Figure 12. The data was fitted to the appropriate rate equations using the reiterative method of Wilkinson (1961). The data for T.K. was fitted to equation 1, which includes a term for substrate inhibition. Control data was fitted to equation 2, the equation for hyperbolic kinetics.

$$V = \frac{V_{max}.S}{K_m + S + S^2/K_i} \qquad (1)$$

$$V = \frac{V_{max}.S}{K_m + S} \qquad (2)$$

where V is the initial velocity, V_{max} the maximum velocity, S the substrate concentration, K_m the Michaelis constant and K_i the dissociation constant of the second substrate molecule.

The control enzyme exhibited normal hyperbolic dependence on the substrate concentration. T.K.'s enzyme exhibited substrate inhibition as previously described by Steyn and Harley (1984). The data for B.K. did not converge with either equation and therefore the data points were connected with straight lines. B.K.'s enzyme exhibited

intermediate enzyme activity; i.e. activity between that of the control and the proband. A computer simulation was therefore generated for the substrate velocity relationship (equation 3) which would be predicted to exist for a heterozygote possessing a mixture of equal proportions of an enzyme with normal kinetic parameters and an enzyme with similar K_m and V_{max} values but with a K_i value of 80 µmoles for hypoxanthine. This is the value which was determined for T.K. (Steyn and Harley, 1984).

$$V = \left(\frac{V_{max}.S}{K_m + S} + \frac{V_{max}.S}{K_m + S + S^2/K_i} \right) \Big/ 2 \qquad (3)$$

The computer simulated plot, which is depicted in Figure 13, closely resembles the plot constructed from B.K.'s enzyme kinetic data.

This finding is consistent with the study of Emmerson et al. (1972) who demonstrated that heterozygotes for a partial deficiency of HPRT exhibited HPRT activities in erythrocyte haemolysates that ranged between 22% and 75% of normal values.

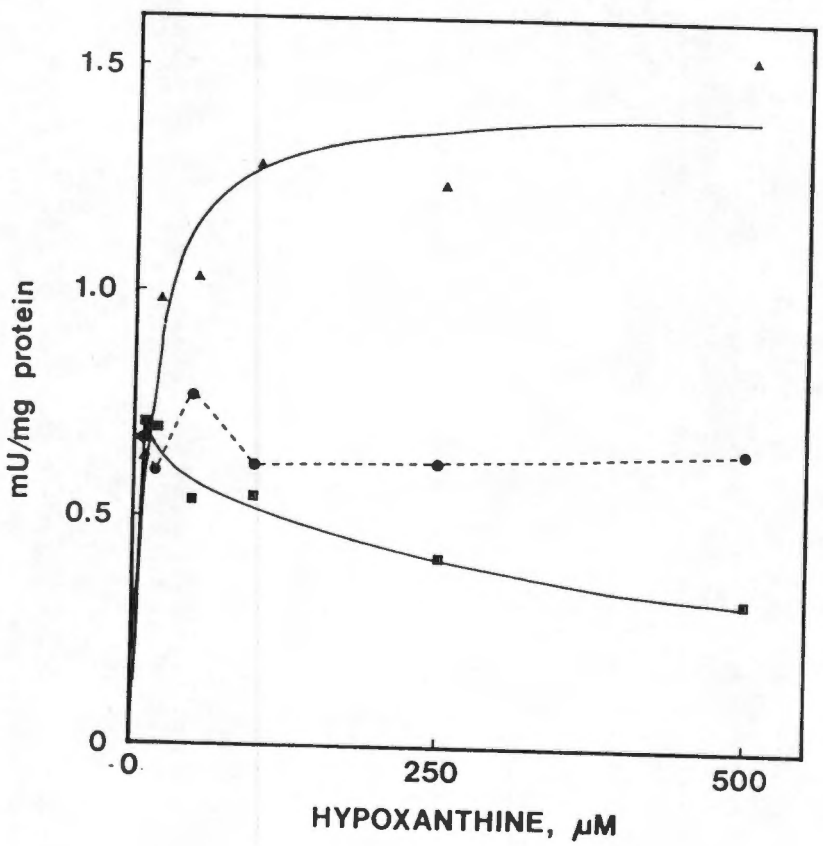
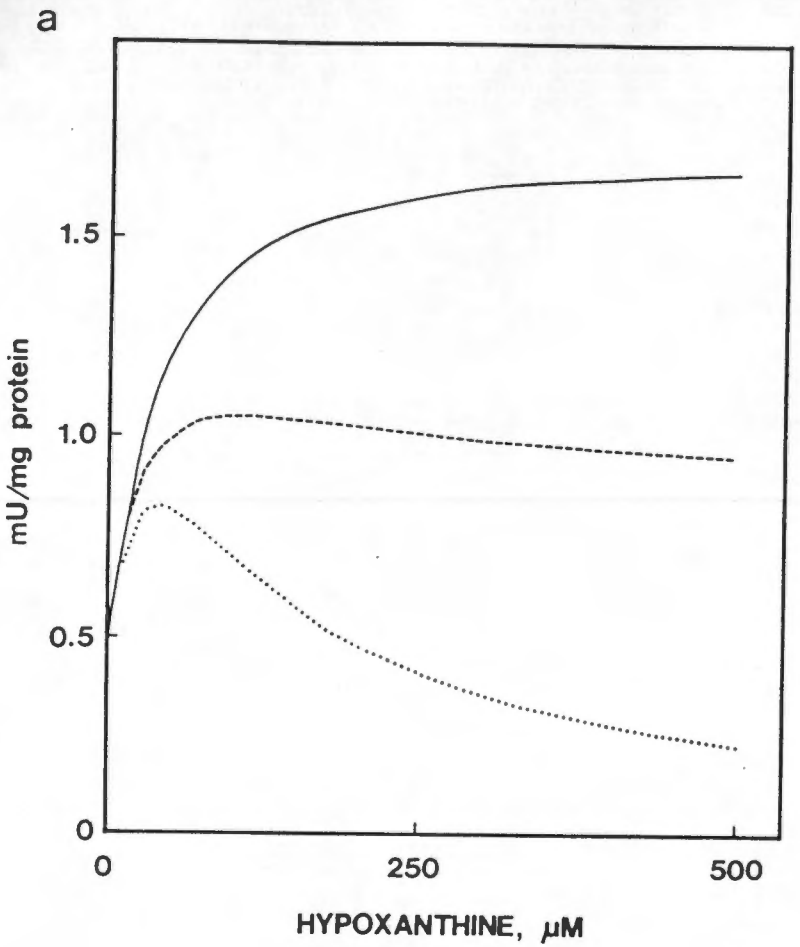


Figure 12 Effect of substrate concentration on the activity of control and variant enzyme prepared from erythrocytes
▲ control; ■ T.K.; ● B.K.



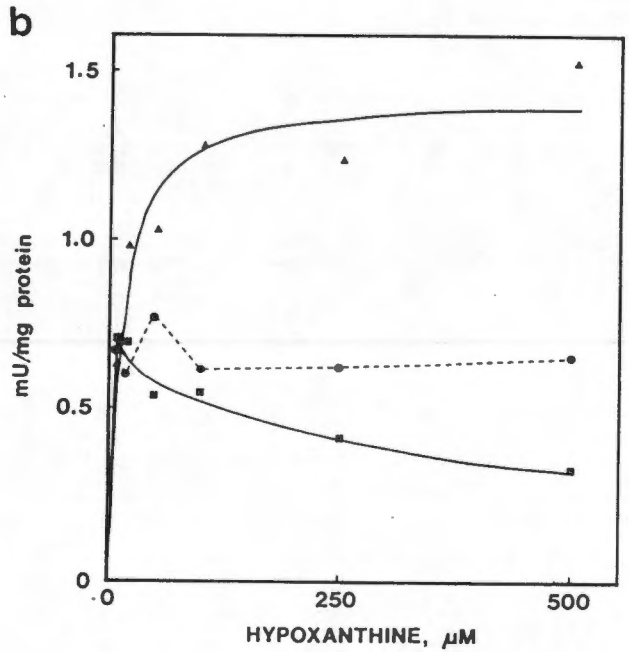


Figure 13a Computer Simulation Curves
—— control; · · · substrate inhibition; - - - simulation of heterozygote situation assuming equal proportions of normal and variant enzyme

Figure 13b Replica of Figure 12 shown in comparison to the computer simulation curves

2.2.2 Selective media experiments

The effect of selective agents on the growth of lymphoblasts from a normal individual, T.K., B.K. and a Lesch-Nyhan lymphoblast line, GM 1899, from the NIGMS Human Genetic Mutant Cell Repository, was studied.

The effect of HAT medium is shown in Figure 14. The control cells proliferated in this medium, whereas the Lesch-Nyhan cells were unable to survive. T.K. and B.K.'s cells were also unable to survive in the presence of this medium. All the cell lines proliferated in the absence of this selective agent. The growth patterns observed were therefore due to the effect of the selective agent and not a characteristic of the cell line being studied.

This result implies that T.K. and B.K.'s cells are deficient in hypoxanthine salvage.

The effect of 6-thioguanine is shown in Figure 15. The control cells were unable to proliferate, whereas the Lesch-Nyhan cells continued to proliferate and were therefore immune to the cytotoxic effects of 6-thioguanine. T.K. and B.K.'s cells were initially able to proliferate, and then growth of the cells slowed down. This can be attributed to the residual HPRT activity present in $HPRT_{Cape\ Town}$ which is probably sufficient for thioguanine to be salvaged to the extent that it starts exerting its cytotoxic effects.

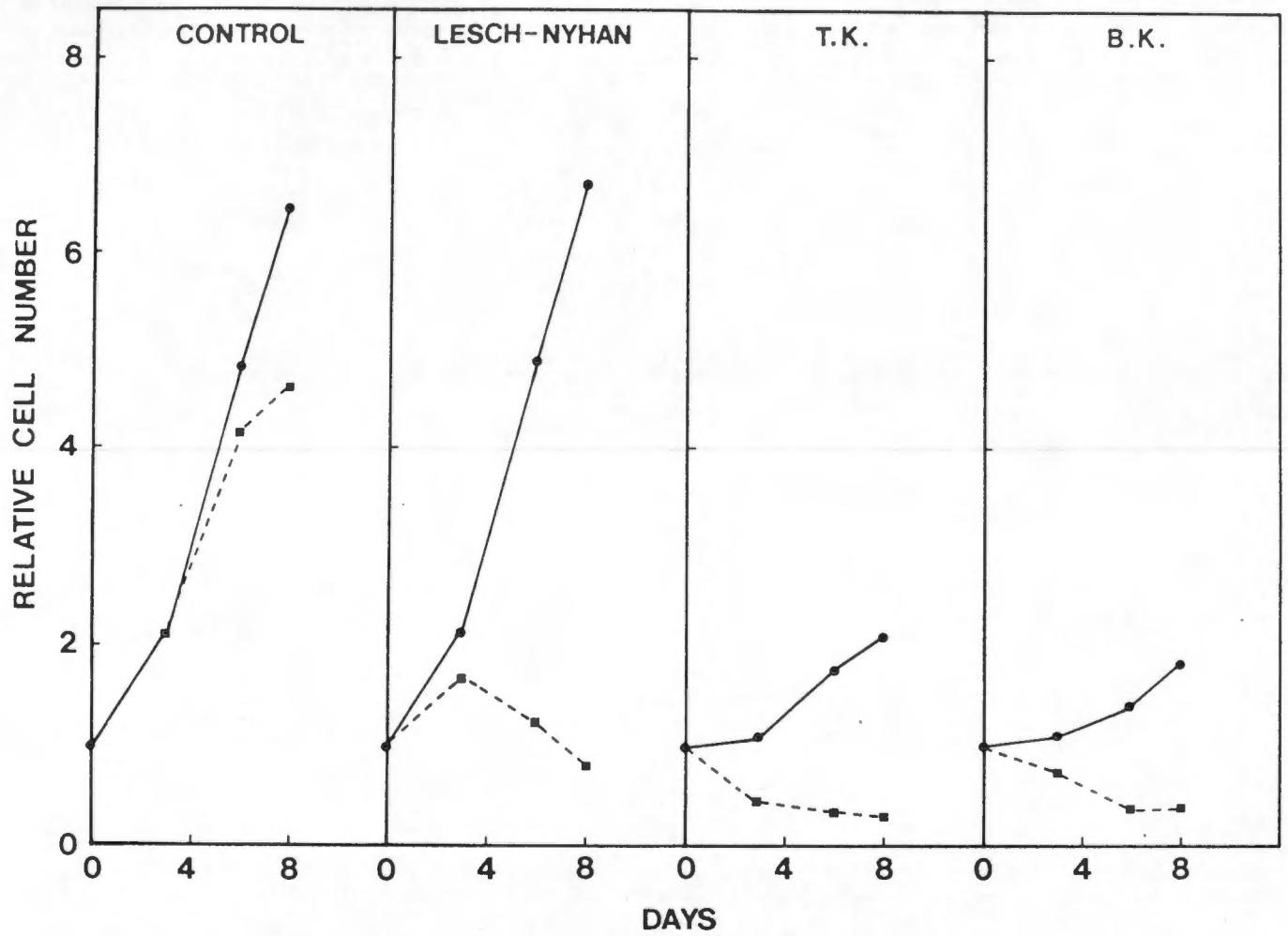


Figure 14 Cell growth in HAT medium

Transformed lymphoblasts were cultured in medium containing
● no additions; ■ HAT medium
Each point represents the mean value from duplicate flasks of cells

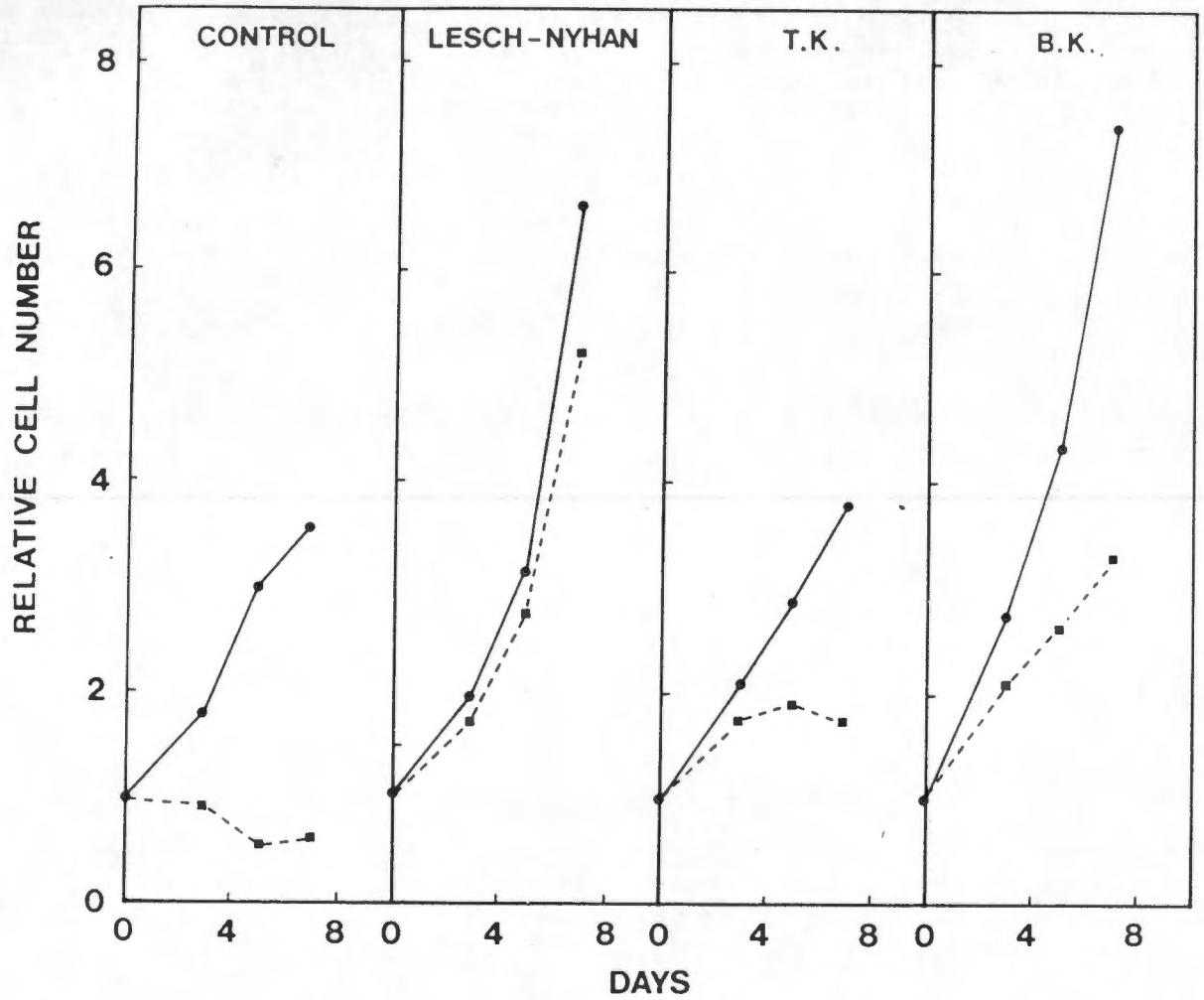


Figure 15 Cell growth in 6-thioguanine-containing medium

Duplicate flasks of transformed lymphoblasts were cultured in medium containing
● no additions; ■ 6-thioguanine

Photographs of the growth of T.K.'s cells in the absence of a selective agent and in the presence of HAT medium are shown in Figure 16. These provide a visual demonstration of the absence of growth in the presence of HAT medium. These cells lack the characteristic projections observed in normal, healthy cells which can be seen in the photograph of T.K.'s cells grown in the absence of a selective agent.

