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Cloning of a human galactokinase gene (GK2) on chromosome 15 by complementation in yeast

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ABSTRACT

Galactokinase deficiency is inherited as an autosomal recessive trait with a heterozygote frequency estimated to be 0.2% in the general population. The major clinical manifestation of galactokinase deficiency is neonatal and possibly presenile cataract formation.

The genetic locus responsible for clinical galactokinase deficiency has not been determined, and thus the precise molecular alterations responsible for this disease are uncharacterized. We have therefore sought to clone a human galactokinase gene as a first step in our effort to identify the molecular defects that cause this deficiency.

A human cDNA encoding a galactokinase was isolated by complementation of a galactokinase-deficient (gal1$^-$) strain of Saccharomyces cerevisiae. This cDNA encodes a predicted protein of 459 amino acids with 29% overall identity to galactokinase of Saccharomyces carlsbergensis. Previous studies have mapped a human galactokinase gene (GK1) to chromosome 17q23-25, closely linked to thymidine kinase. The galactokinase gene that we have isolated (GK2) is located on chromosome 15. The relationship between the disease locus for galactokinase deficiency galactosemia and the two galactokinase loci is unknown.

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INTRODUCTION

Overview

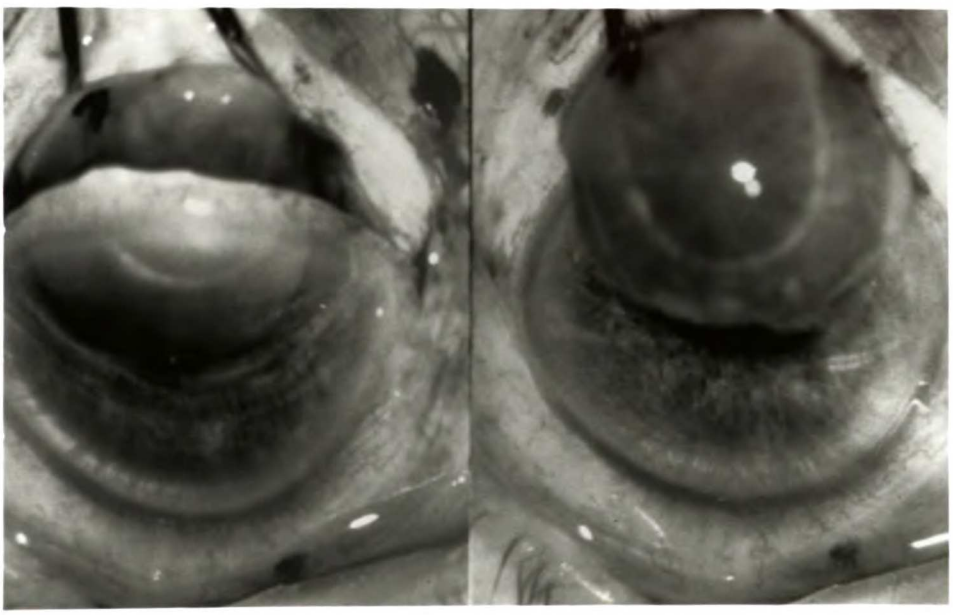
A cataract is any opacity occurring in the lens and is not considered to be of clinical significance unless it interferes with vision (Figure 1). Currently, the only effective treatment is surgical cataract extraction (Figure 2). Despite the success of surgery, cataracts remain the most common cause of treatable blindness in the world, as not all affected individuals have access to this procedure (1). It has been estimated that 5 to 10 million individuals in the United States become visually impaired each year because of cataract formation (2).

Starting in the middle ages, uneducated persons in the barber-surgeon guilds, referred to as "oculists" and "cataract couchers," performed crude surgical manipulations on cataract patients with limited success (3). With the refinement of surgical instruments and skill over time, cataract extraction in the modern era has become an extremely effective curative procedure. Surprisingly, no other effective treatment has been found.

Partly due to the multiplicity of etiologies, the mechanism of cataract formation is not clearly understood. Theoretically, if one could understand the pathophysiology of cataract formation at the molecular level, pharmacologic intervention could be possible. One approach would be to study a single gene disorder which is known to cause cataracts prematurely to serve as a simplified model for cataract formation. A useful

Figure 1. Cataract formation in the lens

Figure 2. Extracapsular cataract extraction procedure



feature of this stategy is that we would be able to isolate a precise molecular alteration and examine its pathologic manifestations. Given the wide range of sources often cited for the cause of cataract formation (i.e., the aging process, exposure to UV radiation, diabetes, etc.), this single-gene approach can clarify an otherwise very complicated and cumbersome picture.

I have, therefore, chosen to study galactokinase deficiency, one of three known forms of the disease galactosemia, the main clinical manifestations of which is neonatal cataract formation (4). This deficiency may also be responsible for pre-senile cataract formation in adults (5,6,7). Understanding galactokinase deficiency will yield insights into how cataracts develop. As a first step to this end, my thesis research focused on cloning a human galactokinase gene in an effort to identify the molecular defects that cause galactokinase deficiency galactosemia. Once we can characterize the precise molecular alterations responsible for galactokinase deficiency, we can effectively screen newborns to determine if they are heterozygote carriers or homozygotes with this disorder using PCR technology. A long-term goal beyond this thesis would be to develop a transgenic mouse model that carries the precise defect responsible for galactokinase deficiency galactosemia, which would enable us to test the effectiveness of new therapeutic modalities. This simplified model would also provide insights into how cataract forms in general.

Galactosemia

Galactosemia is a general term for a hereditary disorder in humans characterized by the inability to convert galactose into glucose. The three known toxicity syndromes associated with this disorder are transferase deficiency galactosemia, galactokinase deficiency galactosemia, and epimerase deficiency galactosemia (4).

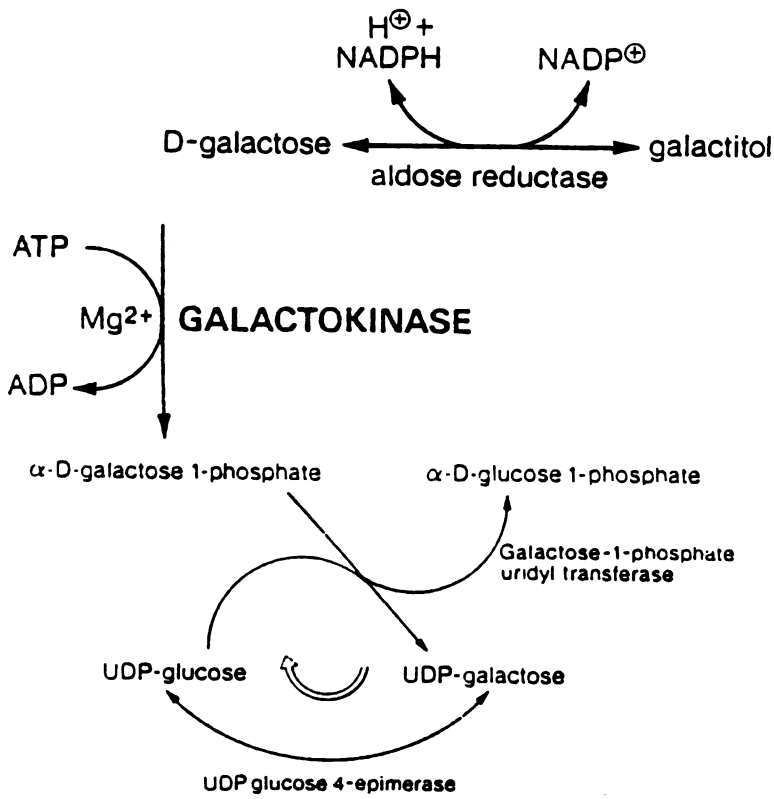
The Basic Physiology and Biochemistry of Galactose Metabolism in Humans

Galactose is ingested as lactose, the main carbohydrate in human milk. Lactose is hydrolyzed at the small intestinal brush border into its two monosaccharide components--galactose and glucose. Following absorption, glucose and galactose are circulated to the liver in the portal vein. Hepatic galactose can be incorporated into glycolipids and glycoprotein or converted into glucose (8).

The studies of Leloir and Kalckar in the 1940's and 1950's showed that the biochemical steps required to convert galactose into glucose involve the presence of at least three specific enzymes (Figure 3) (9,10). Galactokinase catalyzes the phosphorylation of Galactose using adenosine triphosphate (ATP) to produce galactose-1-phosphate (Gal-1-P) . Next, galactose-1-phosphate uridyl transferase transfers the uridyl moiety of diphosphoglucose (UDPG) to Gal-1-P to form uridine diphosphogalactose (UDPGal) . The enzyme uridine diphosphogalactose-4-epimerase regenerates UDPG by inverting the orientation of the hydroxyl group at

Figure 3. Galactose Metabolism in Human (Ref. 14)

GALACTOSE CATABOLISM IN HUMANS



position C4 in the galactosyl portion of UDPGal. Clinical syndromes associated with an inherited disorder of galactose utilization result from a defect in any of these enzymes needed to metabolize galactose.

To place my work on galactokinase deficiency galactosemia into perspective, I will also describe in this introduction transferase and epimerase deficiency galactosemia.

Transferase Deficiency Galactosemia

Clinical manifestation. Although first noted by von Reuss in 1908, it was not until 1956 that a deficiency in the enzyme galactose-1-phosphate uridyl transferase (GALT) was found to be the cause of this disease (11). GALT is inherited as an autosomal recessive trait with a homozygote frequency of 1 in 50,000 newborns (12). Infants with this condition appear to be normal at birth, but within a few days of a milk diet they begin to vomit, fail to gain weight, appear lethargic, and show enlargement of the liver and spleen (13). Prolonged jaundice during the neonatal period is common, and ascites and edema may develop. In severe cases, death occurs because of malnutrition and wasting. Cataracts have been noted within a few days of birth. Because they consist of punctate lesions in the fetal lens nucleus, they can be observed only on slit-lamp examination by the ophthalmologist. Mental retardation may ensue. When the milk or milk product is removed from the infant's diet, signs and symptoms regress.

Cloning of the human GALT gene. Reichardt and Berg (1988) isolated a cDNA encoding GALT (14). A pool of oligonucleotide hybridization probes were synthesized based on regions of amino acid identities between homologous enzymes from E. coli and S. cerevisiae. After screening a human cDNA library with these probes, Reichardt and Berg identified a 1.4 kb cDNA coding for an open reading frame (ORF) with 25 % overall similarity to the amino acid sequences of E. coli and S. cerevisiae GALT. In order to prove that the cDNA encodes a transferase, the investigators introduced the putative GALT cDNA into monkey kidney cells and demonstrated a 40- to 60-fold increase in transferase activity in the recipient cell. The cloned GALT gene was mapped to human chromosome 9 by southern hybridization analysis. Only those human-chinese hamster somatic cell hybrids carrying chromosome 9 had a human specific band pattern that was detected with the GALT cDNA as a hybridization probe. This result is consistent with a study by Khan and Robson which had previously mapped the GALT gene to human chromosome 9 band p13 by detecting GALT activity from cell extracts of various human-chinese hamster somatic hybrid cell lines containing subset of human chromosome (15). Recent studies have shown that in patients with transferase deficient galactosemia, the GALT cDNA was found to have missense mutations at two sites within a highly conserved region between species (16). Thus, the GALT gene identified by Reichardt and Berg appears to be affected in patients with transferase deficiency galactosemia.

Polymorphisms of the human GALT gene. Since Beutler described the Duarte variant of the GALT protein (17), over 10 other variants of the enzyme have been identified by means of parameters such as red cell

enzyme activity, electrophoretic mobility, and immunologic cross-reactivity (18). In almost every case, affected individuals have no clinical symptoms. The Duarte variant gene is allelic with the gene for normal GALT and is transmitted as an autosomal recessive trait. Hemolysates from Duarte variant homozygote have approximately half the normal transferase activity. Reichart and Woo have performed molecular characterization of two GALT polymorphisms from a cell line of a Duarte variant individual and have demonstrated the replacement of leucine 62 by methionine and asparagine 314 by aspartate (16). Interestingly, GALT polymorphisms affected only highly variable residues of the enzyme.