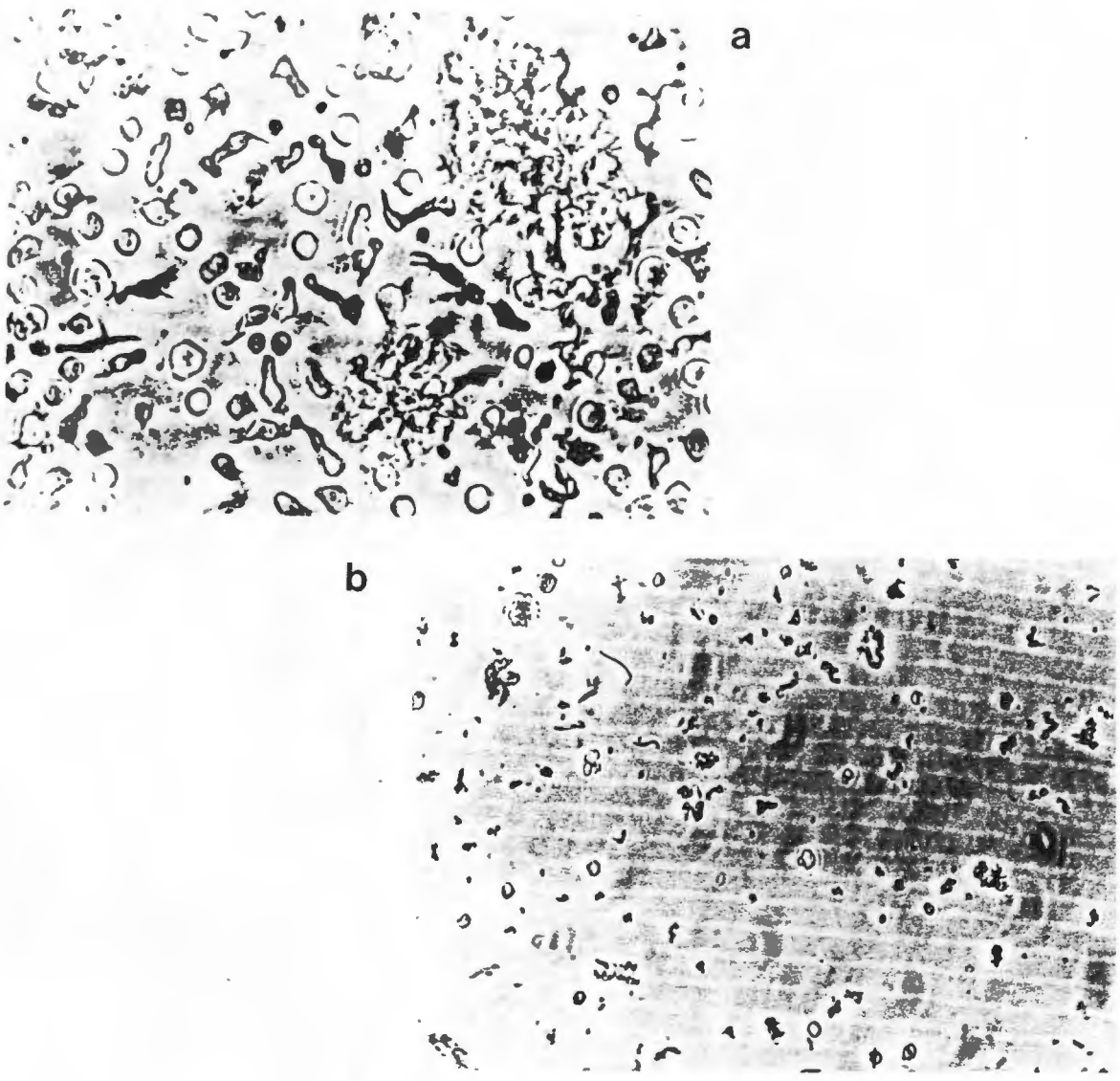


Figure 16 Photographs of the growth of T.K.'s cells in
a) medium without the addition of a selective agent
b) HAT medium

The cells observed are lymphoblasts which grow in suspension as individual cells or in colonies.

There are two interpretations for the observation that B.K.'s cells exhibited growth characteristics in selective media which resemble those of her father's cells. Firstly, because B.K.'s cells are expressing predominantly the variant enzyme, it is possible that this cell line represents a clone, or clones, which have suppressed the function of the X chromosome carrying the maternal, and presumably normal HPRT allele. This is not an unusual phenomenon in cell lines from patients heterozygous for X-linked mutations (Booth and Nadler, 1974).

A second interpretation is that B.K. has inherited an autosomal dominant or X-linked gene from her father which produces a factor which modifies normal HPRT activity. B.K. would therefore demonstrate the characteristics of a partial HPRT deficient enzyme. Ogasawara et al. (1984) reported a case of a female with the Lesch-Nyhan syndrome, thus suggesting a defect separate from a defective gene coding for the HPRT enzyme.

However, these selective media results should not be studied in isolation, but interpreted together with the erythrocyte enzyme reults. These results suggest that B.K. is a heterozygote and because erythrocytes are a more representative population of cells than transformed lymphoblasts, it is most likely that the first interpretation is correct and that the defect is probably in the HPRT gene itself. However, there is still a remote possibility that the substrate inhibition is due to a dominantly inherited, or X-linked, mutant gene constitutively producing a factor influencing HPRT activity.

2.2.3 Enzyme kinetics in cell-free extracts of lymphoblasts

The effect of hypoxanthine concentration on HPRT activity in dialysed lymphoblast extracts is shown in Figure 17. The lymphoblast extracts were dialysed prior to assaying the enzyme activity. This was necessary in the proband in order to remove the inhibitory substrates which were present in the lymphoblasts. If the extracts were not dialysed, then the enzyme activity, at even the lowest substrate concentration, would be very low due to the intracellular, inhibitory substrates.

The control enzyme exhibits hyperbolic dependence on the substrate concentration. The enzyme activity from both T.K. and B.K.'s extracts exhibited substrate inhibition. The data was fitted to the appropriate rate equations, but the data for B.K. did not fit either equation. The data points have therfore been connected by straight lines.

The substrate inhibition exhibited by T.K. shows that this phenomenon is not confined to erythrocytes, but is a more generally expressed phenomenon. The proband's clinical phenotype is therefore compatible with decreased HPRT activity, with physiological levels of guanine (and hypoxanthine) causing substrate inhibition.

Cell-free extracts from B.K. exhibited substrate inhibition of HPRT that resembled that of her father. The likely explanation for this is that the lymphoblast cell line is lyonised in favour of the mutant HPRT.

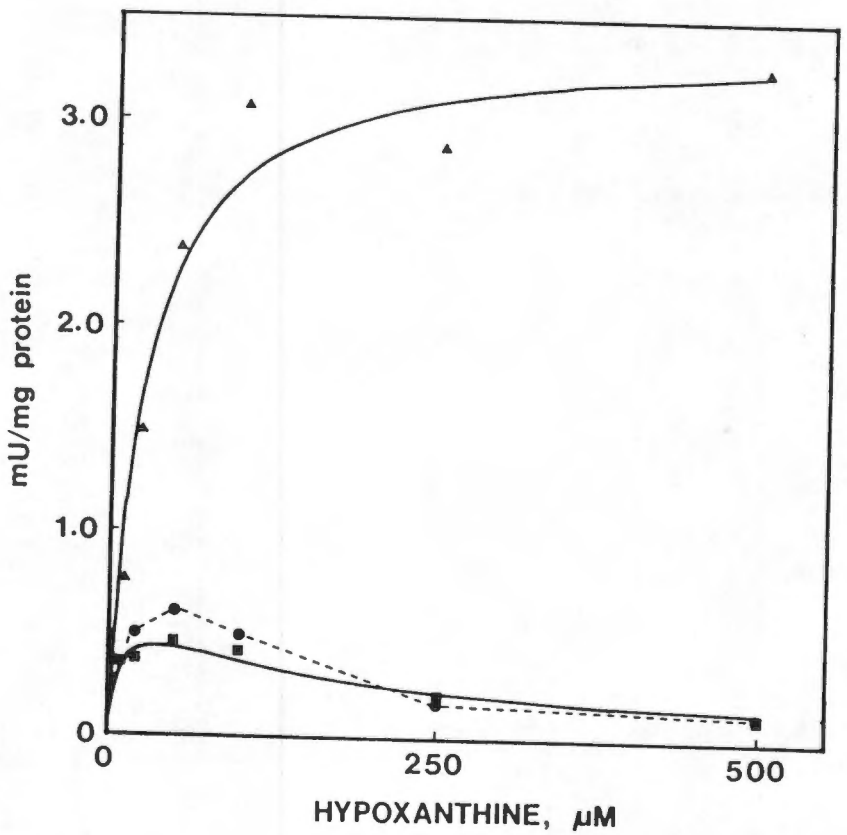


Figure 17 Effect of hypoxanthine concentration on the activity of control and variant enzyme prepared from transformed lymphoblasts

▲ control; ■ T.K.; ● B.K.

3.0 GENETIC PROPERTIES

3.1 INTRODUCTION

It is known that the mutant enzyme is labile in the partially purified form (Steyn and Harley, 1984), thus the defect could not be characterized by purification of the enzyme to homogeneity. The defect, was therefore studied at the gene level.

The proband's DNA was studied by Southern blotting analysis. Genomic DNA was digested to completion with various restriction enzymes and fractionated in agarose gels. The fragments were transferred to Hybond-N membrane and hybridized to a ^{32}P-labelled full-length HPRT cDNA probe. The patterns were compared to those of a control in order to determine whether there were any major gene alterations (insertions or deletions). It is also a remote possibility that a restriction site might have been lost or gained, which would indicate the presence of a single base substitution.

The proband's RNA was studied by two approaches. Firstly, the RNA was studied by Northern blotting to determine whether the length of the message was normal.

Secondly, the RNA was studied by transcription mapping. Approximately 83% of Lesch-Nyhan patients, whose DNA was studied by Southern blotting, appeared normal. This suggests that the mutations probably result from single base mutations (Yang et al., 1984). This has been shown in four variants where tryptic peptides have been mapped and the aberrant peptides sequenced (Wilson and Kelley, 1983; Wilson et al., 1983b; Wilson et al., 1983c; Wilson and Kelley, 1984).

Ribonuclease A is able to recognize and cleave some single-base mismatches in RNA heteroduplexes (Winter et al., 1985) and in RNA:DNA hybrids (Myers et al., 1985), whereas very few mismatches are cleaved by S1 nuclease in DNA:DNA hybrids. An antisense RNA probe, generated from HPRT cDNA cloned into in vitro transcription vectors, is required for this assay. The HPRT cDNA was therefore subcloned into a pGEM-3 vector which contains RNA polymerase promoters. The transcribed RNA was then hybridized to the proband's RNA and digested with S1 nuclease. The resulting fragments were analyzed by electrophoresis and compared with fragments produced by hybridization of the probe to control RNA.

Finally, an attempt was made to prepare cDNA from the proband's RNA and to clone this cDNA into a vector, with the intention of sequencing the cDNA and comparing it to the known sequence of normal HPRT cDNA.

A bacteriophage vector (lambda gt10) was chosen to take advantage of the high efficiency and reproducibility of in vitro packaging as a method of introducing DNA sequences into E.coli. This is especially important when cloning cDNAs of rare messages. HPRT mRNA represents 0.01% of the mRNA population (Melton et al., 1981). Lambda gt10 contains a single Eco RI site within the phage repressor gene (cI). This site is used as the insertion site of the cDNA. The cI protein plays a role in determining whether the bacteriophage enters the lytic pathway which results in the lysis of the host cell, or whether the bacteriophage enters the lysogenic pathway where the lambda DNA is inserted into the host genome and is stably maintained (Echols, 1986). The parental lambda gt10 favours the lysogenic pathway and produces turbid plaques on wild type hosts. The recombinant phages, which

therefore have an inactive cI gene, favour the lytic pathway and produce clear plaques. This provides a visual screen for recombinant phages, and lambda gt10 is therefore referred to as an immunity insertion vector. When E.coli strains with a high frequency lysogeny mutation, are infected with parental lambda gt10, the cI^+ phage is repressed so that plaque formation is suppressed. However, cI^- phage are able to form plaques. This host therefore provides a selection against non-recombinant phage, and therefore screening of the library for a specific insert is easier. This host is referred to as a selective host (Huynh et al., 1985).

3.2 RESULTS AND DISCUSSION

3.2.1 Southern analysis of the mutant DNA

Genomic DNA was extracted from T.K.'s blood and from EBV-transformed lymphoblasts. The DNA was digested to completion with various restriction enzymes and fractionated on 0.8% agarose gels. The fragmented DNA was transferred to Hybond-N membrane and hybridized to a ^{32}P-labelled HPRT full-length HPRT cDNA probe.

No variation in the banding pattern was observed between T.K. and control DNA when digested with Eco RI (Figure 18).

Control DNA, T.K. DNA and B.K. DNA, extracted from EBV-transformed lymphoblasts, were digested with restriction enzymes Hind III and Msp I. No variation was observed in either of the banding patterns (Figure 19). The Bam HI banding pattern is shown in Figure 20. The faint arrowed band is only visible in T.K. The relevance of this is unclear. If this represented a gene alteration, it would be expected to be reflected in the digests with the other enzymes. Overall, these results suggest that there are no major gene alterations (insertions or deletions). This result is to be expected since the proband has a partial HPRT deficiency. Generally, patients who lack the neurological abnormalities that are associated with the Lesch-Nyhan syndrome, have mutations that have less deleterious effects on the function of the enzyme in vivo e.g. amino acid substitutions that affect enzyme function but not enzyme concentration (Wilson et al., 1986).

Figure 18 Autoradiograph of a Southern blot of control and T.K. DNA digested with Eco RI and hybridized to a ^{32}P-labelled HPRT cDNA probe

lane a: DNA from control; lane b: DNA from T.K.

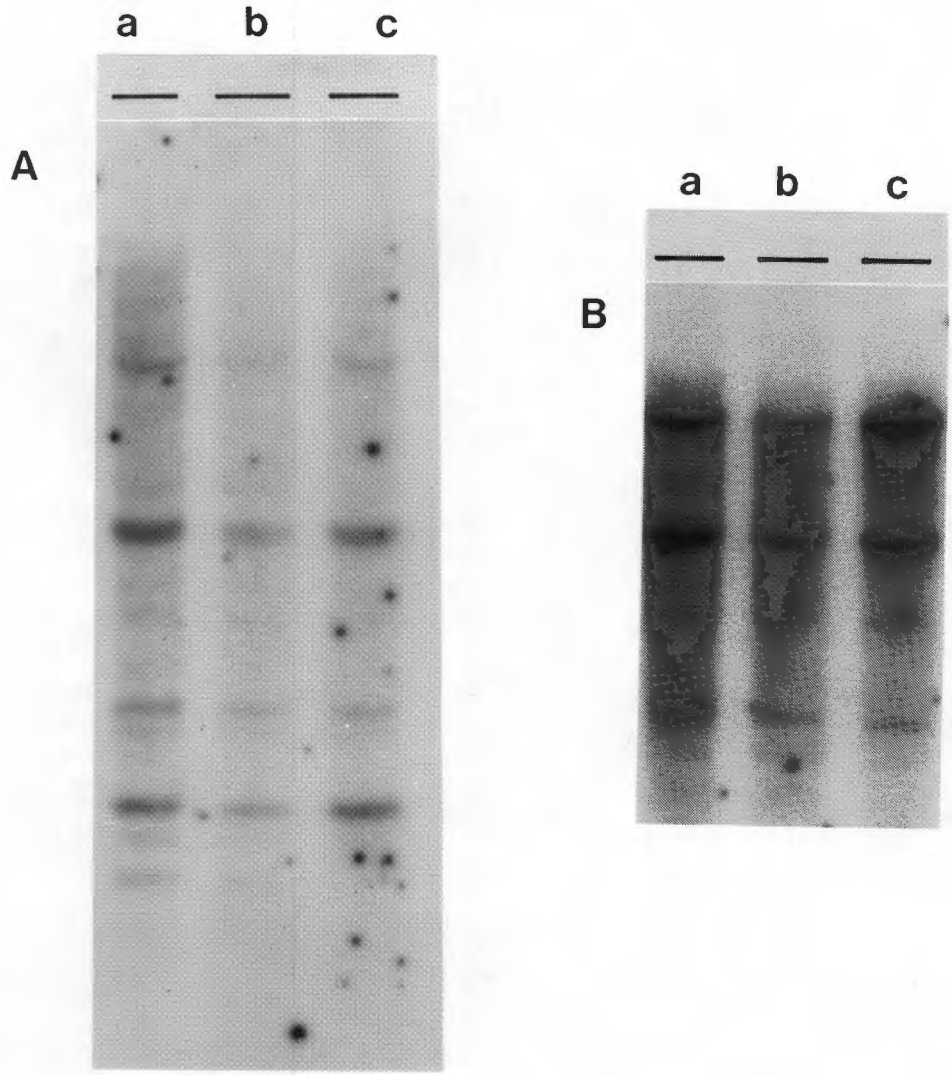


Figure 19 Autoradiograph of a Southern blot of control, T.K. and B.K. DNA digested with Msp I (A) and Hind III (B) and hybridized to a ^{32}P-labelled HPRT cDNA probe

lane a: DNA from T.K.; lane b: DNA from control;
lane c: DNA from B.K.

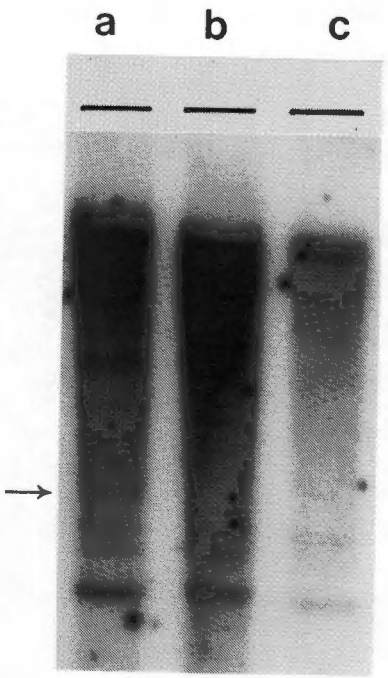


Figure 20 Autoradiograph of a Southern blot of control, T.K. and B.K. DNA digested with Bam HI and hybridized to a ^{32}P-labelled HPRT cDNA probe

lane a: DNA from T.K.; lane b: DNA from control;
lane c: DNA from B.K.

These Southern blotting results do not exclude the possibility that the mutation is the result of a single base substitution. A single base substitution would only be detected by Southern blotting analysis if the base change created or destroyed a restriction enzyme recognition site. Of the four HPRT mutants that have been shown to have single amino acid changes that are the result of single nucleotide changes (Table 2), only one of them occurs within a restriction enzyme recognition site. The HPRT deficiency in $HPRT_{Toronto}$ is caused by a single nucleotide change at the position coding for arginine-50. This change destroys a Taq I recognition site and can be detected by Southern blotting analysis (Wilson et al., 1983d).

3.2.2 Northern analysis of the mutant RNA

RNA was extracted from T.K., B.K. and control lymphoblasts. The RNA was electrophoresed on denaturing agarose gels and blotted onto Hybond-N membrane. The immobilised RNA was hybridized to ^{32}P-labelled full-length HPRT cDNA. The plasmid, containing the HPRT cDNA insert, was digested with the restriction enzymes Msp I and Rsa I, which digest the DNA 5' and 3', respectively, to the HPRT coding region (Figure 21). Thus only the HPRT insert, without any adjoining vector DNA, was used as a probe, in order to prevent cross-hybridization.

No difference was observed in the size of the HPRT mRNA (Figure 22). This result confirms the Southern blotting result; that there are no major gene alterations. It also implies that there is no mutation in the transcription of the gene. The result, again does not exclude the possibility of a single base change, which would not be detected by Northern blotting.