Although GALT deficiency has a higher rate of incidence than galactokinase deficiency galactosemia and has been more extensively studied, pleiotropic clinical consequences of transferase deficiency galactosemia make it a less appropriate model for understanding the pathophysiology of cataract formation.

Epimerase Deficiency Galactosemia

Among ten documented cases of patients with epimerase deficiency galactosemia, eight patients were asymptomatic (19). In these patients, the enzyme deficiency was limited to red blood cells alone. Two infants with generalized deficiency of epimerase presented with clinical manifestations very similar to those seen in neonates with transferase deficiency galactosemia (20,21). Because of the relatively rare incidence of epimerase deficiency galactosemia and the uncertainty of its clinical symptoms, this form of galactosemia also does not serve as the best model to study the process of the opacification of the lens.

Galactokinase Deficiency Galactosemia

Clinical manifestations. Galactokinase deficiency is also inherited as an autosomal recessive trait and has a heterozygote frequency estimated to be 0.2 % in the general population (12, 22, 23, 24). Note that this is a very crude estimate of gene frequency for galactokinase deficiency. In Mayes and Guthrie's study (1968), 642 blood samples were obtained from donors at the Roswell Park Memorial Institute, employees of Children's Hospital, university students, and children at a school for the blind. When assayed for galactokinase activity, six individuals had activity about one-half the normal value and were considered to be heterozygotes for galactokinase deficiency. The study of Levy and Hammersen in 1978 reported only 6 out of 6 million infants screened was homozygous for galactokinase deficiency.

The notable absence of inanition (a debilitated condition due to lack of sufficient food material), gastrointestinal dysfunction, and jaundice in the newborn period, as well as the appearance of cataracts only on an unrestricted diet, differentiate this disorder from transferase deficiency galactosemia.

Galactokinase deficiency was first described in 1965 (25). H.K., a blind 9-year-old gypsy boy with cataracts was referred to the University Children's Hospital in Zurich for pediatric examination. Fanconi diagnosed H.K.'s condition as mellituria and noted the curious finding that elimination of milk from the boy's diet caused relief of the mellituria. Gitzelman located this man again 32 years later living in a home for the

blind and observed total absence of galactokinase enzymatic activity in his blood (26). Hence, cataract formation was attributed to galactokinase deficiency.

In patients lacking this enzyme, galactose accumulates in the lens of the eye and is converted by aldose reductase to galactitol, a sugar to which the lens is impermeable (27). As a result, it is thought that the lens begins to accumulate water in response to the osmotic effects of galactitol, and lens permeability increases. The lens fibers swell and eventually rupture, forming vacuoles. Neonatal presentation is a uniform zonular cataract that has been described as an "oil drop."

Relationship between heterozygotes and pre-senile cataracts: a hypothesis. Beutler and Monteleone (1971) were the first to suggest that heterozygotes for galactokinase deficiency might have increased susceptibility to early cataract formation (5). Subsequent studies have been contradictory and inconclusive. Skalka and associates (1979) studied 50 patients considered to have pre-senile cataract (onset before age 50) and found that 12 patients had decreased levels of galactokinase activity (6). Of 39 cataract patients over 50 years of age tested for galactokinase activity, only 2 demonstrated reduced galactokinase activity. Stevens and associates studied 46 patients found to have idiopathic presenile cataract before age 55, and observed that none had galactokinase enzyme levels of less than 2 standard deviations from the median(23). A few other studies have also addressed this issue, all yielding contradictory and inconclusive results (7,28,29).

Polymorphisms of a human galactokinase. Tedesco has observed a racial polymorphism for red blood cell galactokinase, with galactokinase activity significantly lower in American black populations than in comparable groups of white subjects (30). The allele responsible for this polymorphism was named the Philadelphia variant allele of galactokinase. Almost a decade following Tedesco's study, Soni and associates noted that individuals with the Philadelphia variant form of galactokinase exhibited reduced GK activity in erythrocytes but normal activity in their white blood cells (31). Further, all individuals with Philadelphia variant allele were found to be asymptomatic.

Mapping of a human galactokinase gene. The genetic locus responsible for galactokinase deficiency galactosemia has not been determined, and thus the precise molecular alterations responsible for this disease are uncharacterized. A human galactokinase gene GK1 was previously mapped to human chromosome 17 by assaying galactose phosphorylation activity in cell extracts from various mouse-human hybrid cell lines (32, 33, 34,35). Galactose phosphorylation activity was measured by autoradiography after precipitating ^{14}C galactose-1-phosphate with lanthanum chloride in starch gels. It is not known if GK1 is the gene that is affected in patients with galactokinase deficiency.

Other features of galactokinase deficiency. There are several other key issues regarding galactokinase deficiency galactosemia that need to be resolved. It is unclear why there is such a discrepancy in clinical sequelae of patients with different enzymatic defects (e.g., defective in GK vs. GALT) that affect the same metabolic pathway. It is also quite intriguing that

apparently only the lens is affected in galactokinase deficiency. Clearly, there are many gaps in our understanding of human galactokinase deficiency. The identification and isolation of a human galactokinase should provide a starting point to answer the questions raised above and offer insights into how cataracts form at the molecular level.

Cloning strategy for a human galactokinase gene. Several strategies can be used to clone a human homologue of a known yeast or bacterial gene: PCR amplification with degenerate primers designed from structural similarities, low stringency hybridization to detect similar nucleotide sequences, and antibody screening of expression libraries. We have employed a more novel method that is amenable to genetic analysis and manipulation-- complementation of a yeast mutant to isolate a human gene with similar function.

Galactokinase serves the same function in yeast as it does in bacteria and humans, the phosphorylation of galactose leading to its utilization as a carbon source (36). In yeast, the GAL1, GAL7, and GAL10 genes encode the galactokinase, transferase, and epimerase enzyme (37,38). We have therefore sought to clone the human galactokinase gene by its ability to substitute for the yeast enzyme encoded by GAL1.

Several other mammalian cDNAs have recently been identified by complementation or other functional activity in yeast. Lee and Nurse cloned a human homologue of the fission yeast cell cycle control gene CDC2 by expressing a human cDNA library in S. pombe and selecting those clones which could complement a mutation in the cdc2 gene (39). Subsequently, the same strategy was utilized to clone human DNA ligase I cDNA and others (40, 41, 42,43).