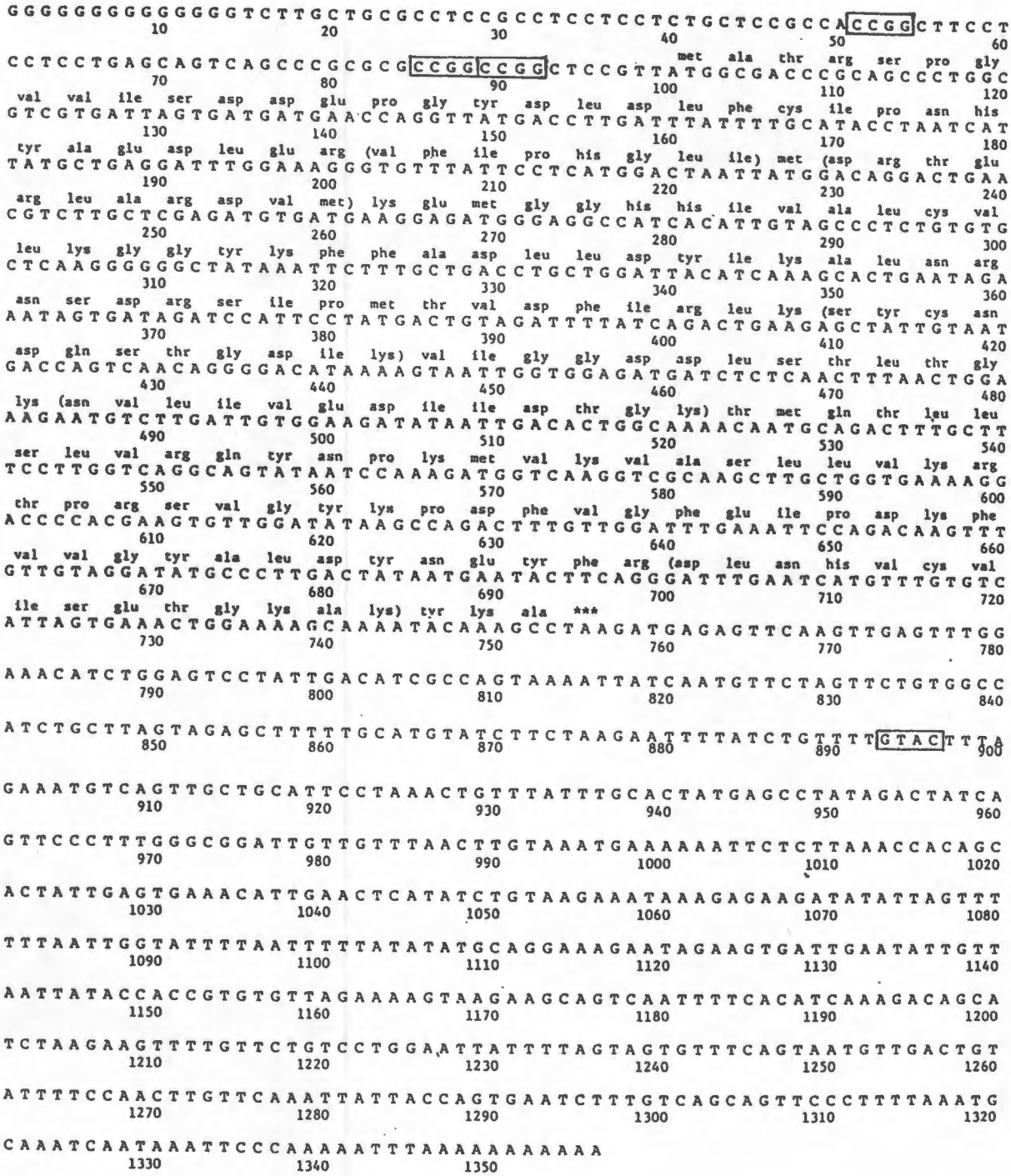


Figure 21 Nucleotide sequence of human HPRT cDNA and inferred amino acid sequence

(Jolly et al., 1983)

Msp I (CCGG) and Rsa I (GTAC) sequences are highlighted in blocks

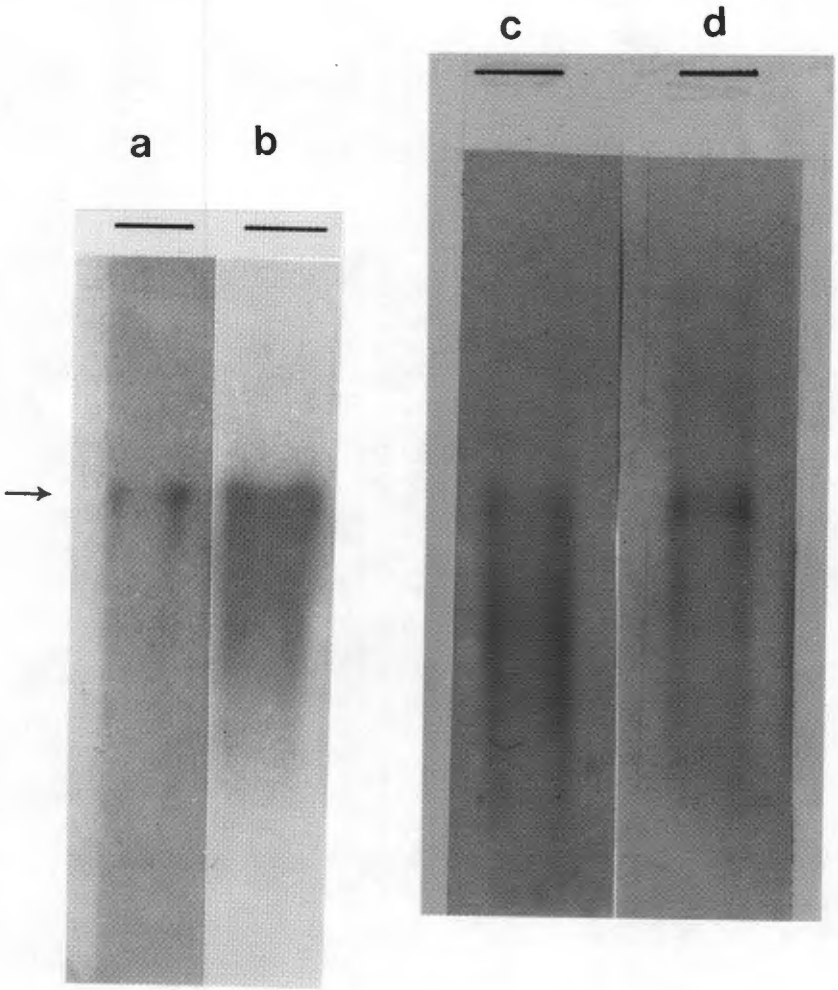


Figure 22 Autoradiograph of a Northern blot of RNA extracted from T.K., B.K. and control lymphoblasts, which was hybridized to a ^{32}P-labelled HPRT cDNA insert

lane a: RNA from T.K.; lane b: RNA from control
lane c: RNA from control; lane d: RNA from B.K.

3.2.3 S1 nuclease analysis

3.2.3.1 Clarification of the HPRT probe

A full-length HPRT cDNA cloned into the Okayama and Berg (1983) pcD vector, was obtained from Jolly et al. (1983). pcD is a recombinant of a segment of pBR322 and two segments of SV40. The cDNA was ligated into a Pst I site and a Bam HI site. Because the sequence or restriction map of the vector was not available, the sizes of the various fragments were not known, and therefore this was determined before subcloning the HPRT fragment (approximately 1 500 base-pairs).

The plasmid, which is named p4aA8 (Figure 23a), was digested with either Pst I or Bam HI, or a double digest was performed using both these enzymes. The fragments were electrophoresed on a 2% agarose gel alongside a lambda/BstE II molecular weight marker (Figure 23b). Two fragments are produced when p4aA8 is digested with Pst I (lane c). The larger fragment (approximately 3 350 base-pairs) contains the HPRT insert. When the plasmid is digested with both enzymes, the 3 350 fragment is split into two fragments (approximately 1 700 and 1 500 base-pairs) and the Pst I smaller fragment (approximately 1 300 base-pairs) is split into a 1 200 base-pair fragment (which can be seen to migrate slightly faster than the 1 300 fragment) and a 100 base-pair fragment, which is too small to be visualized on an agarose gel.

When p4aA8 is digested with Bam HI (lane d), two fragments are

produced. The smaller fragment (approximately 1 600 base-pairs) contains the HPRT insert. When the plasmid is digested with both the enzymes, the 1 600 fragment is split into a 1 500 base-pair fragment (which can be seen to migrate slightly faster than the 1 600 fragment) and a 100 base-pair fragment. These results suggest that the middle band of the double-digested plasmid contains the insert. This was confirmed by Southern blotting this gel and hybridizing it to a ^{32}P-labelled HPRT probe (insert only as described in section 3.2.2). In the double-digest, only the 1 500 band has hybridized (Figure 23c) and in the Pst I digest, only the larger fragment has hybridized. In the Bam HI digest, both fragments have hybridized. This is probably due to cross-hybridization between the guanine-cytosine rich regions of SV40 and HPRT.

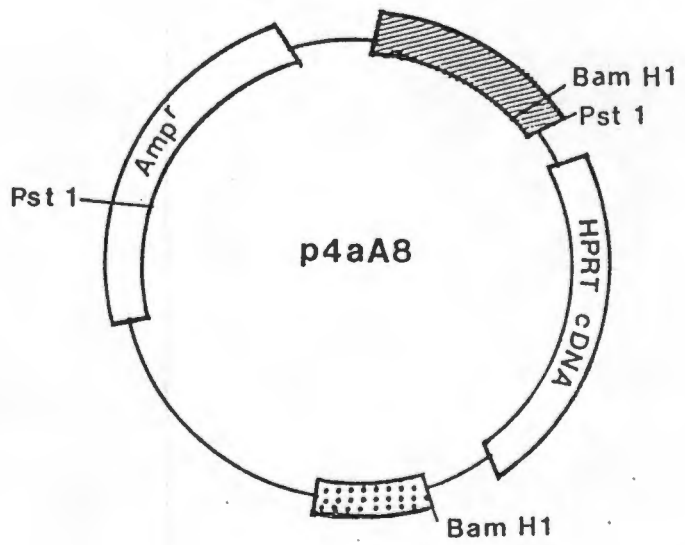
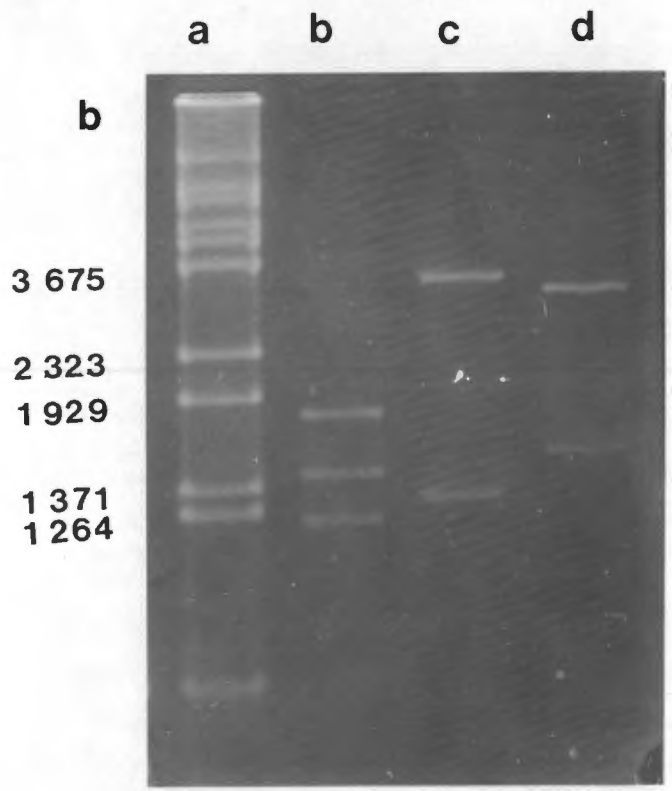


Figure 23a Diagrammatic representation of the p4aA8 plasmid

SV40 DNA SV40 DNA



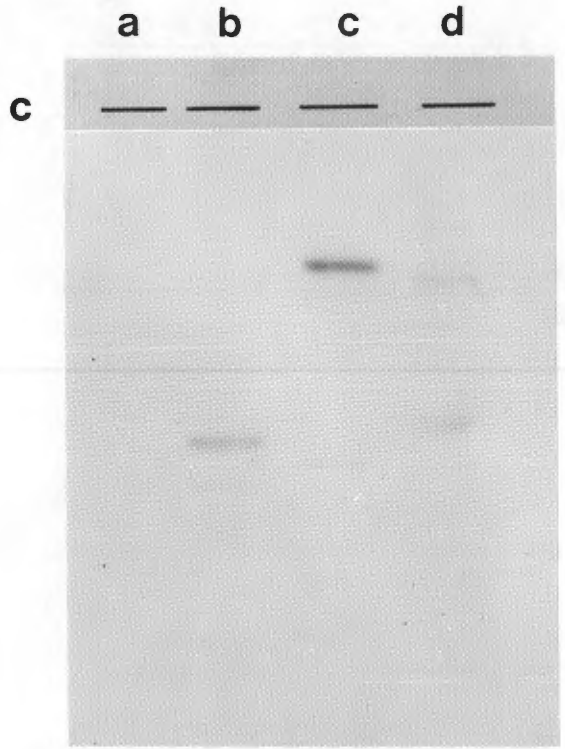


Figure 23b Gel electrophoresis of p4aA8 digested with Pst I and Bam HI

lane a: lambda/BstE II molecular weight marker (fragment sizes are shown on the left); lane b: p4aA8 digested with Pst I and Bam HI
land c: p4aA8 digested with Pst I; lane d: p4aA8 digested with Bam HI

Figure 23c Autoradiograph of hybridization of the HPRT insert to a Southern blot of the gel shown in Figure 23b

3.2.3.2 Subcloning of the HPRT insert into pGEM-3

pGEM-3 is a riboprobe vector (Promega-Biotec). It has a gene which confers ampicillin resistance and this is used for selection purposes. It has both a SP6 and a T7 promoter, which enables one to synthesize the antisense and sense RNA sequences from the same plasmid. It has a multiple cloning site, which is situated between the two promoters. The size of the vector is 2 867 base-pairs (Figure 24).

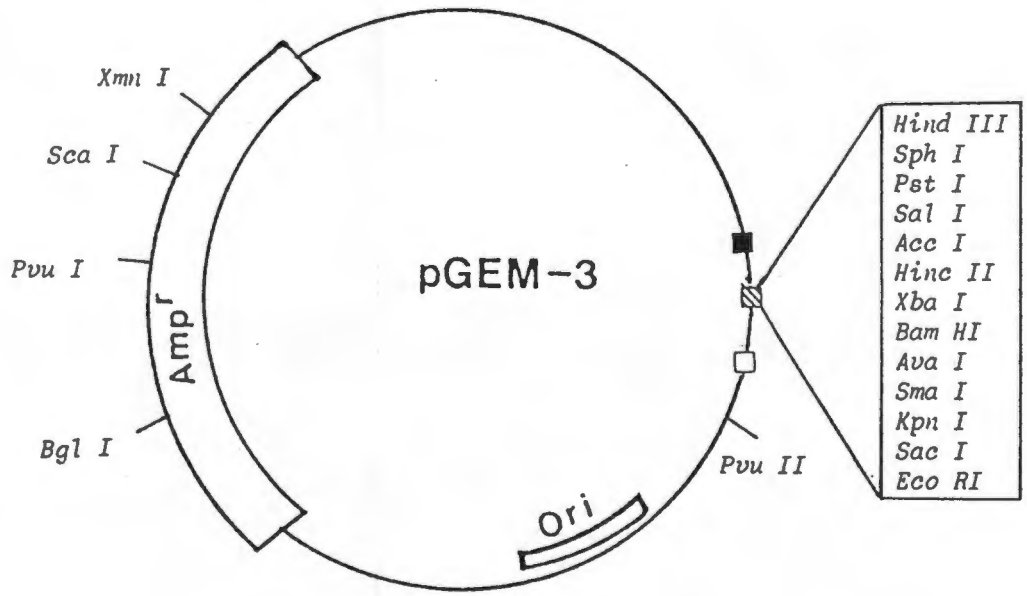


Figure 24 Diagrammatic representation of the pGEM-3 vector

■ SP6 promoter; ▨ multiple cloning site;
□ T7 promoter

pGEM-3 and p4aA8 were digested with Bam HI and Pst I. This digest cleaves p4aA8 into 4 fragments as described above. pGEM-3 is linearised and possesses two sticky-ends. The digested fragments were then ligated together. pGEM-3 cannot recircularize because its sticky-ends are not complementary.

E.coli DK1 was transformed with the ligated DNA and plated onto ampicillin plates in order to select for those bacteria containing plasmids. Fifteen of the colonies were selected and mini-preparations prepared from them. These plasmid preparations were then digested with Pst I and Bam HI and electrophoresed on a 1.2% agarose gel. pGEM-3 (undigested) was also used to transform E.coli as a control for the transformation. A single colony was also extracted as a control for the mini-preparation procedure and digested and electrophoresed together with the recombinants. The electrophoresis provided a method to select for those plasmids containing the HPRT insert.

A photograph of the electrophoresed plasmid DNA is shown in Figure 25. Uncut plasmid was electrophoresed in the first lane of each pair, and Pst I-Bam HI-digested plasmid was electrophoresed in the second lane of each pair. Pair 1 contains the pGEM-3 control. Eight of the recombinants (3, 4, 6, 7, 9, 10, 14 and 16) do not have a band corresponding to pGEM-3, but have two bands corresponding to the 1 700 and 1 200 base-pair fragments of p4aA8. Both these fragments have a Bam HI and a Pst I sticky-end and can thus ligate together. The gene conferring ampicillin resistant is coded for in these fragments, and thus the E.coli hosts transformed with this newly created plasmid, would be able to grow in the presence of ampicillin. The faint, largest band seen in

recombinants 6, 14 and 16 is due to a partial digest of the DNA, resulting in a single, full-length, linear molecule. The plasmids which appear to have no inserts (2 and 13), possibly contain the 100 base-pair fragment, which cannot be resolved on this agarose gel. However, any traces of single-cut pGEM-3 would be able to recircularize and would be expected to give rise to transformants lacking inserts.

One of the plasmids (5) contains three bands corresponding to the three largest bands of p4aA8 and therefore this recombinant represents fragments entirely derived from p4aA8. The 100 base-pair fragment must have also been ligated or else this would not have been possible.

Two of the plasmids (8 and 11) contain the HPRT insert. The faint, largest band represents a partial digest of the DNA. This can be seen from the size of the uncut DNA which is larger than uncut pGEM-3 without an insert.

Plasmid DNA was prepared from one of the colonies containing the HPRT insert, as well as from one of the colonies containing the condensed p4aA8 plasmid. These two plasmids, as well as pGEM-3 and p4aA8, were digested with Pst I and Bam HI and electrophoresed on a 1.2% agarose gel in order to verify the conclusions reached from the mini-preparations.

Figure 26a shows that plasmid 11 (lane c) contains the 1 500 base-pair fragment of p4aA8 (HPRT insert) cloned into pGEM-3. Plasmid 13 (lane d) contains both the 1 700 and 1 200 base-pair fragments of p4aA8 ligated together. To provide final proof that plasmid 11 did contain

the HPRT insert, the gel was Southern blotted and hybridized to the ^{32}P-labelled HPRT insert (described in section 3.3.2). Figure 26b shows the autoradiograph. It clearly shows that only the 1 500 base-pair fragment of p4aA8 and the smaller fragment of plasmid 11 hybridize to the HPRT probe.

Plasmid 11 was named pGEM-3-HPRT and was used to transcribe RNA for use in the S1 nuclease assay. When pGEM-3-HPRT is digested with Pst I, it is linearized as depicted in Figure 27. The antisense RNA strand can thus be transcribed by using the T7 promoter. When pGEM-3-HPRT is digested with Eco RI, the plasmid is linearized as depicted in Figure 27. The sense strand can be transcribed by using the SP6 promoter, although there would be no purpose in doing this for this S1 experiment.

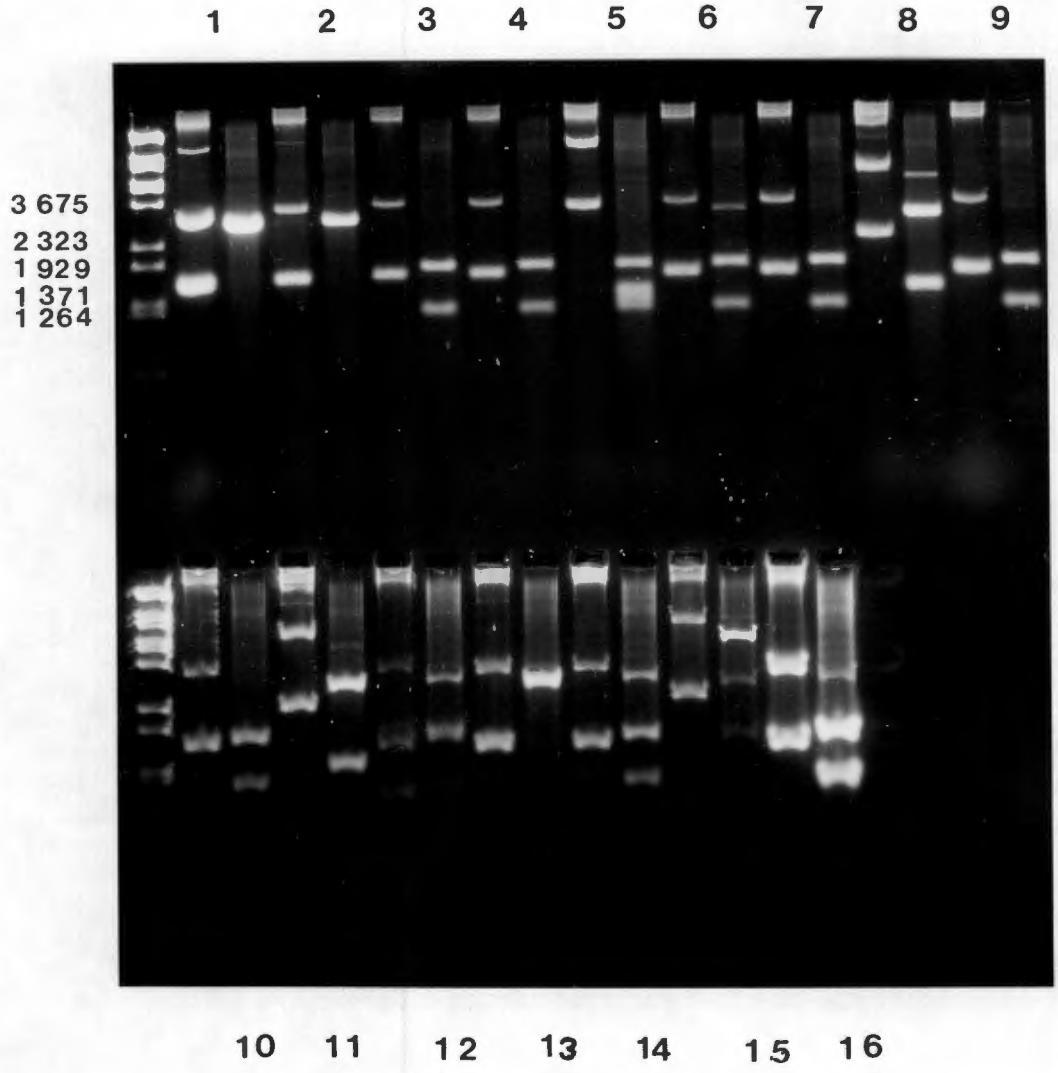


Figure 25 Gel electrophoresis of recombinant plasmid DNA

The first lane of each gel contains a lambda/BstE II molecular weight marker with fragment sizes indicated on the left.

The first lane of each pair contains uncut plasmid DNA and the second lane contains Pst I-Bam HI cut DNA.

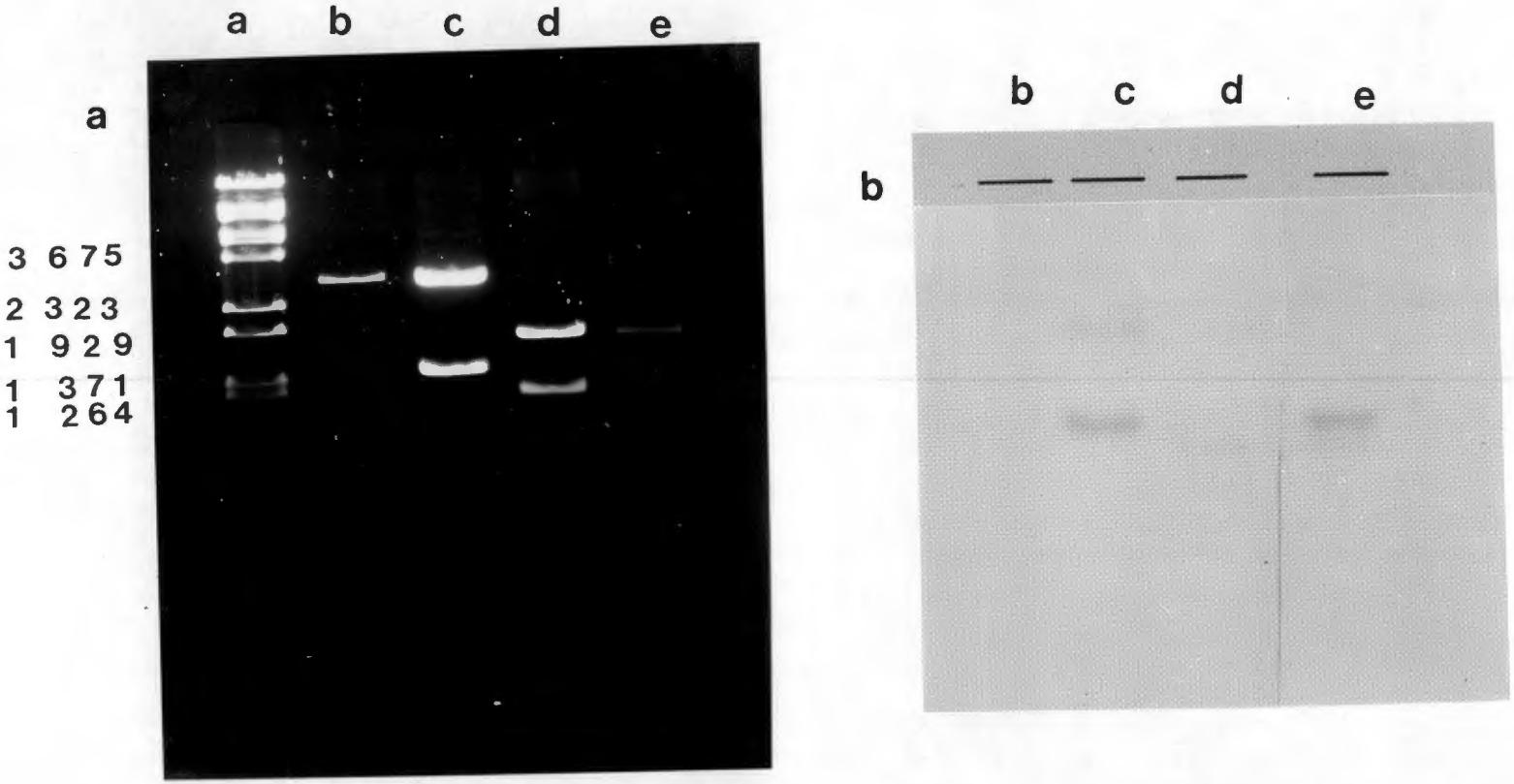


Figure 26a Gel electrophoresis of Pst I-Bam HI cut plasmid 11 (lane c), plasmid 13 (lane d), pGEM-3 (lane b) and p4aA8 (lane e)

Lambda/BstE II molecular weight marker is electrophoresed in lane a. Fragment sizes are shown on the left.

Figure 26b Autoradiograph of hybridization of the HPRT insert to a Southern blot of the gel shown in Figure 26a (lanes are the same as in Figure 26a)

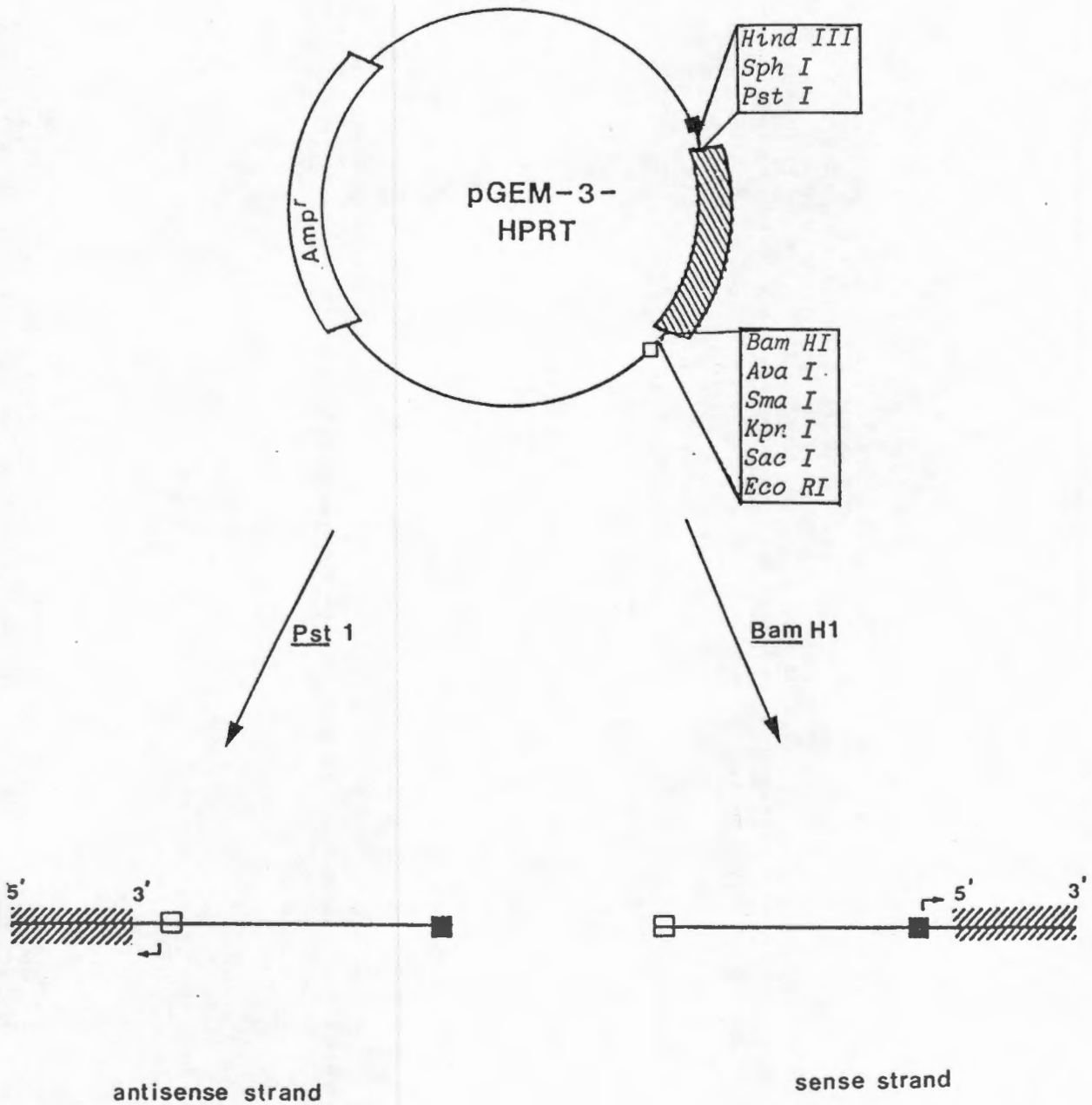


Figure 27 Diagrammatic representation of the pGEM-3-HPRT plasmid and the transcription of antisense and sense RNA strands

▨ HPRT cDNA; ■ SP6 promoter; □ T7 promoter

3.2.3.3 S1 nuclease assay

^{32}P-labelled antisense HPRT RNA was transcribed from the pGEM-3-HPRT plasmid using T7 polymerase. This was hybridized to T.K., B.K. and control RNA. A reaction was set up without any RNA as a control for the efficiency of the S1 nuclease enzyme. After hybridization was completed, S1 nuclease was added to the reaction. S1 nuclease is a single-strand-specific endonuclease and hydrolyzes single-stranded RNA and DNA. The antisense RNA forms a RNA:RNA hybrid with the HPRT mRNA and thus protects it from digestion by S1 nuclease. The rest of the RNA in the RNA population will be single-stranded and therefore susceptible to digestion by S1 nuclease. Certain point mutations in the RNA:RNA hybrid will be recognized by S1 nuclease as single-stranded and will therefore be digested.

No difference was observed in the size of the protected fragment between control, T.K. and B.K. RNA (Figure 28). There was no protected fragment in the minus RNA reaction (lane e), which implies that the S1 nuclease has digested the RNA to completion. The size of the RNA protected fragment is approximately 800 base-pairs (calculated from ribosomal markers that were electrophoresed alongside the RNA) in contrast to the expected size of 1 500 base-pairs. The reasons for this are uncertain. The possibilities are firstly, that the cloned cDNA contains a base change relative to all the samples tested, resulting in S1 cleavage. This base change might be due to a normal polymorphism or a base change that arose subsequent to cloning. A second possibility is that there is premature termination of transcription of the riboprobe.

Although this result does not demonstrate a single base substitution, it does not in any way exclude it, since S1 nuclease will not always cleave all single base mismatches. Gibbs and Caskey (1987) hybridized a RNA probe to RNA extracted from 14 Lesch-Nyhan patients and digested the hybrids with ribonuclease A. Nine of these were not cleaved by the enzyme, which suggests that they contain single base substitutions that do not result in base mismatches that are susceptible to cleavage by ribonuclease A.

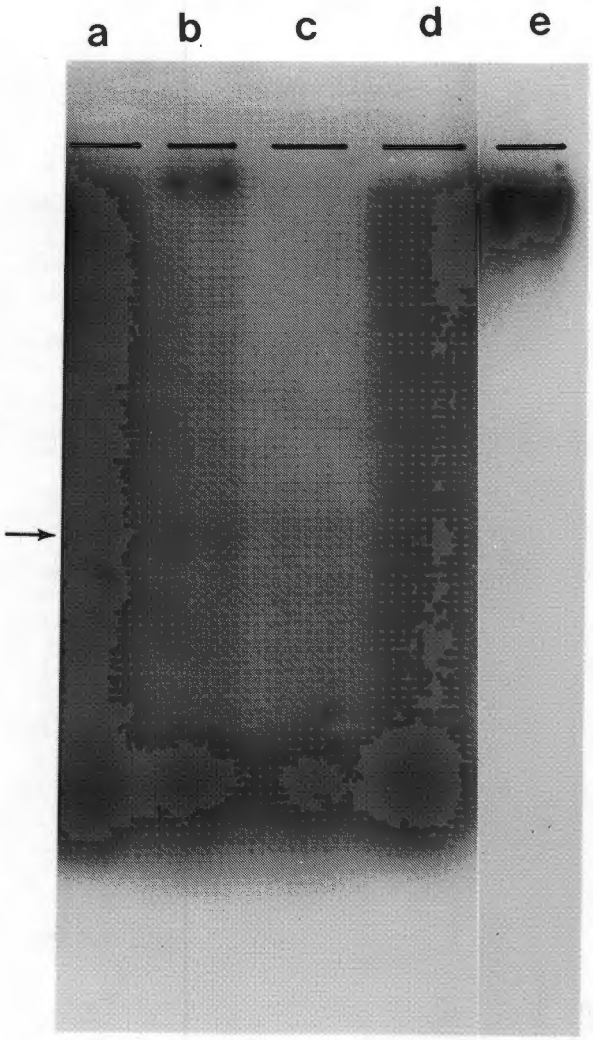


Figure 28 Autoradiograph of S1 nuclease assay

lanes a and d: RNA from control; lane b: RNA from T.K.
lane c: RNA from B.K.; lane e: minus RNA control

3.2.4 Attempt to clone the mutant cDNA

Total RNA was extracted from T.K.'s lymphoblasts and used as a template to reverse transcribe cDNA. The cDNA was then digested with the restriction enzymes Msp I and Rsa I which digest HPRT cDNA 5' and 3', respectively, to the coding region (Figure 21). The digested cDNA was then electrophoresed on a 1.2% agarose gel and cDNA fragments between 603 and 1 078 base-pairs, were eluted from the gel. These fragments were used for cloning. This size selection increases the proportion of HPRT cDNA in the total cDNA population. This lessens the number of recombinants which have to be screened when attempting to clone rare messages.

The cDNA was cloned into the Eco RI site of lambda gt10 (Figure 29) by ligating Eco RI linkers to the cDNA and then digesting the linkers with Eco RI, which yields sticky-ends. Any Eco RI sites within the cDNA sequence must be protected from digestion by Eco RI. This is accomplished by methylating any Eco RI sites with Eco RI methylase. It is known that normal HPRT cDNA does not contain any Eco RI sites and a Southern blot of the mutant DNA cut with Eco RI showed there was no difference from normal (Figure 18), thus no Eco RI site has been created in the mutant gene. Therefore, the Eco RI methylase step was not performed.

After the cDNA had been digested with Eco RI, it was separated by column chromatography from the unligated linkers. The cDNA was then ligated to Eco RI-digested lambda gt10 arms. The lambda gt10 containing the cDNA was then packaged and used to infect

E.coli.