I report here on the main goal of my thesis research: the cloning of a cDNA that encodes human galactokinase by complementation of a galactokinase-deficient (gal1$^-$) strain of Saccharomyces cerevisiae. I have made the unexpected finding that this new gene, GK2, does not map to chromosome 17. It is thus not clear whether the locus responsible for galactokinase deficiency disease affects GK1 or GK2.

METHODS

Yeast Strains, Media, and Transformation

The recipient yeast strain used in transformations was Saccharomyces cerevisiae YM20 (MATa ura3-1 ura3-2 trpl-289 his3-532 ade2-1(ochre) gal1-del111 GAL2+ CRY1s), strain 1947 from the Herskowitz laboratory collection. A human hepatoma cell line HepG2 cDNA library was constructed in the yeast expression vector pAB23BXN by Anthony Brake at Chiron Corporation (Emeryville CA) (40). This expression vector contains a yeast origin of replication, the yeast URA3 gene, and the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter which governs expression of the human cDNA in yeast cells. The cDNA inserts are flanked by Bgl II sites. PBM48 (44) contains the yeast GAL1 gene in the vector YCp 19, and YCp 50 is a yeast low-copy vector.

Screening

A modification of the lithium acetate method was used for yeast transformation (45). Most transformants were selected on a standard yeast synthetic medium lacking uracil and containing galactose as the sole carbon source (46). The total number of transformants tested was determined by plating on yeast synthetic medium lacking uracil but containing glucose as the sole carbon source. Approximately 150,000 transformants were tested, 200 colonies per plate.

Subcloning and DNA Sequencing

Plasmid pJJGK was isolated by first extracting total yeast DNA and then transforming E. coli strain DH5a via electroporation, selecting for ampicillin resistance. pJJGK was found to contain a 1.5kb cDNA insert. pJJGK was digested with Bgl II, which divided the cDNA insert into 1.0kb and 0.5kb fragments. Each of these two cDNA fragments was subcloned into M13 mp19 (47) and propagated in JM 107. DNA sequencing was carried out by the dideoxy chain termination method (48). Four oligonucleotide primers were designed from the determined sequence to complete the sequencing on both strands, which included determining the sequence across the Bgl II site used for subcloning.

DNA and Protein Sequence Analysis

A FASTA program for searching amino acid sequence data bases (49) was carried out using the EUGENE sequence analysis program (Baylor University). GENBANK (release 68.0, June 1991), PIR-Protein (release 28.0, March 1991), PIR-Nucleic (release 36.0, March 1990), EMBL (release 27.0, May 1991) gene and protein sequence databases were searched for homology to the GK2 gene sequence (50). Phylogenetic tree sequence analysis was performed using the Doolittle Multiple Alignments Program (51).

Northern Hybridization Analysis.

Human fibroblast cell line (Hs27) was grown in Minimal Eagle's Medium and dialysed fetal calf serum. These cells were grown under two

separate conditions: in the presence of galactose (0.1%), and glucose (0.1%). Poly (A)+ RNA was isolated from total mRNA by binding to oligo (dT)-cellulose. Samples were electrophoresed on a 1% agarose gel containing formaldehyde and blotted onto nylon filters (Hybond N, Amersham). The blots were hybridized with ^{32}P-labeled 1.0kb cDNA fragments labeled by random priming. Hybridization conditions included 5x SSC, 1x Denhardt's solution, 0.1% SDS, 0.05M NaPO4 pH 6.5, 50% (v/v) formamide, and denatured herring sperm DNA (100 ug/ml) at 42°C for 20 hours. After incubation, the blot was washed four times for 5 minutes in 2x SSC/0.1%SDS at room temperature, then two times for 15 minutes in 0.1x SSC/0.1% SDS at 55°C. Transcript sizes were determined by migratory patterns of synthetic RNA fragments (0.16-1.77 kilobase (kb) and 0.24-9.5 kb RNA ladder, BRL) visualized by ethidium bromide staining.

Polymerase Chain Reaction (PCR) Technique

Amplification by PCR used an Ericomp thermal cycler and a modified version of the standard protocol provided with the Perkin-Elmer/Cetus GeneAmp DNA kit (52). The cDNA insert in pJJGK was amplified with primers hG5'-25 (5' GCGGACAAGCTGCCCAGCTTTCTAG 3') corresponding to positions 1260 to 1284, and hG3'-26 (5' GTGGCACAGAAAGTCCTGCCAATTCC 3') corresponding to position 1464 to 1439. After 10 min at 94°C to denature the genomic DNA, reactions ran for 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. The reaction mixture contained 67 mM Tris (pH 8.8), 6.7 mM Mg Cl 2, 16.6 mM (NH4)2 SO4 , 10mM beta mercaptoethanol, 50 pM of each primer, 0.1 ug of uncut genomic DNA or 1 ng of pJJGK and 2.5 units

of Ampli-Taq DNA polymerase (Perkin-Elmer/Cetus). A well-characterized panel of human-mouse somatic cell hybrids retaining subsets of human chromosomes was purchased from NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research (53, 54, 55, 56, 57, 58). Total human genomic DNA was a generous gift of Margit Burnmeister, Department of Human Genetics, University of Michigan. DNA samples from human-mouse hybrid cell lines A59-3Aaz10a and MH 22.4 were obtained from Huntington F. Willard (Stanford University) and Pragna Patel (Baylor University), respectively.

To characterize our somatic cell hybrid panel for the presence of human chromosomes 15 and 17, the same PCR conditions and templates were used as described above to amplify a part of the human b2-microglobulin gene (B2M) and the human gastrin gene (GAS) (59). B2MI (5'CACCCAGTCTAGTGCATGCCTTCT 3') and B2M2 (5'TGAGAAGGAAGTCACGGAGCGAGA 3') and GAS1 (5'ATGCTAGTCGGTGTAGAGCCATG 3') and GAS2 (5'TTGTACCTCATAGGGCTGCGTGA 3') were designed to produce a 357-bp and a 297-bp fragment from human chromosomes 15 and 17 respectively.

RESULTS

Isolation of a Human cDNA Clone by Complementation in Yeast

A HepG2 cDNA expression library was introduced into yeast strain YM21, which contains a deletion of the gene encoding galactokinase (GAL1). Transformants were plated on selective media containing galactose as the sole carbon source. Since galactokinase is required to phosphorylate galactose to galactose-1-phosphate, growth on this medium requires that the human cDNA expresses functional galactokinase activity. One yeast transformant with the ability to grow on this medium was identified in approximately 150,000 transformants tested.

We first determined whether growth on galactose medium was plasmid dependent by selecting for cells which had lost this plamid by growth on plates containing 5-fluoro-orotic acid (5-FOA) (60). Cells cured of the plasmid in this manner were no longer able to grow on galactose medium (data not shown). A plasmid, designated pJJGK, was isolated from the original transformant by transformation into bacteria and then reintroduced into strain YM21. All Ura^+ retransformants were able to grow on galactose medium, suggesting that this plasmid encoded a galactokinase.

The cDNA insert of pJJGK was sequenced, and a single large open reading frame (ORF) was identified which has the potential to encode a 458 amino acid protein (Figure 4). The amino acid sequence of this ORF shows extensive similarity to galactokinase from yeasts and bacteria: 29% overall identity is observed to the GAL1 protein of *Saccharomyces carlsbergensis*,

Figure 4. Nucleotide sequence and deduced amino acid sequence of a human galactokinase cDNA. Primer used for PCR analysis were the following: positions 1260-1284 (hG5'-25; GCGGACAAGCTGCCCAGCTTTCTAG 3') and positions 1464-1439 (hG3'-26; 5' GTGGCACAGAAAGTCCTGCCAATTCC 3') .

```
                                                                                Met Ala Thr Glu Ser Pro Ala Thr Arg Arg Val      11
                                                AGATCTGAATTCGGCGAAAT ATG GCT ACA GAG AGC CCT GCT ACG CGT CGG GTC      53

Gln Val Ala Glu His Pro Arg Leu Leu Lys Leu Lys Glu Met Phe Asn Ser Lys Phe Gly Ser Ile Pro Lys Phe Tyr Val Arg      39
CAG GTG GCA GAA CAT CCT AGG TTA CTG AAG CTA AAG GAG ATG TTT AAC TCC AAG TTT GGA TCT ATT CCC AAG TTT TAT GTT CGA     137

Ala Pro Gly Arg Val Asn Ile Ile Gly Glu His Ile Asp Tyr Cys Gly Tyr Ser Val Leu Pro Met Ala Val Glu Gln Asp Val      67
GCA CCA GGA AGA GTC AAC ATA ATA GGA GAG CAT ATA GAT TAT TGT GGA TAT TCT GTT CTT CCT ATG GCT GTA GAA CAA GAT GTG     221

Leu Ile Ala Val Glu Pro Val Lys Thr Tyr Ala Leu Gln Leu Ala Asn Thr Asn Pro Leu Tyr Pro Asp Phe Ser Thr Ser Ala      95
CTA ATA GCT GTA GAA CCT GTG AAA ACG TAC GCT CTC CAA CTG GCC AAT ACA AAT CCC TTG TAT CCG GAC TTC AGT ACT AGT GCT     305

Asn Asn Ile Gln Ile Asp Lys Thr Lys Pro Leu Trp His Asn Tyr Phe Leu Cys Gly Leu Lys Gly Ile Gln Glu His Phe Gly     123
AAT AAC ATC CAG ATT GAT AAA ACC AAG CCT TTG TGG CAC AAC TAT TTC TTA TGT GGA CTT AAA GGA ATT CAG GAA CAC TTT GGT     389

Leu Ser Asn Leu Thr Gly Met Asn Cys Leu Val Asp Gly Asn Ile Pro Pro Ser Ser Gly Leu Ser Ser Ser Ser Ala Leu Val     151
CTT AGT AAC CTG ACT GGA ATG AAC TGC CTG GTA GAT GGA AAT ATC CCA CCA AGT TCT GGC CTC TCC AGC TCC AGT GCT TTG GTC     473

Cys Cys Ala Gly Leu Val Thr Leu Thr Val Leu Gly Arg Asn Leu Ser Lys Val Glu Leu Ala Glu Ile Cys Ala Lys Ser Glu     179
TGT TGT GCT GGC TTG GTG ACG CTC ACA GTG CTG GGA AGG AAT CTA TCC AAG GTG GAA CTT GCA GAA ATC TGT GCC AAG AGT GAG     557

Arg Tyr Ile Gly Thr Glu Gly Gly Gly Met Asp Gln Ser Ile Ser Phe Leu Ala Glu Glu Gly Thr Ala Lys Leu Ile Glu Phe     207
CGT TAC ATT GGC ACT GAA GGA GGA GGC ATG GAC CAG TCT ATA TCA TTT CTT GCA GAA GAA GGA ACT GCC AAG TTG ATA GAA TTT     641

Ser Pro Leu Arg Ala Thr Asp Val Lys Leu Pro Ser Gly Ala Val Phe Val Ile Ala Asn Ser Cys Val Glu Met Asn Lys Ala     235
AGT CCT CTG AGG GCA ACC GAT GTA AAA CTC CCA AGT GGA GCA GTG TTT GTG ATT GCC AAC AGT TGT GTG GAG ATG AAT AAG GCA     725

Ala Thr Ser His Phe Asn Ile Arg Val Met Glu Cys Arg Leu Ala Ala Lys Leu Leu Ala Lys Tyr Lys Ser Leu Gln Trp Asp     263
GCA ACT TCC CAT TTC AAT ATC AGG GTG ATG GAG TGT CGG CTG GCT GCG AAG CTC CTG GCT AAA TAC AAA AGC TTG CAA TGG GAC     809

Lys Val Leu Arg Leu Glu Glu Val Gln Ala Lys Leu Gly Ile Ser Leu Glu Glu Met Leu Leu Val Thr Glu Asp Ala Leu His     291
AAA GTA CTG AGG CTG GAG GAG GTG CAG GCT AAA CTA GGG ATT AGT CTA GAA GAA ATG CTG TTG GTC ACA GAA GAT GCC CTT CAT     893

Pro Glu Pro Tyr Asn Pro Glu Glu Ile Cys Arg Cys Leu Gly Ile Ser Leu Glu Glu Leu Arg Thr Gln Ile Leu Ser Pro Asn     319
CCT GAA CCC TAT AAC CCT GAG GAG ATC TGC AGG TGT CTG GGA ATT AGC CTG GAG GAA CTC CGA ACC CAA ATC CTG AGT CCA AAC     977

Thr Gln Asp Val Leu Ile Phe Lys Leu Tyr Gln Arg Ala Lys His Val Tyr Ser Glu Ala Ala Arg Val Leu Gln Phe Lys Lys     347
ACT CAA GAT GTG CTC ATC TTC AAA CTC TAT CAG CGG GCA AAG CAT GTG TAC AGC GAG GCT GCG CGA GTG CTC CAG TTT AAG AAG    1061

Ile Cys Glu Glu Ala Pro Glu Asn Met Val Gln Leu Leu Gly Glu Leu Met Asn Gln Ser His Met Ser Cys Arg Asp Met Tyr     375
ATA TGT GAA GAA GCA CCT GAA AAC ATG GTC CAG CTG CTG GGA GAG TTG ATG AAC CAG AGC CAC ATG AGC TGC CGG GAC ATG TAT    1145

Glu Cys Ser Cys Pro Glu Leu Asp Gln Leu Val Asp Ile Cys Arg Lys Phe Gly Ala Gln Gly Ser Arg Leu Thr Gly Ala Gly     403
GAG TGC AGC TGC CCC GAG CTG GAT CAG CTG GTG GAC ATC TGT CGG AAG TTT GGG GCT CAA GGG TCA CGA CTT ACT GGA GCA GGA    1229

Trp Gly Gly Cys Thr Val Ser Met Val Pro Ala Asp Lys Leu Pro Ser Phe Leu Ala Asn Val His Lys Ala Tyr Tyr Gln Arg     431
TGG GGA GGC TGT ACA GTA TCA ATG GTA CCT GCG GAC AAG CTG CCC AGC TTT CTA GCA AAT GTG CAC AAA GCT TAT TAC CAG AGG    1313

Ser Asp Gly Ser Leu Ala Pro Glu Lys Gln Ser Leu Phe Ala Thr Lys Pro Gly Gly Gly Ala Leu Val Leu Leu Glu Ala  *     458
AGT GAT GGA AGC TTA GCA CCG GAG AAG CAA AGT TTG TTT GCT ACC AAA CCT GGA GGT GGG GCT TTG GTT TTG CTT GAG GCC TGA    1397

AAAAATGTAAAAAGTCTGAGAGAAACTACTTAGGGCACTTAGGAATTGGCAGGACTTTCTGTGCCACAGTAAATTAATCTTCCTTCTGTTTTGTATTATGATGAACGGTTG    1508
```