Turbid plaques (non-recombinant phage) and clear plaques (recombinant phage) were observed on the non-selective host. Clear plaques (recombinant phage) were observed on the selective host (*E.coli* strain with a high frequency lysogeny mutation). The library was amplified using the selective host and was used for screening. Replica filters were prepared of the plaques and hybridized to ^{32}P-labelled HPRT cDNA probe. Positive spots were obtained on autoradiographs (Figure 30) and plaques appearing to underlie these spots were picked and used to prepare DNA. This DNA was digested with *Eco* RI to release the insert, electrophoresed on an agarose gel, Southern blotted and hybridized to ^{32}P-labelled HPRT cDNA probe. No bands corresponding to 800 base-pairs (size of the HPRT insert) were observed (Figure 31). Some hybridization was seen to the lambda gt10 arms which was due to cross-hybridization between complementary sequences. After a large number of phage had been analyzed in this way, some of the *Eco* RI-digested phage DNA was end-labelled and electrophoresed to determine whether the phage contained any inserts. The only bands observed were those corresponding to the lambda gt10 arms (Figure 32).

The screened library thus appeared to have no inserts. The "positive" spots observed on autoradiographs were probably artefacts.

The appearance of clear plaques on the non-selective and the selective hosts implied that the phage contained inserts, yet preparation of DNA from some of them, showed that no inserts were present. It is possible that some of the *Eco* RI linkers ligated together and were not fully

digested by Eco RI (even though an excess of enzyme was used) and were then inserted into the vector, thus inactivating the cI gene.

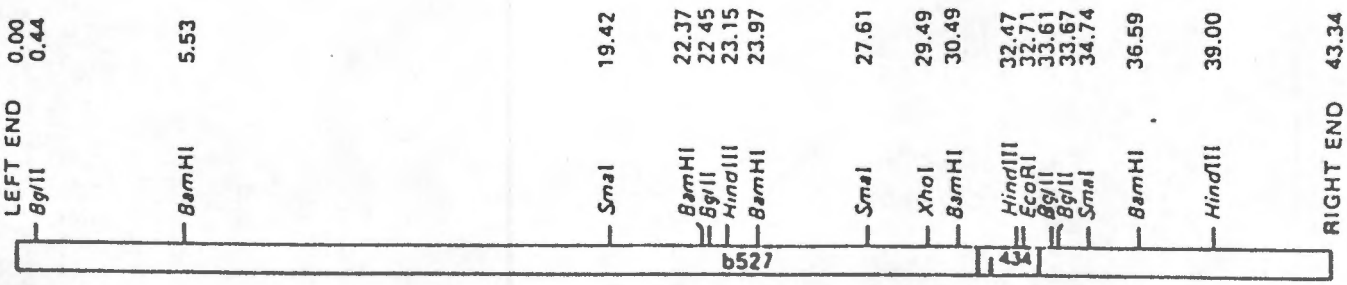


Figure 29 Map of lambda gt10

Restriction enzyme cleavage sites are designated in kilobase pairs from the left end.
(Huynh et al., 1985)

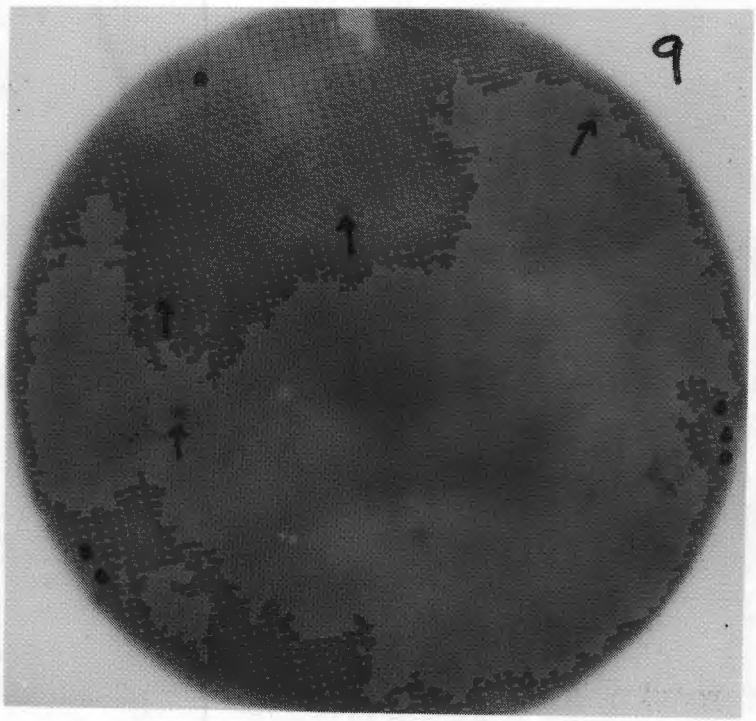


Figure 30 Autoradiograph of HPRT cDNA hybridized to a replica filter

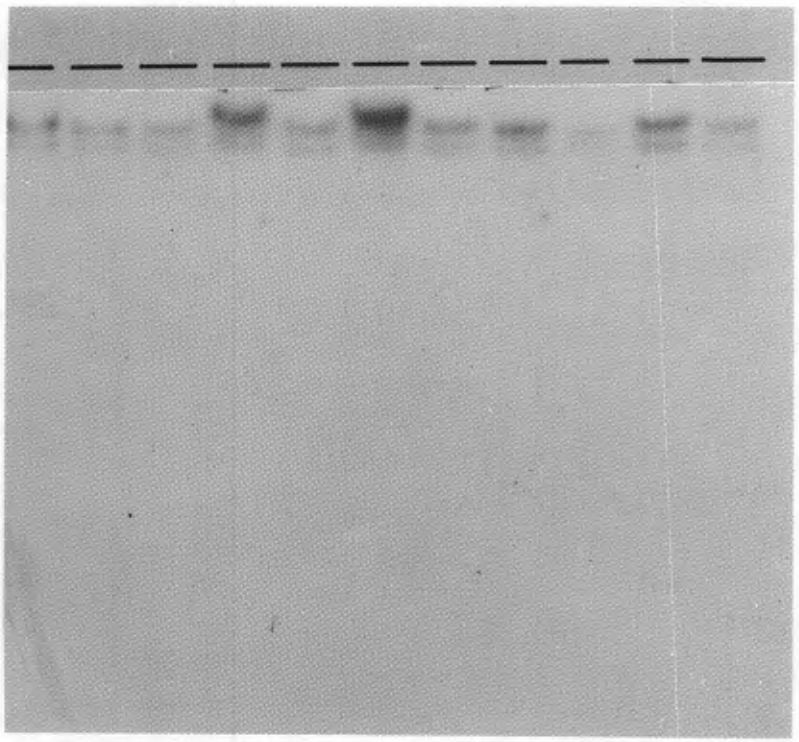


Figure 31 Autoradiograph of ^{32}P-labelled HPRT cDNA hybridized to phage DNA digested with Eco RI

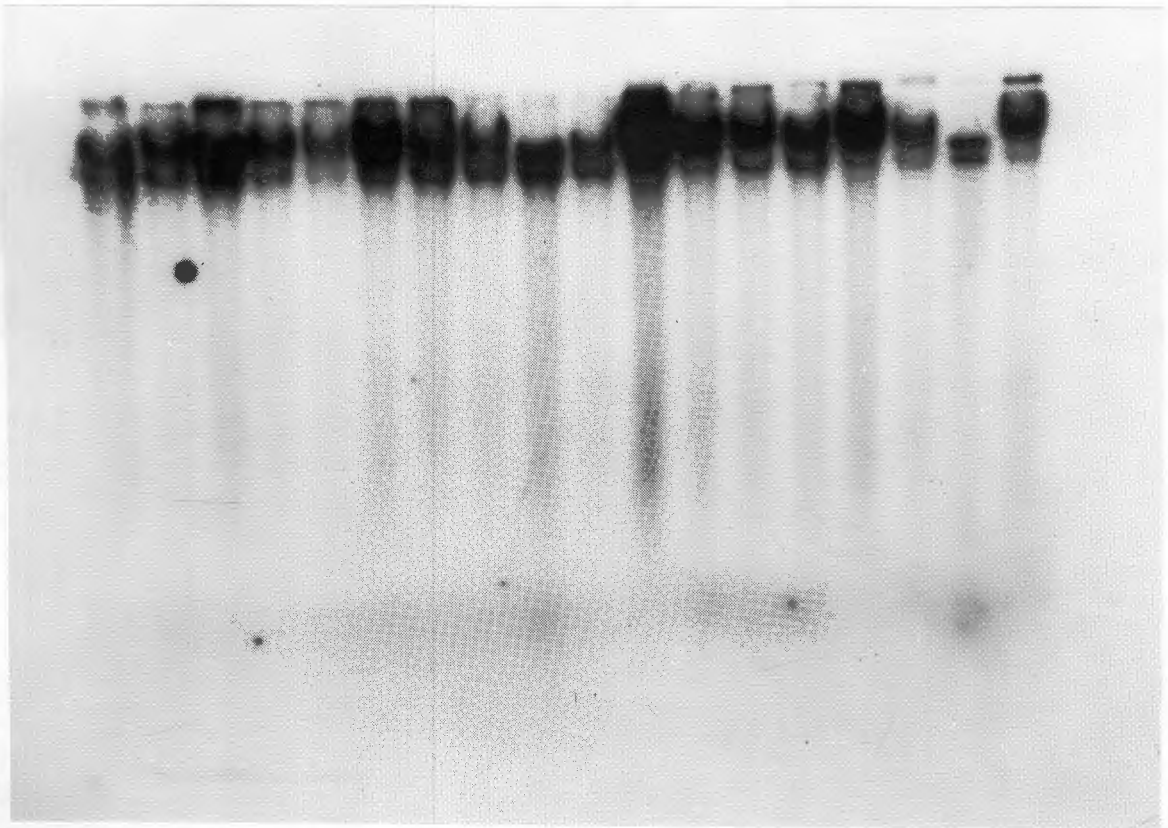


Figure 32 Autoradiograph of ^{32}P end-labelled phage DNA digested with Eco RI

4.0 CONCLUSION

The partial HPRT deficient mutant, $HPRT_{Cape\ Town}$ had been previously shown to have a low activity in fibroblasts and erythrocyte haemolysates. This low activity was not associated with changes in the V_{max} or K_m for any of the substrates. However, the enzyme exhibited low activity in erythrocyte haemolysates at high concentrations of the purine substrates, hypoxanthine and guanine. This substrate inhibition was not observed with PPRP as the substrate (Steyn and Harley, 1984).

Wohlhueter (1975) observed substrate inhibition in normal rat liver HPRT, whereas rat hepatoma HPRT exhibited hyperbolic kinetics, implying that post-translational events can modify HPRT kinetics. HPRT activity in the proband's lymphoblasts was therefore studied in order to determine whether the substrate inhibition observed was a generalized or a secondary phenomenon occuring only in erythrocyte haemolysates. Substrate inhibition was observed in lymphoblast extracts which indicates that this is a generalized phenomenon. The proband's clinical phenotype is therefore compatible with decreased HPRT activity, with physiological levels of guanine (and hypoxanthine) causing substrate inhibition.

It was not known whether the substrate inhibition was the result of a mutation in the gene coding for the HPRT enzyme, a mutation resulting in altered post-translational modification or an absence or alteration of a factor influencing normal HPRT kinetics. The HPRT enzyme is coded for on the X chromosome (Pai et al., 1980), thus if the mutation was in the gene coding for the enzyme, the proband's daughter would be an obligate heterozygote.

The daughter's HPRT in erythrocyte haemolysates exhibited intermediate activity i.e. activity between that of the control and her father. The daughter's enzyme in transformed lymphoblasts exhibited growth patterns similar to those of her father i.e. growth in 6-thioguanine-containing medium and absence of growth in HAT medium. The kinetics of the daughter's enzyme prepared from lymphoblast extracts exhibited the characteristic substrate inhibition of the proband. These results of the daughter's lymphoblasts could be interpreted as a selection of a clone or clones that have suppressed the function of the X chromosome carrying the maternal, and presumably normal HPRT gene. The daughter's results in erythrocytes, which are a more representative population of cells than transformed lymphoblasts, imply that she is an obligate heterozygote and that the mutation is in the gene coding for the HPRT enzyme. The lymphoblast results are compatible with this. However, there is a remote possibility that the substrate inhibition is not due to a HPRT gene mutation, but due to a dominantly inherited or X-linked mutant gene constitutively producing a factor that influences normal HPRT kinetics.

It is known that the mutant enzyme is labile in the partially purified form (Steyn and Harley, 1984), thus characterization of the defect by purification to homogeneity was not feasible. The defect was thus studied at the gene level. No difference was observed in the banding patterns between the proband's DNA and control DNA when digested with various restriction enzymes and hybridized to ^{32}P-labelled HPRT cDNA. No difference was observed in the size of the HPRT mRNA of the proband and that of the control. These results imply that there is no major gene alteration. This result is to be expected, since the proband only

has a partial deficiency of the enzyme. Generally, patients who lack the neurological abnormalities that are associated with the Lesch-Nyhan syndrome, have mutations that have less deleterious effects on the function of the enzyme in vivo (Wilson et al., 1986).

The HPRT cDNA was subcloned into a riboprobe vector, pGEM-3. HPRT RNA antisense strands were transcribed using the T7 promoter. This ^{32}P-labelled RNA was hybridized to the proband's RNA and control RNA. The double-stranded RNA was digested with S1 nuclease. No difference was observed in the size of the HPRT protected fragment between the proband and the control. However, the analysis may not have covered the full length of the mRNA. This result does not exclude the possibility of a base-pair mismatch, but suggests that if there is a mismatch, it is not susceptible to digestion by S1 nuclease. S1 nuclease does not recognize all base-pair mismatches as single-stranded.

The attempt at cloning the mutant cDNA was not successful. In order to complete the characterization of this mutant enzyme, this should be repeated, possibly using oligonucleotides corresponding to regions of the HPRT cDNA to select the HPRT mRNA.

It would be interesting, for a number of reasons, to know exactly where the mutation is in the gene or whether the mutation is not in the HPRT gene itself. Firstly, it would provide information relevant to explaining the phenomenon of substrate inhibition i.e. which region of the protein is altered. Secondly, it might provide some insight into the mode of binding of substrates in the active site of an enzyme.

Thirdly, it might provide more information on the role of HPRT in purine metabolism.

5.0 MATERIALS AND METHODS

Composition of commonly used buffers and media

Gel buffer: 10 x: 0.2 M morpholinopropanesulfonic acid, pH 7.0, 50 mM sodium acetate, 10 mM EDTA, pH 8.0

L-agar plates: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar in 1 litre water, pH 7.0

LB medium: 10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 litre water, pH 7.0

Loading buffer: 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol

Medium-salt buffer: 10 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 1 mM DTT, 50 mM NaCl

RSB: 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$

SM buffer: 0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin

SSC: 20 x : 3 M NaCl, 0.3 M trisodium citrate, pH 7.6

SSPE: 20 x: 3.6 M NaCl, 0.2 M sodium phosphate, pH 7.7, 0.002 M Na_2EDTA

STE: 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA

TAE buffer: 40 mM Tris-HCl, p H 7.7, 40 mM acetic acid, 1 mM EDTA

Top agar: 1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.25 g $MgSO_4$, 0.7 g agar in 1 litre water, pH 7.0

TE: 10 mM Tris-HCl, 1 mM EDTA

5.1. TRANSFORMATION OF LYMPHOCYTES

Lymphocytes were prepared according to the method of van der Westhuyzen et al. (1984). Medium containing Epstein-Barr virus (EBV) was prepared from semi-confluent cultures of the EBV-producing marmoset cell line B95/8 (Miller and Lipman, 1973). Ham's F10 medium (Gibco or Flow Laboratories) containing 15% foetal calf serum (Gibco or Flow Laboratories) was added to the cells and removed 4 days later. Any B95/8 cells in this medium were removed by centrifugation followed by passage through a 0.45 um membrane filter (Millipore). The medium was mixed with an equal volume of Ham's F10 medium containing 15% foetal calf serum. This mixture constituted the transforming medium and was effective for at least 3 months when stored at 4°C.

10 ml of heparinised blood was diluted 1:2 with Dulbecco's Modified Eagle's Media (DMEM, Gibco or Flow Laboratories), layered onto Ficoll-Paque (Pharmacia Fine Chemicals) and centrifuged at 1 000x g for 20 minutes in a MSE bench top centrifuge. The lymphocyte layer was removed and washed twice with DMEM to remove any platelets, Ficoll-Paque and plasma. The lymphocytes were then resuspended in 2-3 ml EBV-transforming medium (the volume depended on the concentration of cells) and transferred to a 25 cm^2 tissue culture flask. During the transformation period (1-3 weeks), the cells were examined regularly and the medium changed when necessary. The cells were incubated in a humidified 95% air-5% CO_2 incubator. Once the transformed lymphoblasts were multiplying rapidly, they were transferred into 75 cm^2 tissue culture flasks and passaged approximately twice a week at a split ratio of 3.

These cells were used for the preparation of enzyme extract, for selective media experiments and for the extraction of DNA and RNA.

5.2. SELECTIVE MEDIA EXPERIMENTS

Stock cultures of lymphoblasts from a normal individual, a Lesch-Nyhan lymphoblast line GM 1899, from the NIGMS Human Genetic Mutant Cell Repository, and from T.K. and B.K., were grown. The number of cells in each stock culture was determined by counting 0.1 ml of cells in 10 ml of isoton (Coulter Electronics) in a Coulter Counter, model Z_F (Coulter Electronics,Inc. Hialeah, Florida) using settings appropriate for lymphoblasts (amplification = 2, threshold = 22, aperture current = 2). Approximately 200 000 cells were centrifuged at 1 000x g in a MSE bench top centrifuge. The cells were resuspended in 5 ml of selective media or RPMI 1640 (Flow Laboratories) without the addition of selective media. This was to show that the growth characteristics observed in the presence of the selective agent were due to the agent and not a feature of that particular cell line. All media contained 10% foetal calf serum (Gibco or Flow Laboratories) and PSN (100 mg/l sodium benzylpenicillin, 100 mg/l streptomycin sulphate and 60 mg/l neomycin sulphate). The initial number of cells in each culture flask was then determined as described. Every 2 to 3 days the cells were centrifuged and the pellet resuspended in 5 ml of fresh medium and the number of cells counted. The cultures were maintained for 7 or 8 days. All cultures were performed in duplicate. The number of cells in each flask was expressed relative to the initial cell number.

5.2.1 HAT selective media

HAT medium (1x RPMI 1640 medium with HAT, Flow Laboratories) consists

of 4×10^{-7} M aminopterin, 10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine.

5.2.2 6-thioguanine

6-thioguanine (Sigma) was used at a final concentration of 10 µg/ml.

5.3. ENZYME ASSAYS

5.3.1 Preparation of enzyme extracts from heparinised blood

Enzyme extracts were prepared according to the method of Steyn and Harley (1984). 10 ml of fresh, heparinised blood was obtained from T.K., B.K. and a normal control. The plasma and buffy coat were removed after centrifugation at 1 000x g for 10 minutes in a MSE desk top centrifuge. The red blood cells were washed twice with 1.5 volumes of distilled water at 4°C. The stroma was then removed by centrifugation. The haemolysate was dialysed for 16 hours at 4°C against two changes of 5 litres of 50 mM Tris-HCl, pH 7.8, containing 1 mM dithiothreitol (DTT). The dialysed lysate was frozen in 500 µl aliquots and stored at -20°C.