31% to GAL1 of Kluyveromyces lactis, 22% to galK of Escherichia coli, 21% to galK of Lactobacillus helveticus, and 16% to the GK gene of Streptomyces lividans (Figure 5). Alignment of these sequences revealed at least six regions (A-F) with particularly striking amino acid identity. For example, the human protein is identical to the two yeast enzyme in 20 of 28 positions in Region A (positions 39-66 of GK2). The human protein is identical to the yeast enzymes in 14/14 positions in Region F (positions 396-409 of GK2). These regions contains many matches to the E. coli galactokinase sequence and to the yeast GAL3 sequence as well. One striking difference between the human and yeast sequences is the region corresponding to positions 75-96 of GK2, which matches the highly-conserved yeast sequences in only 2 of 22 positions. These identities leave little doubt that the GK2 gene that we have cloned complements the yeast galactokinase-deficient mutant by providing galactokinase activity.

A phylogenetic tree-structure comparison between GK2, galactokinases from yeasts and bacteria, and GAL3 of yeast shows a clear distinction in the structural relationship between prokaryotes and eukaryotes (Figure 6). Human GK2 has a completely independent branching point from other known yeast galactokinases. GAL3 protein's similarity to yeast galactokinase was indicated by the very close association to GAL1 of S. carlsbergensis.

Expression of GK2 in Human Fibroblast Cell Line (Hs27)

Northern blot hybridization analysis with the 1.0kb cDNA insert of pJJGK as a probe identified a single 2 kb transcript (Figure 7). Human fibroblasts

Figure 5. Alignment of the deduced amino acid sequences from a human (H.s.), Saccharomyces carlsbergensis (S.c.), Kluyveromyces lactis (K. l.), E. coli (E. c.), and Streptomyces lividans (S. l.) galactokinase genes, and Saccharomyces cerevisiae GAL3 gene. Numbers indicate positions in the GK2 open reading frame. Amino acid identities between the human sequence and the other sequences are indicated by black background with white letters; other identities are shaded in gray. Six distinct regions of similarity (A-F) are indicated. Gaps(dashes) were introduced for optimal alignment. * indicates translation termination codon.

Figure 2

```
                                                                                                                A
H. s.                MATESPATRRVQVAEHPRLLKLKEMFNSKFGSIPKFYVRAPGRVNIIGEHIDYCGYSVLPMAVEQDVLIAVEPVKTYALQLA   82
S. c.   MTKSHSEEVIVPEFNSSAKELPRPLAEKCPSIIKKFISAYDAKPDFVARSPGRVNLIGEHIDYCDFSVLPLAIDFDMLCAVKVLNEK.NPSI
K. l.                MSVPIVPTDFAHAPKLEQLKNEFVECYDTDDSRKFFITRSPGRVNLIGEHIDYCQFSVLPMAIENDLMLACRLTSESENPSI
L. h.                          MNKEELLKKYEATSNEKAKDVFF...SPGRINVIGEHTDYNGGHVFPAPISLGVYGVYGPRDDKKVRLY
E. c.                            MSLKEKTQSLFANAFGYPATHTIQAPGRVNLIGEHTDYNDGFVLPCAIDYQTVISCAPRDDRKVRVM
S. l.                          MGEAVGEPSASGSGSCTGRSRRGCGRRAGRENLIGEHTDYNDGFVMPSPCRTRSRPSPGANDGILRLHS
GAL 3          MNTNVPIFSSPVRDLPRSFEQKHLAVVDAFFQTYHVNLILSLGLLGRVNLIGEHIDYCDFSVLPLAIDVDMLCAVKILDEK.NPSI

                                                B                                                   C
H. s.   NTNPLYPDFSTSAN.......NIQIDKTKPLWHNYFLCGLKGIQEHFGLSNLTGMNCLVD........GNIPPSSGLSSSSALVCCAGLVTL  159
S. c.   TLINADPKFAQRKFDLPLDGSYVTIDPSVSDWSNYFKCGLHVAHSFLKKLAPERFASAPLAGLQVFCEGDIPTCSGLSSSAAFICAVALAVV
K. l.   TLTNHDSNFAQRKFDLPLDGSLIEIDPSVSDWSNYFKCGLLVACQFLQEKYNFKGPVHGMEIYVK...GDIPSGGGLSSSAAFICAVSLAII
L. h.   SGNVDGDIVEFDI......DDTNVEKDEDRFWANYFK.GMITYLREKYDGIDHGFNLYI........EANLPSGSGLSSSAAIEMLMGIILK
E. c.   AADYENQLDEFSL.......DAPIVAHENYQWANYVRGVVKHLQ.....LRNNSFGGVDMVIS.....GNVPQGAGLSSSASLEVAVGTVLQ
S. l.   ADVDADPVELRV.........ADLAPASDKSWTAYPSGVLWALREAG..HELTGADVHLA........STVPSGAGLSSSAALEVR.PLAMN
GAL 3   TLTNADPKFAQRKFDLPLDGSYMAIDPSVSEWSNYFKCGLHVAHSYLKKIAPERFNNTPLVGAQIFCQSDIPTGGGL..SSAFTCAGRLATI

H. s.   TVLGRNLSKVELAEICAK...SERYIGTEGGGMDCSISFLAEEGTAKLIEFSPLRATDVKLPSGAVFVIANSCVEMNKAAT.......  237
S. c.   KANMGPGYHMSKQNLMRITVVADIMLVLTMAVWIRL.PLFAVRKIMLYTLSSNAVEATPFKFPQLKNHEISFVIANTLVVSNKFETAP
K. l.   YSNVPAGTPILKDELTKTTAVAEHHVGVNNGGMDCAASICGIEGHALYVEFKPELKATPFKFPEDLP..ISFLIANTLVVSNKAETGP
L. h.   DEFNLDVDRVSLAKMGQRT..ENEFIGLNSGIMDCFACIMGKKNSAIFLDCNTLKYEYLPLALGDYEIIIMATNNPHTLAD.......
E. c.   QLYHLPLDGAQIALNGQE..AENQFVGCNCGIMDCLISALGKKDHALLIDCRSLGTKAVSMPKGVAVVIINSNFKRTLVGS.......
S. l.   DLYALALRGWCLARLCQR..AENVYVGAPVGIMDCTASACCEAGTPSSSTPATSPSGRSPSTSPPRGCACWSSTPGSS..T.......
GAL 3   RANMGKNFDISKKDLTASQRLRSTMLESIMVVWIKQTSVYGEEDHALYVEFMAKTKWPHFQVSSIENHEISFVIAILCT.........

H. s.   SHFNIRVMECRLAAKLLAKYKSLQWDKVLRLEEVQAKL.......................GISLEE.ML.LVTEDALHPEPYN  296
S. c.   TNYNLRVVEVTTAANVLAATYGVVLPSGKEGSSTNKGNLRDFMNVYYARYHNISTPWNGDIESGIERLTKMLVLVEESLANKKQGF
K. l.   VNYNLRVVEVTVAANVLAQKFGVTLQTEGNLGKGTLRNFMDSYYTKYDKSCRKPWD..GEIQTGIERLNKMLQLVEETLDPNGYTL
L. h.   SAYNNRVAECGRALKKLQQKLDIKALGELDNDTFDEY.....................................................
E. c.   E.YNTRREQCETGARFFQQPALRDVTIEEF.....................................................
S. l.   PTARASTASAARAARRAPRCWASTRCDVPYADLDAAL.....................................................
GAL 3   .RSNNRTLLHISLSCSLLTLLYISTFAREPCGPDTLGLTISQGQSNSERGNLEIYGCLLRPDTKTKPNHGMEISELVLNVYSRCYN

                                                                D
H. s.    PEEI...CRC...........LGISLEELRT...QILSPNTQDVLIFKLYQRAKHVYSEAARVLQFKKICEEAPENMV.....  357
S. c.    SVDDVAQS.............LNCSREE.FTRDYLTTSPVRFQVL..KLYQRAKHVYSESLRVLKAVKLMTTASFTADEDFFK
K. l.    DHAVELCGCE...........SISQFTEL....YLTNFPVRFQRL..KLFQRAKHVYSEALRVLKALQLFQKGESNFF.....
L. h.    ..........................................SYLINDETEIKRARHAVSENQRTLRATQAMKDGDLEK......
E. c.    ..........................................NAVAHELDPIVAKRVRHILTENARTVEAASALEQGDLKR......
S. l.    ..........................................ERLGDEEEVRRLVRHVVTEDERVERVVALLESATP........
GAL 3    WYEESFSRKKSGFTVHEASTALNCSREE.FTRDYLTTFPVRFQVL..KLYQRAKHVYSESLRVLKALKMMTSAIFTRTRFLYR
```