5.3.2 Preparation of enzyme extracts from EBV transformed lymphoblasts

Enzyme extracts were prepared by a modification of the method of Wilson et al. (1982b). Lymphoblast cultures were pelleted and washed twice with 0.9% saline. The cells were resuspended in 50 mM Tris-HCl, pH 7.8, 150 mM NaCl in a volume which was equivalent to 0.7 times the cell pellet volume. The cells were lysed by six cycles of freeze-thawing. Cells were frozen in an ethanol-dry ice bath and thawed in a 37°C waterbath. Membrane-free extracts were obtained by centrifuging the cell lysates at 10 000 rpm for 30 minutes at 4°C in a 12001 rotor in a Sigma 2MK centrifuge. The supernatant was dialysed against two

changes of 200 ml of 50 mM Tris-HCl, pH 7.8, 150 mM NaCl at 4°C in order to remove intracellular hypoxanthine and guanine. The time required for dialysis was determined using [8-^{14}C] hypoxanthine (Amersham). Less than 1% of the hypoxanthine remained after overnight dialysis. Enzyme activity was determined in the presence of 1 mg/ml bovine serum albumin (BSA).

5.3.3 Determination of HPRT activity in enzyme extracts

Hypoxanthine-guanine phosphoribosyltransferase activity was assayed according to the method of Steyn and Harley (1984). The assay was performed at 37°C. Each assay contained the following in a final volume of 100 µl: 50 mM Tris-HCl, pH 7.8, 14 mM $MgCl_2$, 1.4 mM PPRP and [8-^{14}C] hypoxanthine at appropriate concentrations. All assays were performed in duplicate. The assay tubes were pre-incubated at 37°C for 1 minute. The reaction was started by the addition of 20 µl of enzyme preparation to each of the assay tubes. Four aliquots of 20 µl were taken at 1 minute intervals (for the haemolysates) and at 4 minute intervals (for the lymphoblast extracts) and applied to PEI-cellulose plates (Merck) which were preheated to 70°C on a heating block (to terminate the reaction). The PEI-cellulose plates were pre-drawn into circles (2 cm diameter) with one circle per aliquot. The aliquot was unable to spread outside the circle. In this way, 8 duplicate assays could be accommodated on each plate.

The unconverted hypoxanthine was removed by placing the plates in a chromatography tank containing 4 litres of tap water for 5 minutes.

The plates were removed before the water was changed. 8 washes were required to remove all the unused hypoxanthine. (The number of washes required was determined by spotting [8-^{14}C] hypoxanthine on a PEI-cellulose plate and comparing the measured radioactivity in washed and unwashed plates). Less than 1% of unconverted hypoxanthine was retained on the plates and less than 10% of the product was lost. After the plates had dried, the individual circles were cut out and placed in scintillation vials. 5 ml of Hionic fluor (Packard) was added, which was sufficient to completely cover the circle and the radioactivity was counted in a Beckman LS-250 scintillation counter. The amount of quenching was determined by counting a known volume of [8-^{14}C] hypoxanthine placed either directly in a vial containing scintillation fluid or spotted onto a PEI-cellulose plate, which was then washed and placed in a vial containing scintillation fluid. Quenching amounted to a mean of 26% and this was corrected for when the amount of enzyme activity was calculated.

The unit of enzyme activity was defined as that which catalyzed the formation of 1 µmol of product, IMP, per minute.

5.3.4 Determination of protein concentration in enzyme extracts

The protein concentration in the enzyme extracts was determined by a modification of the method of Lowry et al. (1951). The assay was performed in a volume of 200 µl. 5 µl of the enzyme extract was added to 195 µl of distilled water. The blank consisted of 200 µl of distilled water. A standard curve was constructed by setting up known

concentrations of bovine serum albumin in the range 10 µg to 200 µg. 1 ml of Lowry reagent (25 ml of 2% Na_2CO_3 in 0.1 N NaOH, 0.25 ml 2% K^+Na^+ Tartrate, 0.25 ml 1% $CuSO_4$) was added to all the tubes and mixed immediately and the tubes allowed to stand at room temperature for 10 minutes. 100 µl of Folin and Ciocalteu's phenol reagent (diluted 1:2 with distilled water just before use) was added to each tube and mixed immediately. The absorbance at 750 nm was read on a Hewlett Packard 8450A diode array spectrophotometer after a 60 minute incubation at room temperature. The unknown protein concentrations were calculated from the standard curve.

5.3.5 Data analysis

The enzyme kinetic data was fitted to the appropriate rate equations by the reiterative method of Wilkinson (1961) using a Hewlett Packard 85 computer. The data for T.K. was fitted to equation 1 which includes a term for substrate inhibition and the control data was fitted to equation 2 for hyperbolic kinetics.

$$V = \frac{V_{max}}{K_m + S + S^2/K_i} \qquad (1)$$

$$V = \frac{V_{max}.S}{K_m + S} \qquad (2)$$

where V is the initial velocity, V_{max} the maximum velocity, S the substrate concentration, K_m the Michaelis constant and K_i the dissociation constant of the second substrate molecule.

5.4. PREPARATION OF RECOMBINANT PLASMID

5.4.1. Isolation of plasmid DNA

Plasmid DNA was isolated according to the method of Maniatis et al. (1982). A single colony of p4aA8 (Jolly et al., 1983) which is a 1.6kb human HPRT cDNA cloned into a pcD vector (Okayama and Berg, 1983) was grown in the presence of ampicillin (Sigma) and was used to inoculate a 25 ml starter culture of LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 litre water, autoclaved at 15 psi for 15 minutes) containing 50 µg/ml ampicillin. The starter culture was incubated overnight at 37°C in a rotary incubator. The starter culture was used to inoculate 500 ml of this same medium which was incubated at 37°C with vigorous shaking until the OD_{600} reached 0.6 units.

The bacterial cells were harvested by centrifugation at 1 500x g for 10 minutes at 4°C. The pellets were resuspended in 20 ml of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing 5 mg/ml lysozyme (Sigma) and allowed to stand at room temperature for 5 minutes. 40 ml of solution II (0.2 N NaOH, 1% (w/v) SDS) was added and the contents mixed by gentle inversion of the tube. The tube was allowed to stand on ice for 10 minutes. 30 ml of ice-cold 5 M potassium acetate, pH 4.8 (prepared by mixing 60 ml of 5 M potassium acetate with 11.5 ml of glacial acetic acid and 28.5 ml of water. The solution is 3 M with respect to potassium and 5 M with respect to acetate.) was added and the contents mixed by inverting the tube sharply. The contents were allowed to stand on ice for 10 minutes.

The cellular DNA and bacterial debris was pelleted by centrifugation at 31 000x g for 20 minutes at 4°C. 0.6 volumes of isopropanol was added to the supernatant. The contents were mixed well and allowed to stand at room temperature for 15 minutes. The DNA was recovered by centrifugation at 7 800x g for 30 minutes at room temperature. The pellets were washed in 70% ethanol and dried under vacuum. The pellets were resuspended in 2 ml of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

The plasmid DNA was then purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients. 2 g of cesium chloride (BRL) was added to 2 ml of DNA solution and mixed gently until all the salt was dissolved. 80 µl of ethidium bromide (10 mg/ml) per ml of cesium chloride solution was added and mixed well. The final density of the solution was measured. The cesium chloride solution was transferred to a Beckman polyallomer tube and the remainder of the tube was filled with cesium chloride-ethidium bromide solution of the same density. The tube was heat-sealed and centrifuged in a Beckman type 65 rotor in a Beckman L8-M ultracentrifuge at 50 000 rpm for 20 hours at 20°C.

The plasmid DNA band was pumped out of the tube into a microfuge tube. The ethidium bromide was removed by adding an equal volume of amyl alcohol, mixing well and centrifuging in a microfuge. The upper, organic (pink) phase was discarded and the extraction repeated on the lower phase until all the pink colour disappeared.

The cesium chloride was diluted by adding 2 volumes of sterile, distilled water. The DNA was precipitated overnight at 4°C by the

addition of 2 volumes of absolute ethanol. The DNA was recovered by centrifugation, washed in 70% ethanol and dried under vacuum. The DNA pellet was resuspended in 1 ml of TE, pH 7.5.

The purity of the DNA sample (estimated from the 260/280 ratio) was determined by scanning the sample from 220-310 nm on a Hewlett Packard 8450A diode array spectrophotometer. The DNA concentration was calculated from the absorbance at 260 nm. A 1 mg/ml solution of DNA has an absorbance of 20 units at 260 nm. The average yield was 100 µg/500 ml bacterial culture.

5.4.2 Isolation of HPRT cDNA sequences from p4aA8

The restriction endonuclease Msp I cleaves HPRT cDNA 5' to the start codon and Rsa I cleaves 3' to the termination codon producing a 806 base-pair fragment (Figure 21). By using both these enzymes it is possible to cleave out the HPRT cDNA without any adjoining vector DNA which could possibly cross-hybridize with the sequences being probed.

The plasmid DNA was digested with Msp I (Boehringer Mannheim) at 37°C for 60 minutes in the presence of low-salt buffer (10 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 1 mM DTT). At the end of the digestion period, NaCl was added to a final concentration of 50 mM (Rsa I requires a medium-salt buffer: 10 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 1 mM DTT, 50 mM NaCl). Rsa 1 (Biolabs) was then added and the sample incubated at 37°C for a further 60 minutes. At the end of the digestion time, the DNA was extracted with phenol:chloroform (1:1) (TE-saturated) and then

extracted with chloroform:octanol (24:1) and precipitated overnight at -20°C with 2 volumes of absolute ethanol. The DNA was recovered by centrifugation, washed in 70% ethanol and dried under vacuum. The DNA was then resuspended in TE, pH 7.5.

The DNA was electrophoresed on a 1.2% agarose gel alongside a ϕX174/Hae III molecular weight marker (Table 3). The 804 base-pair fragment was eluted from the gel using DEAE membrane (Schleicher and Schuell). A strip of the membrane was placed in an incision just ahead of the 804 base-pair band and another in an incision just behind the band in order to trap the larger fragments. Electrophoresis was then continued until binding was complete. This was judged by ethidium bromide fluorescence using an ultraviolet light box. The strip ahead of the band was removed and freed of residual agarose by thorough shaking in a microfuge tube containing NET buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris, pH 8.0). The DNA was eluted from the membrane by incubating it in 250 µl of high salt NET buffer (1.0 M NaCl, 0.1 mM EDTA, 20 mM Tris, pH 8.0) at 60°C for 45 minutes. The microfuge tube contents were swirled occasionally. The buffer was pipetted into a clean microfuge tube and the membrane washed with a further 50 µl of high salt NET buffer. Residual ethidium bromide was extracted with 3 volumes of water-saturated n-butanol and the DNA then precipitated at -20°C with 2.5 volumes of ethanol. The DNA was then reprecipitated from 0.3 M sodium acetate to remove any NaCl residue.

This eluted HPRT cDNA was used to probe Southern and Northern blots and to screen the recombinant library.

5.5 DNA STUDIES

5.5.1 Extraction of DNA from blood

Genomic DNA was isolated from 10 ml of T.K.'s blood and 10 ml of blood from a normal individual according to the method of Kunkel et al. (1977).

The blood was homogenized at 4°C in 60 ml of lysis buffer (0.32 M sucrose, 5 mM $MgCl_2$, 1% (v/v) Triton X-100 and 10 mM Tris-HCl, pH 7.6). The leukocyte nuclei were pelleted by centrifugation at 2 500x g for 20 minutes at 4°C. The pellets were resuspended in a total volume of 8 ml of 75 mM NaCl, 25 mM EDTA, pH 8.0 and then disrupted by the addition of 0.8 ml of 10% (w/v) SDS. Pronase (100 ul of 10 mg/ml stock, Sigma) was added to the samples, which were then incubated at 37°C for at least 3 hours. The digestion was terminated by placing the samples on ice. 0.5 ml of 5 M sodium perchlorate solution was added and the sample extracted with 8 ml of phenol:chloroform (TE-saturated). The aqueous and organic phases were separated by centrifugation at 2 500x g for 10 minutes at 10°C. The upper, aqueous phase was re-extracted with an equal volume of chloroform:octanol (24:1). The two phases were separated by centrifugation at 2 500x g for 10 minutes at 10°C. The aqueous phase was pipetted into a clean tube and 2 volumes of ice-cold absolute ethanol were added to precipitate the nucleic acid. The precipitate was lifted out into 1 ml of TE, pH 7.6 and allowed to dissolve with gentle mixing at 4°C overnight.

0.1 ml 4 M NaCl and 0.05 mg heat-treated RNase A (Sigma) were added to the nucleic acid solution which was then incubated at 37°C for 1 hour. Upon completion of the digestion, 2 ml sterile, distilled water was added and the solution extracted with an equal volume of chloroform:octanol (24:1) until the interface was clear. The samples were centrifuged at 2 500x g for 10 minutes at 10°C to separate the two phases. The DNA was precipitated from the aqueous phase by the addition of 2 volumes of ice-cold absolute ethanol. The precipitate was transferred into a small volume of 70% ethanol and washed twice to remove any remaining salt. The DNA pellet was dried under vacuum and redissolved by gentle mixing in 1 ml sterile, distilled water at 4°C overnight.

The DNA concentration was determined as described in section 5.4.1. The average yield was 200 µg.

5.5.2 Extraction of DNA from lymphoblasts

DNA was isolated according to the method of Davis et al. (submitted).

Cultured lymphoblasts (1 x 10^7 cells) were harvested by centrifugation at 2 000x g for 10 minutes in a MSE bench top centrifuge and washed twice with phosphate-buffered saline. The cells were suspended in 200 µl of cold RSB (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$) containing 2% (v/v) Triton X-100 and transferred to sterile microfuge tubes. The cells were suspended by gentle pipetting. The cells were left on ice for 5 minutes and then the nuclei were pelleted for

1 minute at 4°C in a microfuge. The supernatant was transferred to a clean microfuge tube. The pellet was resuspended in RSB-Triton X-100 and centrifuged as before. This supernatant was added to the previous supernatant.

The pellet was resuspended in 450 µl of 2x SSC (20x SSC is 3 M NaCl, 0.3 M trisodium citrate, pH 7.6). 50 µl of 10% (w/v) SDS and 50 µl of 1 mg/ml proteinase K (Boehringer Mannheim) was added. The samples were then incubated at 50°C for 20-24 hours. The extract was placed in a 30 ml Corex tube and 500 µl of phenol (TE-saturated) was added and the tubes were then shaken gently for one hour at room temperature. 100 µl of 3 M sodium acetate, pH 5.5 and 10 ml of cold 95% ethanol were added and the tube inverted several times to mix the contents. The DNA was spooled onto a pasteur pipette, transferred to a microfuge tube and washed in 70% ethanol. The residual ethanol was removed in a vacuum dessicator. 500 µl of TE, pH 7.5 was added to the pellet and the DNA was left at 4°C for 16-20 hours to dissolve. The DNA was extracted with an equal volume of phenol (TE-saturated) and then with chloroform-octanol. 1/10 volume 3 M sodium acetate and 2.5 volumes of cold ethanol were added to the aqueous phase. The DNA was collected by spooling, washed in 70% ethanol, dried and resuspended in 500 µl of TE as described above.

The nucleic acid concentration and the purity of the DNA were determined as described in section 5.4.1.

5.5.3 Southern analysis of genomic DNA

5.5.3.1 Restriction enzyme digestion

Genomic DNA (15 µg) was digested to completion with 30-40 units of restriction enzyme (Amersham, Boehringer Mannheim, Biolabs) in the presence of 1/10 volume restriction enzyme buffer (according to manufacturer's specifications) and 100 ug/ml nuclease-free bovine serum albumin (Sigma). The reaction was incubated at 37°C for a minimum of 6 hours. The reaction was terminated by the addition of 1/10 volume 100 mM EDTA, pH 8.0. The DNA was precipitated by the addition of 1/10 volume 4 M NaCl and 2 volumes of ice-cold absolute ethanol. The DNA was recovered by centrifugation, washed in 70% ethanol and dried under vacuum. The DNA was dissolved in 34 µl sterile distilled water with gentle mixing at 4°C.

5.5.3.2 Agarose gel electrophoresis of DNA

A 0.8% agarose gel was prepared by boiling 1.2 g agarose in 150 ml TAE buffer (40 mM Tris-HCl, pH 7.7, 40 mM acetic acid and 1 mM EDTA). Ethidium bromide (final concentration 1 µg/ml) was added prior to pouring the gel into the gel apparatus (MAX Submarine agarose gel unit, Hoefer Scientific Instuments). The gel was allowed to set for at least one hour.

The DNA samples were incubated at 37°C for 15 minutes to ensure that

the DNA was completely dissolved. 6 ul orange-G/Ficoll (1% (w/v) orange-G, 20% Ficoll and 20mM EDTA) was added to the DNA samples.

The gel apparatus was filled with TAE buffer and the samples loaded into the wells. The samples were run into the gel at 15 volts. The voltage was then adjusted to 40 volts and electrophoresis performed for at least 18 hours or until the orange-G front had disappeared off the end of the gel.

When electrophoresis was completed, the gel was viewed on an ultraviolet light box and photographed with a ruler adjacent to the molecular weight markers. Lambda/Hind III fragments were used as molecular weight markers (Table 3). The reciprocal of the migration distance of the fragments was plotted against the known sizes of the fragments. This curve was used to determine the sizes of the HPRT DNA fragments after autoradiography.

5.5.3.3 Transfer of DNA

The gel was removed from the electrophoresis apparatus and the DNA denatured by soaking the gel with gentle shaking for 1 hour in 300 ml of 0.5 M NaOH, 1.5 M NaCl. The gel was rinsed twice in distilled water and then neutralized with gentle shaking for 1 hour in 300 ml of 0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl, 1 mM EDTA.

The gel was placed on a piece of gel bond (hydrophilic side) and a

piece of Hybond-N membrane, with the same dimensions as the gel, placed on the gel, ensuring that no air bubbles were trapped in between. Two pieces of Whatman 3MM filter paper, which were presoaked in 2x SSC (20x SSC is 3 M NaCl, 0.3 M trisodium citrate, pH 7.6), were placed on top of the Hybond-N membrane, again ensuring that no air bubbles were trapped. Tissues (3-5 cm in height) were placed on top of the filter paper and finally a glass plate with weights (approximately 0.5 kg) was placed on top of the tissues. The transfer was allowed to occur overnight and then the tissues and filter paper removed. The position of the wells was marked on the Hybond-N membrane which was then rinsed in 2x SSC for 10 minutes. The membrane was then placed on an ultraviolet light box for 3-5 minutes to cross-link the DNA to the membrane.

5.5.3.4 Nick translation of the HPRT probe

The HPRT recombinant DNA was labelled to a specific activity of 5×10^7-1×10^8 dpm/µg DNA by incubating 0.5 µg intact plasmid (or eluted insert) with 20 µl nucleotide buffer solution (100 µM dATP, 100 µM dGTP, 100 µM TTP in a concentrated buffer solution containing Tris-HCl, pH 7.8, $MgCl_2$ and 2-mercaptoethanol, Amersham nick translation kit), 70 µCi [alpha-^{32}P] dCTP (Amersham, specific activity 3 000 Ci/mmol) and 10 µl enzyme solution (5 units DNA polymerase 1 and 100 pg DNase 1 in a buffer solution containing Tris-HCl, pH 7.5, $MgCl_2$, glycerol and bovine serum albumin, Amersham nick translation kit) in a total volume of 100 µl. The reaction was allowed to proceed at 16°C for 90 minutes. At the end of the reaction, the labelled DNA was

separated from the unincorporated nucleotides on a 8 x 0.5 cm Sephadex G-50 (medium) column. 12 fractions (150 µl each) were collected using TE, pH 7.5 as the eluting buffer. 1/100 volume aliquots of each fraction were counted by Cerenkov radiation (Williams and Wilson, 1975). The fractions containing the DNA peak were pooled.

Alternatively, the labelled DNA was separated from the unincorporated nucleotides using the spun column procedure (Maniatis et al., 1982). A 1 ml disposable syringe was plugged with sterile glass wool and a column prepared using Sephadex G-50 equilibrated with STE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100mM NaCl). The syringe was placed in a centrifuge tube and centrifuged at 1 600x g for 4 minutes. Sephadex was added until the column volume was 0.9 ml. 0.1 ml STE was added and the column recentrifuged at exactly the same speed and for exactly the same time as before. This equilibration step was repeated before applying the DNA sample to the column. The column was centrifuged at exactly the same speed and for exactly the same time as before. The effluent (labelled DNA) was collected in a decapped cryotube. The unincorporated nucleotides remain on the column. The DNA was denatured by incubating it in a boiling waterbath for 5 minutes and was immediately placed on ice to prevent reannealing of the strands.

5.5.3.5 Hybridization of the membrane

The membrane was wet by flotation in 2x SSC and then washed in 3x SSC, 0.1% (w/v) SDS, 10 µg/ml polyadenylic acid, 50 µg/ml sonicated salmon sperm DNA and 0.25% (w/v) BLOTTO at 65°C with shaking for at least 2

hours.

BLOTTO (Bovine Lacto Transfer Technique Optimizer, Johnson et al. (1984)) is nonfat dry milk which is used to block non-specific reactions instead of Denhardts solution (0.2% BSA (fraction V), 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400).

The membrane was hybridized with shaking at 65°C for 18 hours in fresh prehybridization solution with addition of the ^{32}P-labelled probe.

The membrane was then washed in the following solutions to remove non-specifically bound probe:

3x SSC, 0.1% (w/v) SDS at 65°C for 2x 2 minutes

3x SSC, 0.1% (w/v) SDS at 65°C for 2x 30 minutes

0.2x SSC, 0.1% (w/v) SDS at 65°C for 2x 30 minutes.

The washes were monitored with a handheld Geiger counter and if the amount of radioactivity remaining on the membrane was significantly above background, the final, stringent wash was repeated. The membrane was sealed in a plastic bag while still damp.

5.5.3.6 Autoradiography

The membrane was autoradiographed using either Kodak X-omat MA X-ray film or Amersham Hyperfilm MP and a Dupont intensifying screen at -70°C for 1-10 days as required.

The film was developed in either Kodak X-ray developer or Dupont X-ray developer for 3 minutes, rinsed in 2% glacial acetic acid for 20 seconds and fixed in Amfix fixative (Maybaker) with hardener for 10 minutes. The autoradiograph was then washed in running water for 15 minutes and dried.

5.6 RNA STUDIES

5.6.1 Extraction of RNA from cultured lymphoblasts

RNA was prepared from cultured lymphoblasts using three different methods.

5.6.1.1 Guanidinium chloride method

RNA was isolated using a modification of the method of Cox (1968).

Cultured lymphoblasts (1 x 10^7 cells) were harvested by centrifugation at 2 000x g for 10 minutes in a MSE bench top centrifuge and washed twice with normal, sterile saline. The cell pellet was resuspended in 4.75 ml of guanidinium chloride and 250 µl of potassium acetate, pH 8.0 and then sonicated to disrupt the cells. The cellular debris was then pelleted at 2 000x g for 15 minutes at 4°C. The supernatant was decanted and 2.5 ml of 95% ethanol added to precipitate the nucleic acid at -20°C overnight.

The precipitate was recovered by centrifugation at 32 000x g for 15 minutes at -10°C. The pellet was resuspended in 475 µl of guanidinium chloride, 50 µl of EDTA, pH 6.0 and 25 µl of potassium acetate, pH 5.0 by vortexing and sonicating. The supernatant was recovered by centrifugation at 8 000x g for 10 minutes at -10°C. The remaining pellet was re-extracted in the same way and the supernatant combined

with that of the previous step. Half the volume ice-cold 95% ethanol was added to the combined supernatants which were then incubated for 1-2 hours at -20°C. The precipitate was recovered by centrifugation at 8 000x g for 15 minutes at -10°C and resuspended in 475 µl of guanidinium chloride, 50 µl of EDTA, pH 6.0 and 25 µl of potassium acetate, pH 5.0 by vortexing and sonicating. The RNA was precipitated by the addition of half the volume of 95% ethanol and incubated at -20°C overnight. The RNA was recovered by centrifugation at 10 000x g for 15 minutes at -10°C. The pellet was resuspended in 200 ul of ice-cold 95% ethanol by vortexing. The RNA was recovered by centrifugation at 10 000x g for 15 minutes at -10°C. The pellet was drained by placing the tube upside down on paper towels for 20 minutes at -20°C. The RNA was resuspended in 200 µl of 20 mM EDTA, pH 7.0 and the purity determined by scanning a dilution of the sample from 220 to 310 nm. The concentration was calculated from the absorbance at 260 nm (40 µg/ml RNA solution has an OD = 1).

5.6.1.2 RSB Method

RNA was isolated according to the method of Davis et al. (submitted).

Cultured lymphoblasts (1 x 10^7 cells) were harvested by centrifugation at 2 000x g for 10 minutes in a MSE bench top centrifuge and washed twice with phosphate buffered saline. The cells were suspended in 200 µl of cold RSB (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$) containing 2% (v/v) Triton X-100 and transferred to sterile microfuge tubes. The cells were suspended by gentle pipetting. The cells were

allowed to stand on ice for 5 minutes and then the nuclei were pelleted for 1 minute at 4°C in a microfuge. The supernatant was transferred to a clean microfuge tube and kept on ice. The pellet was resuspended in RSB-Triton X-100 and centrifuged as before. This supernatant was added to the previous supernatant.

400 µl of TE, pH 7.5 and 500 µl of phenol (TE-saturated) were added to the combined supernatants. After gentle shaking at room temperature for 3 minutes, the phases were separated by centrifugation in a microfuge. The aqueous phase was extracted twice with phenol:chloroform (TE-saturated) and then twice with chloroform:octanol (24:1). The RNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate, pH 5.5 and 2.5 volumes of ethanol at -20°C overnight. The RNA was recovered by centrifugation in a microfuge. The pellet was washed twice in 70% ethanol, dried under vacuum and dissolved in 100 µl sterile, distilled water. The concentration was determined as described in section 5.6.1.1.

5.6.1.3 Guanidinium thiocyanate method

RNA was isolated according to the method of Chomczynski and Sacchi (1987).

Cultured lymphoblasts (1 x 10^7 cells) were harvested and washed as described previously. The pellet was resuspended in 1 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Sequentially, 0.1 ml of 2 M sodium

acetate, pH 4, 1 ml of phenol (water-saturated) and 0.2 ml of chloroform:isoamyl alcohol (49:1) were added to the suspended pellet with inversion of the tube after the addition of each reagent. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 minutes.

Samples were centrifuged at 10 000x g for 20 minutes at 4°C. After centrifugation, RNA was present in the aqueous phase and the DNA and proteins in the interphase and phenol phase. The aqueous phase was transferred to a clean tube and 1 ml of isopropanol added. The tube was placed at -20°C for at least 1 hour to precipitate the RNA. The RNA was recovered by centrifugation at 10 000x g for 20 minutes at 4°C. The pellet was dissolved in 0.3 ml of solution D and precipitated with 1 volume of isopropanol at -20°C for 1 hour. The RNA was recovered by centrifugation at 10 000x g for 10 minutes at 4°C. The pellet was resuspended in 75% ethanol, sedimented, vacuum dried and dissolved in 50 µl 0.5% (w/v) SDS at 65°C for 10 minutes.

The concentration of RNA was determined as described in section 5.6.1.1.

5.6.2 Northern analysis of RNA

5.6.2.1 Electrophoresis of RNA

Electrophoresis was performed according to the method of Maniatis

et al. (1982).

A 1.5% gel was prepared by melting the agarose in water and when it had cooled to 60°C, 10x gel buffer (0,2 M morpholinopropanesulfonic acid (MOPS), pH 7.0, 50 mM sodium acetate, 10 mM EDTA, pH 8.0) and formaldehyde were added to final concentrations of 1x and 2.2 M respectively.

The RNA sample was prepared by adding 50% (v/v) deionized formamide, 2.2 M formaldehyde and 1x gel buffer (final concentrations) and incubating at 55°C for 15 minutes. 2 µl loading buffer (50% glycerol, 1 mM EDTA, 0.4% xylene cyanol, 0.4% bromophenol blue) was added to the RNA sample which was then loaded into the gel. The gel was electrophoresed in 1x gel buffer at 30 volts overnight. Once the samples had run into the gel, the gel buffer was circulated continuously. The gel was either stained or the RNA transferred onto Hybond-N membrane (Amersham).

5.6.2.2 Staining of RNA gel

The gel was washed in distilled water and then washed in 0.1 M ammonium acetate for 30 minutes. It was then stained in 0.1 M ammonium acetate, 0.5 µg/ml ethidium bromide for 60 minutes and destained in 0.1 M ammonium acetate for 45 minutes. The gel was then viewed on an ultraviolet light box. The 18S and 28S rRNA fragments were visible and used as molecular weight markers.

5.6.2.3 Transfer of RNA to Hybond-N membrane

The gel was placed on 2 pieces of Whatman 3MM paper which had been presoaked in 20x SSC. Hybond-N membrane, with the same dimensions as the gel, was placed on the gel ensuring that there were no air bubbles trapped in between. Two pieces of Whatman 3MM paper were then placed over the Hybond membrane. Tissues (3-5 cm in height) were placed over the 3MM paper and finally a glass plate with weights (approximately 0.5 kg) was placed on top. Once blotting was completed (overnight), the weights, tissues and 3MM paper were removed and the positions of the wells marked on the Hybond membrane. The membrane was rinsed in 2x SSC, air dried and wrapped in cling film. It was then placed RNA side down on an ultraviolet light box for 2-5 minutes to cross-link the RNA to the membrane.

5.6.2.4 Hybridization of RNA blots

The membranes were washed in 5x SSPE (20x SSPE is 3.6 M NaCl, 0.2 M sodium phosphate, pH 7.7, 0.002 M Na_2EDTA), 50% (v/v) formamide, 0.5% (w/v) SDS, 0.25% (w/v) BLOTTO, 0.5 mg/ml sonicated salmon sperm DNA at 42°C for at least 2 hours.

The membranes were hybridized with shaking at 42°C for 18 hours in fresh prehybridization solution with addition of the ^{32}P-nick translated cDNA probe (described in section 5.5.3.4).

The membranes were washed in the following solutions to remove the

non-specifically bound probe:

2x SSPE, 0.1% (w/v) SDS at 42°C for 2x 15 minutes

1x SSPE, 0.1% (w/v) SDS at 42°C for 30 minutes

0.1x SSPE, 0.1% (w/v) SDS at room temperature for 2x 15 minutes.

The washes were monitored with a handheld Geiger counter and the final high stringency wash was repeated if the filters emitted radiation significantly above background.

5.6.2.5 Autoradiography

These procedures were carried out as described in section 5.5.3.6.

5.7 cDNA CLONING

The cDNA was cloned into a lambda gt10 vector using a cDNA cloning system (Amersham). The enzyme buffers were supplied in the kit. The composition of these buffers was not specified.

5.7.1 Preparation of cDNA for cloning

5.7.1.1 cDNA synthesis

cDNA was prepared according to a modification of Ullrich's lab protocol, California, USA. Total RNA prepared from lymphoblasts was used as a template. Methylmercuryhydroxide was added to 40 ug RNA to a final concentration of 10 mM and heated at 60°C for 5 minutes (to disrupt secondary structures) and then snap-cooled. The RNA was added to a tube containing 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM TTP, 0.5 mM dCTP (final concentrations), 10 μCi [alpha-^{32}P] dCTP, 1x RT buffer (50 mM Tris-HCl, pH 8.3, 100 mM KCl, 6 mM $MgCl_2$), 10 mM DTT (sequesters the mercury ions which would otherwise inhibit the reverse transcriptase enzyme), 100 μg/ml oligo dT_{12-18}, 500 units/ml RNasin (final concentrations) and 40 units of AMV reverse transcriptase (Seikagaku America). The reaction was incubated at 42°C for 60 minutes.

To the first strand reaction, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM TTP, 1 unit RNase H (Amersham), and 2 mM DTT were added. A further 40 units of AMV reverse transcriptase was added and the

reaction incubated at 42°C for 60 minutes. 1x Klenow buffer (10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl) and 20 units of DNA polymerase 1 "Klenow fragment" (Amersham) was added. The reaction was incubated at 15°C for 20 hours.

7.5 µl of 0.5 M EDTA, pH 8.0 was added and the cDNA extracted once with an equal volume of chloroform:octanol (24:1). The cDNA was precipitated with 1/4 volume of 10 M ammonium acetate and 2 volumes of absolute ethanol on crushed dry ice for 10 minutes and recovered by centrifugation at 0°C. The pellet was resuspended in 250 ul 0.3 M sodium acetate (to remove any remaining ammonium acetate) and the cDNA precipitated with 750 µl of absolute ethanol. The pellet was washed in 95% ethanol, dried under vacuum and resuspended in sterile distilled water.

5.7.1.2 Size selection of cDNA

The double-stranded cDNA was digested with the restriction enzymes Msp I and Rsa I as described in section 5.4.2. This step was included to increase the percentage of HPRT-containing recombinants in the library which is important when cloning rare messages. Only cDNAs which were of a similar size to HPRT were cloned into the vector. (This digestion step removes the necessity for an S1 nuclease step which digests the hairpin loop. The hairpin loop acts as a primer for the synthesis of the second strand).

After the digest was complete, the cDNA was blunt-ended (blunt ends are

required for the addition of linkers) by the addition of 0.1 M dATP, 0.1 M dCTP, 0.1 M dGTP, 0.1 M TTP and 5 units of DNA polymerase 1 "Klenow fragment". The reaction was incubated at room temperature for 30 minutes. A further 5 units of DNA polymerase 1 "Klenow fragment" was added and the reaction continued at 15°C for 60 minutes. The reaction was terminated by the addition of 15 mM EDTA, pH 8.0 (final concentration). The cDNA was extracted once with phenol:chloroform (TE-saturated) and once with chloroform:octanol (24:1). The cDNA was precipitated by the addition of 2 volumes of absolute ethanol and 1/10 volume 3 M sodium acetate. The cDNA was recovered by centrifugation, washed in 95% ethanol, dried under vacuum and resuspended in TE, pH 7.5.

The cDNA was electrophoresed on a 1.2% agarose gel and fragments between 603 and 1078 base-pairs (sizes of two of the ØX174/Hae 111 fragments, Table 3) eluted as described in section 5.4.2.

5.7.2 Cloning of cDNA into the lambda gt10 vector

5.7.2.1 Methylation of cDNA

The cDNA was cloned into the Eco RI site of lambda, thus any internal Eco RI sites must be protected from digestion by Eco RI. This is accomplished using the enzyme Eco RI methylase which methylates the adenine in the Eco RI recognition site (GAATTC). It is known that HPRT cDNA has no internal Eco RI sites and a Southern blot of T.K.'s genomic

DNA digested with Eco RI showed no difference in banding patterns between normal and T.K., thus no Eco RI site has been created in T.K.'s DNA. The methylation was thus not necessary and was not performed.

5.7.2.2 Addition of Eco RI cohesive termini to cDNA

1 ug of phosphorylated Eco RI linkers were ligated to the cDNA in the presence of 1/10 volume L buffer (Amersham cloning kit). 5 units of ligase was added and the reaction incubated at 15°C for 15-20 hours. The reaction was terminated by heating at 70°C for 10 minutes.

5.7.2.3 Eco RI digestion of cDNA

The cDNA was digested with 100 units of Eco RI in the presence of 1/10 volume E buffer (Amersham cloning kit) at 37°C for a minimum of 5 hours. The reaction was terminated by heating at 70°C for 10 minutes.

5.7.2.4 Separation of cDNA from unligated linkers

The cDNA was separated from unligated phosphorylated linkers using the column supplied in the kit. 10 fractions (200 ul) were collected using STE (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) to elute the fractions. The two major fractions, containing the cDNA, were identified by scintillation counting of a small aliquot of each

fraction. These fractions were pooled and the cDNA precipitated with 0.3 M sodium acetate and 2.5 volumes of ethanol. The cDNA was recovered by centrifugation in a microfuge for 30 minutes. The pellet was dried under vacuum and resuspended in 2.5 µl STE, pH 7.5.

5.7.2.5 Ligation to lambda gt10 arms

The cDNA was ligated to 1 µg of lambda gt10 arms in the presence of L buffer (Amersham cloning kit) and 2.5 units of T4 DNA ligase. The ligation reaction was incubated at 15°C for 16-20 hours. At the end of the reaction, the DNA was precipitated with 1/10 volume 3 M sodium acetate and 2.5 volumes of absolute ethanol in an ethanol-dry ice bath for 15 minutes. The DNA was recovered by centrifugation in a microfuge for 15 minutes, the pellet dried under vacuum and resuspended in 2.5 µl of TE, pH 7.5.

A number of control reactions were included. Uncut lambda gt10 vector (0.25 µg) was used to determine the efficiency of the in vitro packaging reactions and the biological selection of the two host cell types. Lambda gt10 arms were used to determine the efficiency of the ligation reaction. Control DNA was treated in the same way as the cDNA (although it was also methylated) and ligated to lambda gt10 arms. This control determines the efficiency of the cloning procedure.

5.7.2.6 In vitro packaging of ligation mixtures

Two cell extracts were used for *in vitro* packaging. They are both derived from induced lambda lysogens whose prophages carry different but complementing mutations in the genes required for assembly of mature phage particles. Extract A is prepared from *E. coli* BHB2688 [N205 *rec A*$^-$ (lambda *imm434 cIts b2 red3 Eam4 Sam7*/lambda] and does not allow the insertion of DNA. Extract B is prepared from *E. coli* BHB 2690 [N205 *rec A*$^-$ (lambda *imm 434 cIts b2 red 3 Dam 15 Sam 7*/lambda] and does not allow the formation of the capsid. When these two extracts are mixed together with DNA, the DNA is packaged into infectious phage particles.

The extracts were removed from the -70°C freezer and allowed to thaw on ice. As soon as they had thawed, 10 µl of extract A was added to the ligation reaction and then 15 µl of extract B was immediately added to this. The contents of the reaction were mixed gently with the pipette tip which was used for the transfer. The packaging reaction was incubated at 20°C for 2 hours. At the end of the incubation, 0.5 ml of SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin) and 10 µl of chloroform were added. This packaged reaction was stored at 4°C. (The growth of bacteria is inhibited by the chloroform).

5.7.2.7 Preparation of phage plating cells

Glycerol stocks of *E.coli* L87 and *E.coli* NM514 (selective host) were

used to streak plates. Individual colonies were picked from each plate and used to inoculate 10 ml of LB medium + 0.2% maltose which was incubated with shaking at 37°C overnight. 1 ml of each overnight culture was added to 50 ml of pre-warmed LB medium + 0.2% maltose and was incubated with shaking at 37°C until the OD_{600} = 0.5 units. After cooling the culture on ice, it was centrifuged at 1 000x g for 10 minutes at 4°C. The pellet was resuspended in 15 ml of ice-cold 10 mM $MgSO_4$ and the cells stored at 4°C.

5.7.2.8 Titration of lambda gt10 recombinants

A dilution series in duplicate was made for each packaged reaction. 100 µl of each dilution was added to 100 µl of plating cells and incubated at 37°C for 20 minutes to allow adsorption of the phage to the cells. 3 ml of liquid top agar (1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.25 g $MgSO_4$, 0.7 g agar in 100 ml distilled water, pH 7.0) was added to each dilution and poured onto L-agar plates (10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g Difco Bacto-agar in 1 litre distilled water, pH 7.0). The top agar was allowed to set and then the plates were incubated at 37°C overnight. The number of plaques was counted and the titre calculated.

5.7.3 Screening of the recombinant library

5.7.3.1 Amplification of the library

Half of the library was amplified. 250 µl of phage stock was added to 200 µl of a fresh, overnight culture of E.coli NM514 and incubated at 37°C for 20 minutes. 7 ml liquid top agarose was added and poured onto 145 mm L-plates + 0.2% glucose. The plates were incubated at 37°C for 6-8 hours or until complete lysis occurred. The plates were stored at 4°C overnight. 7 ml of SM buffer and 100 µl of chloroform were added to each plate which were then incubated with agitation for 2 hours at room temperature. The SM buffer was removed and centrifuged at 3 000x g for 10 minutes. The supernatant was pipetted into a clean tube and 200 µl of chloroform added. This high titre lysate stock was stored at 4°C. The titre was calculated as described in section 5.7.2.8. The titre was calculated to be 10^9 plaque forming units (pfu) per millilitre.

5.7.3.2 Preparation of replica membranes

1.2×10^5 pfu of the amplified library and 6×10^4 pfu of the unamplified library were used for screening.

The phage were plated onto agarose plates at a concentration of 8×10^3 pfu/plate. The phage were mixed with 100 µl of fresh, overnight culture of E.coli NM514 and incubated at 37°C for 20 minutes.

3 ml of liquid top agarose was added and poured onto 85 mm plates. The plates were incubated at 37°C for 6-8 hours and then stored at 4°C overnight. A Hybond-N membrane was placed on the plate for 1 minute. Before removal, the orientation was marked by piercing asymmetrically through the membrane and the agarose with a needle. The membrane was immersed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes, then immersed in 2 rinses of neutralizing solution (0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl) for 5 minutes each and finally rinsed in 6x SSC for 5 minutes. The membrane was then air dried, plaque side up on a sheet of filter paper. It was then UV-irradiated plaque side down for 4 minutes to cross-link the DNA to the membrane.

5.7.3.3 Hybridization of membranes

Membranes were wet in 2x SSC and then prewashed in 6x SSC, 0.1% (w/v) SDS, 50 µg/ml sonicated salmon sperm DNA, 10 µg/ml polyadenylic acid, 0.25% (w/v) BLOTTO, 0.06% (w/v) sodium pyrophosphate at 65°C for 4 hours.

The membranes were hybridized in fresh prehybridization solution containing radioactively labelled probe (method described in section 5.5.3.4) at 65°C for 18 hours.

The membranes were then washed at 65°C in the following solutions to remove nonspecifically bound probe:

6x SSC, 0.1% (w/v) SDS, 0.06% (w/v) sodium pyrophosphate for 30 minutes

2x SSC, 0.1% (w/v) SDS, 0.06% (w/v) sodium pyrophosphate for 30 minutes

0.2x SSC, 0.1% (w/v) SDS, 0.06% (w/v) sodium pyrophosphate for 2x 30 minutes.

5.7.3.4 Autoradiography

The membranes were sealed in plastic bags while still damp and autoradiographed using Hyperfilm-MP (Amersham) and a Dupont intensifying screen. The X-ray film was exposed at -70°C overnight. The film was developed as described in section 5.5.3.6.

5.7.3.5 Identification and picking of positive plaques

The X-ray film was aligned with the corresponding agarose plate using the asymmetric needle stabs. Positive areas were picked using a pipette tip and placed in 200 µl of SM buffer and a drop of chloroform was added. These were stored at 4°C.

5.7.4. Phage DNA mini-preparation

Phage DNA was prepared from plate lysates by a modification of the method of Davis et al. (1980). Complete lysis was allowed to occur as described in section 5.7.3.1. 7 ml of SM buffer and 100 µl of chloroform were added to the plates which were then incubated with

agitation for 2 hours at room temperature. The medium was centrifuged at 3 000x g for 10 minutes and 4 ml of the supernatant was incubated at 37°C for 30 minutes with 10 ug of DNase 1 (Miles Laboratory) and 10 ug of RNase A (Sigma). (The remainder of the supernatant was stored as an amplified stock). EDTA was added to 20 mM to terminate the nuclease digestion. The phage was then lysed by incubation for 5 minutes at 70°C in 150 mM Tris-HCl, pH 8.0, 0.15% (w/v) SDS, 0.15% (v/v) dimethylsulfoxide. Sodium acetate was added to 0.3 M and the lysate incubated at 4°C for 2 hours. After centrifugation at 10 000x g for 10 minutes at 4°C, 1 volume of isopropanol was added to the supernatant.

Phage DNA was then precipitated at -20oC overnight. The DNA was recovered by centrifugation, the pellet dried under vacuum and resuspended in 400 ul TE, pH 8.0. The DNA was extracted twice with phenol (TE-saturated), twice with phenol:chloroform (TE-saturated), once with chloroform and finally with ether. Traces of remaining ether were removed by heating to 65°C. The DNA was reprecipitated with 2 volumes of ethanol and 0.5 M ammonium acetate (final concentration) at -20°C overnight. The DNA was recovered by centrifugation in a microfuge, washed in 70% ethanol, dried under vacuum and resuspended in 100 ul of TE, pH 7.5.

5.7.4.1 Analysis of phage DNA by Southern blotting

1/10 of the phage DNA was digested with 10 units Eco RI in a total volume of 20 ul at 37°C for 2 hours. The DNA was electrophoresed on a 1.2% agarose gel with a ØX174/*Hae* 111 molecular weight marker as

described in section 5.5.3.2. The gel was blotted and hybridized as described in sections 5.5.3.3-5.5.3.6.

5.7.4.2 Analysis of phage DNA by end-labelling

Phage DNA was digested with Eco RI (Amersham) in the presence of 1/10 volume Eco RI buffer (10 mM Tris-HCl, pH 7.5, 60 mM NaCl, 7 mM $MgCl_2$, 7 mM beta-mercaptoethanol) at 37°C for 2 hours. 1 unit of DNA polymerase 1 "Klenow fragment" (Amersham) was added and the DNA incubated for 20 minutes at room temperature. 1 µl of 2 mM dGTP, 2 mM dATP and 2 mM TTP and 1 µCi [alpha-^{32}P] dCTP (Amersham, 3 000 Ci/mmol) were added and the reaction allowed to continue for 15 minutes. The DNA was incubated at 65°C for 10 minutes to terminate the reaction. The DNA was electrophoresed on a 1.2% agarose gel together with a ØX174/Hae III molecular weight marker. When electrophoresis was complete, the gel was dried in a gel dryer and autoradiographed as described in section 5.5.3.6.

5.8 S1 NUCLEASE ANALYSIS

5.8.1 Subcloning of HPRT insert into pGEM-3 vector

pGEM-3 is a 2 867 base-pair plasmid vector containing a SP6 and a T7 promoter and a multiple cloning site (Figure 24).

5.8.1.1 Restriction enzyme digest of the plasmids

5 µg of p4aA8 and 0.5 µg of pGEM-3 were digested with 10 units each of Bam HI and Pst I (Amersham) in the presence of 1/10 volume of medium-salt buffer at 37°C for 2 hours. The DNA was then extracted with phenol (TE-saturated), followed by a chloroform extraction and finally extracted with ether. The DNA was precipitated with 2 volumes of ethanol on dry ice for 10 minutes and recovered by centrifugation, washed in 70% ethanol and dried under vacuum. The pellet was resuspended in TE, pH 7.5.

5.8.1.2 Ligation of the DNA fragments

The DNA fragments were ligated together in the presence of 1/10 volume ligation buffer (0.5 M Tris-HCl, pH 7.4, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10mM spermidine, 1mg/ml BSA, 10mM ATP) and 5 units of ligase (Amersham) in a total volume of 20 µl. The ligation reaction was incubated at 14°C for 20 hours. The DNA was incubated at 65°C for

10 minutes to terminate the reaction.

5.8.1.3 Transformation of the bacteria

The transformation was performed according to the method of Maniatis et al. (1982).

10 ml of LB medium was inoculated with an overnight culture of E. coli DK1 and incubated at 37°C with vigorous shaking until a cell density of 5 x 10^7 was reached. The cells were then centrifuged in 2 ml aliquots at 4 000x g for 10 minutes at 4°C . The pellet was resuspended in 1 ml of 10 mM MOPS, pH 7.0, 10 mM rubidium chloride (RbCl). The cells were recovered by centrifugation at 4 000x g for 10 minutes at 4°C. The pellet was resuspended in 1 ml of 0.1 M MOPS, pH 6.5, 50 mM calcium chloride ($CaCl_2$), 10 mM RbCl and then placed on ice for 15 minutes. The cells were recovered by centrifugation at 4 000x g for 10 minutes at 4°C. The pellet was resuspended in 0.2 ml of 0.1 M MOPS, pH 6.5, 50 mM $CaCl_2$, 10mM RbCl. 3 µl of dimethylsulfoxide and 200 ng of ligated DNA were added and the mixture placed on ice for 30 minutes. The cells were heat-shocked at 44°C for 90 seconds. 1 ml of LB medium was added and the mixture incubated at 37°C for 60 minutes. 50 µl of this mixture was spread onto ampicillin plates (LB plates with 35 µg/ml ampicillin) which were incubated at 37°C overnight.

5.8.2 Analysis of the recombinants

The recombinants were analysed by preparing mini-preps of a few of the colonies according to a modification of the method of Holmes and Quigley (1981).

5 ml of LB medium containing 35 µg/ml ampicillin (Sigma) was inoculated with a single colony and incubated overnight at 37°C with vigorous shaking. The cells were centrifuged at 4 000x g for 10 minutes. The pellet was resuspended in 80 µl STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-HCl, pH 8.0). 5 µl lysozyme (10 mg/ml stock freshly prepared in 10 mM Tris-HCl, pH 8.0) was added and the bacterial suspension vortexed, boiled for 40 seconds and then centrifuged at 10 000x g for 20 minutes. The supernatant was placed in a clean tube and extracted sequentially with phenol (TE-saturated), phenol:chloroform (TE-saturated), chloroform and ether. Any remaining ether was removed by heating to 65°C. The DNA was precipitated with 3 volumes of ethanol, recovered by centrifugation at 10 000x g for 10 minutes at 4°C, washed in 70% ethanol and dried under vacuum. The DNA was resuspended in 20 µl TE, pH 7.5.

10 µl of the DNA was digested with Bam HI and Pst I (Amersham) in the presence of 1/10 volume medium-salt buffer at 37°C for 2 hours. 1 µl of 10 mg/ml RNase A (Sigma) was then added and the reaction incubated at 37°C for 10 minutes.

The DNA was then analysed on a 1.2% agarose gel alongside a lambda/BstE II molecular weight marker (Table 3).

5.8.3 Preparation of the recombinant plasmid

40 ml of LB medium containing 35 µg/ml ampicillin was inoculated with the recombinant plasmid and incubated at 37°C overnight with vigorous shaking. The cells were centrifuged at 1 500x g for 10 minutes. The pellet was resuspended in 2 ml of 20% sucrose, 25 mM Tris-HCl, pH 8.0 and kept on ice. 100 µl of 10 mg/ml lysozyme (freshly prepared in 20% sucrose, 25 mM Tris-HCl, pH 8.0) was added and the suspension left on ice for 10 minutes. 2.5 ml of Triton X-100 lytic mix (0.1 (v/v) Triton X-100, 25 mM Tris-HCl, pH 7.5, 50 mM EDTA) was added and the suspension gently agitated and left on ice for 10 minutes. The suspension was centrifuged at 20 700x g for 20 minutes at 4°C. The supernatant was poured into a clean tube and extracted once with phenol (TE-saturated) and then with chloroform. One volume of cold isopropanol was added to the aqueous phase which was then placed at -20°C for 20 minutes to precipitate the nucleic acids. The nucleic acid was recovered by centrifugation at 7 800x g for 20 minutes at 4°C. The nucleic acid was washed in 70% ethanol, dried and resuspended in 400 µl TE, pH 7.5. 1 µl of 10 mg/ml RNase A (heat-treated) was added and the reaction incubated at 37°C for 15 minutes. The nucleic acid was then extracted sequentially with phenol (TE-saturated), phenol:chloroform (TE-saturated), chloroform and ether. Any remaining ether was removed by heating to 65°C.

Sodium acetate was added to 0.3 M and 2 volumes of ethanol were added and the nucleic acid precipitated on dry ice for 10 minutes. The nucleic acid was recovered by centrifugation at 10 000x g for 10 minutes at 4°C. The pellet was washed in 70% ethanol, dried under

vacuum and resuspended in 400 µl sterile, distilled water.

Supercoiled DNA was precipitated according to the Promega-Biotec technical manual. 100 µl of 4 M sodium chloride was added to the nucleic acid solution and mixed. 500 µl of 13% polyethylene glycol (PEG) 8 000 (Sigma) was added and mixed. The reaction was incubated at 4°C for 60 minutes. The DNA was recovered by centrifugation at 10 000x g for 10 minutes at 4°C. The DNA was washed in 70% ethanol, dried under vacuum and resuspended in 100 µl TE, pH 7.5.

The DNA concentration was determined as described in section 5.4.1.

5.8.4 Transcription of riboprobe

The transcription was performed according to a modification of Melton et al. (1984b).

The antisense strand was transcribed by incubating 1 µg of Pst I cut pGEM-3-HPRT with 1x transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 10 mM dithiothreitol, 23 units RNasin (Amersham), 2.5 mM ATP, 2.5 mM GTP, 2.5 mM CTP, 50 µCi [alpha-^{32}P] UTP and 15 units of T7 polymerase (Promega-Biotec) at 40°C for 60 minutes.

The DNA template was removed by the addition of 23 units of DNase (RNase-free, Boehringer Mannheim) and incubating the reaction at 37°C

for 10 minutes. The RNA was then extracted with phenol:chloroform (TE-saturated) and precipitated by the addition of 1/10 volume 5 M ammonium acetate and 1 volume isopropanol. The RNA was recovered by centrifugation in a microfuge for 20 minutes at 4°C, washed in 70% ethanol and dried under vacuum. The RNA was resuspended in 100 µl of diethylpyrocarbonate-treated water. A 1 µl aliquot was counted to determine the yield of RNA synthesized.

5.8.5 S1 nuclease assay

Aliquots (200 000 cpm) of the ^{32}-P antisense RNA were mixed together with:

a) 50 µg control RNA,

b) 50 µg T.K. RNA,

c) 50 µg B.K. RNA,

d) no RNA

10 ng of carrier tRNA and 1 volume of isopropanol were added to each aliquot and the RNA precipitated on crushed dry ice for 10 minutes. The RNA was recovered by centrifugation in a microfuge for 20 minutes at 4°C, washed in 70% ethanol and dried under vacuum. The RNA was resuspended in 100 µl of hybridization solution consisting of 20 µl 5x buffer (2 M NaCl, 0.2 M Na_2PIPES, pH 6.5, 5 mM EDTA) and 80 µl deionised formamide. The samples were incubated at 65°C for 15 minutes and then incubated at 45°C overnight.

The unprotected RNA was then digested with 100 units of S1 nuclease (Amersham) in 100 µl of S1 buffer (0.25 M NaCl, 30 mM sodium acetate,

pH 4.6, 1 mM $ZnSO_4$, 20 µg/ml sonicated salmon sperm DNA) at 30°C for 60 minutes. The RNA was extracted with phenol:chloroform (TE-saturated) and finally 20 µg of carrier tRNA was added to the aqueous phase. One volume isopropanol was added and the RNA precipitated on crushed dry ice for 10 minutes. The RNA was recovered by centrifugation in a microfuge for 20 minutes at 4°C, washed in 70% ethanol and dried under vacuum. The pellet was resuspended in 4.5 µl diethylpyrocarbonate-treated water. Fifty percent (v/v) formamide, 2.2 M formaldehyde and 1x gel buffer (final concentrations) were added and the samples incubated at 55°C for 15 minutes. 2 µl loading buffer was added and the samples electrophoresed on a 1.5% denaturing agarose gel (described in section 5.6.2.1).

When electrophoresis was complete, the gel was dried on a gel dryer and autoradiographed as described in section 5.5.3.6.

DNA	Fragment number	Number of base pairs	Photograph of electrophoresed fragments
Lambda DNA-*Hind* III digest	1	23 130	
	2	9 419	
	3	6 557	
	4	4 371	
	5	2 322	
	6	2 028	
	7	564	
	8	125	
			1.0% agarose
Lambda DNA-*BstE* II digest	1	8 454	
	2	7 242	
	3	6 369	
	4	5 686	
	5	4 822	
	6	4 324	
	7	3 675	
	8	2 323	
	9	1 929	
	10	1 371	
	11	1 264	
	12	702	
	13	224	
	14	117	
			1.0% agarose
ϕX174 RF DNA-*Hae* III digest	1	1 353	
	2	1 078	
	3	872	
	4	603	
	5	310	
	6b	281	
	6a	271	
	7	234	
	8	194	
	9	118	
	10	72	
			1.7% agarose

Table 3 Fragment sizes of DNA molecular weight markers (Fragment sizes obtained from the New England Biolabs catalogue)

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