Figure 2

```
                                E                                   F
H. s.    QLLGELMNQSHMSCRDMYECSCPELDQLVDICRKF.GAQ..GSRLTGAGWGGCTVSMVPADKLPSFLANVHKAYYQRSDGSLA   437
S. c.    QF.GVLMNESQASCDKLYECSCPEIDKICSIALSN.GSY..GSRLTGAGWGGCTVHLVPGGPNNGNIEKVKEALANEFYKVKY
K. l.    EEFGALMNESQESCDKLYECSCPETDSICEIALKN.GSF..GSRLTGAGWGGCTVHLCSTDTVDSVKSALTEQYYNLRFPELT
L. h.    ..LGRLINASHESLHYDYEVTGKELDTLAEASWKQPGVL..GARMIGGGFGGSAIAIVKKSEAENFKKNVGKIYRDAVGYDAS
E. c.    ..MGELMAESHASMRDDFEITVPQIDTLVEIVKAVIGDK.GGVRMTGGGFGGCIVALIPEELVPAVQQAVAECYEAKTGIKET
S. l.    GASAPSWSRATPAARRLPHLL.PRAGPGRRHGPGLRGPR...RRMTGGGFGGSAIVLVEAAAVDAVTKAVEDAFAAAGLKRPR
GAL 3    FWPTNE*

H. s.    PEKQSLFATKPGGGALVLLEA*                                                                 458
S. c.    PKITDAELENAIIVSKPALGSCLYEL*
K. l.    AEELEDAIIISKPSLGSVLYE*
L. h.    FYDAEIVDQTKRI*
E. c.    FYVCKPSQGAGQC*
S. l.    VFEAVPRRGAAPGLTVSRAASPACTP*
```

Figure 6. Phylogenetic tree-structure comparison between GK2, galactokinases from yeasts and bacteria, and *Saccharomyces cerevisiae* GAL3.

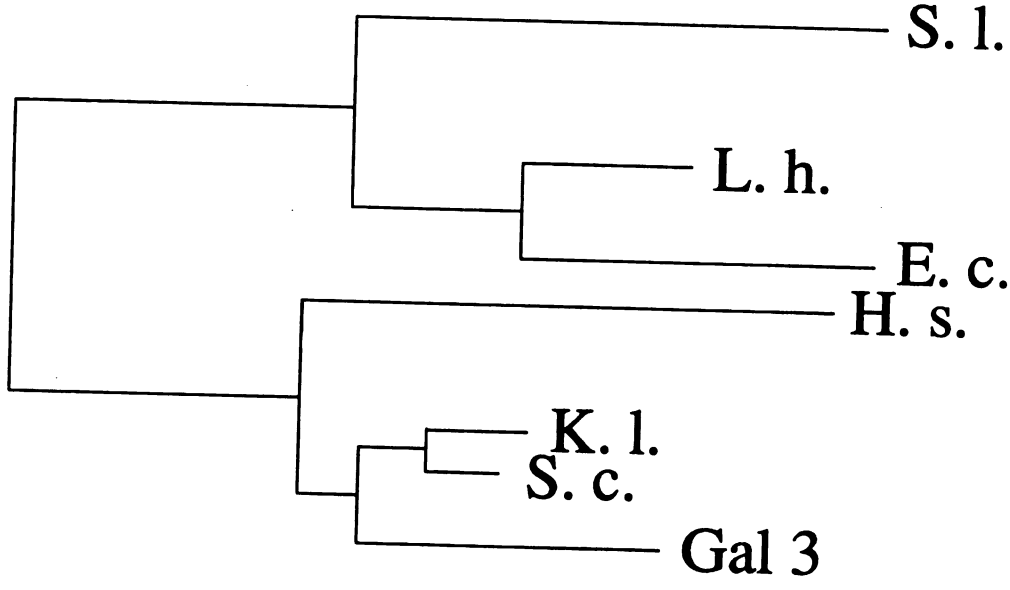
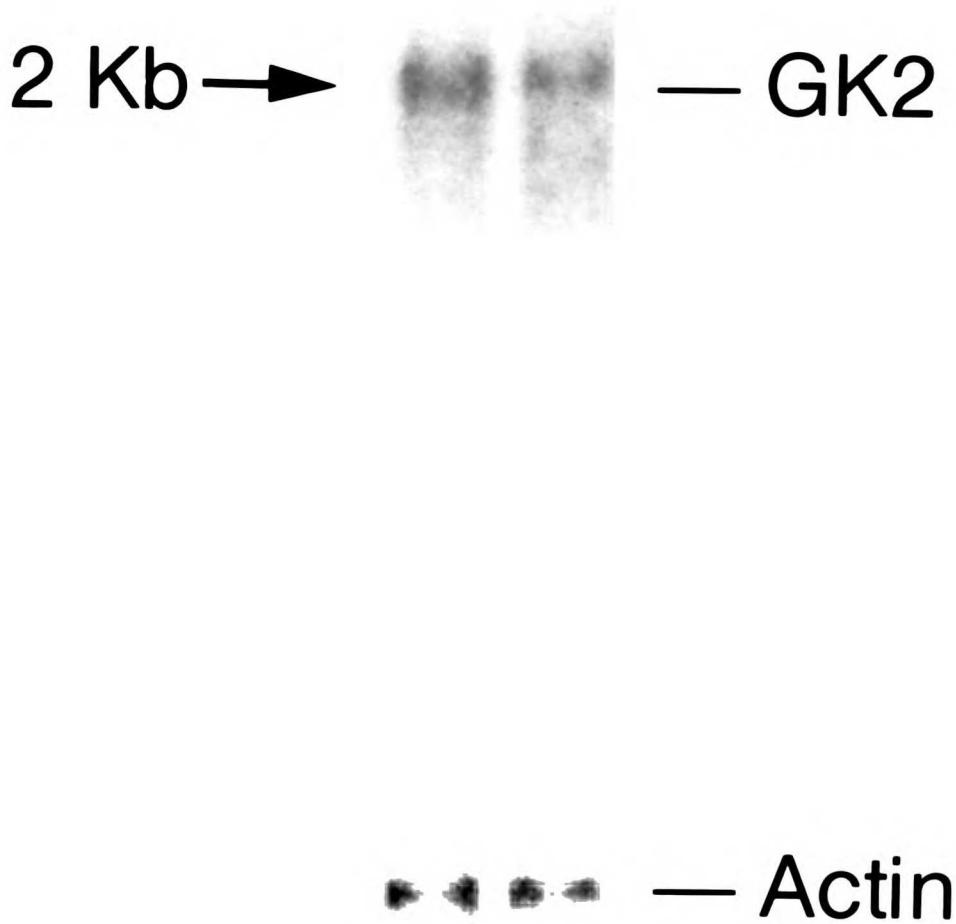

S. l.
L. h.
E. c.
H. s.
K. l.
S. c.
Gal 3

Figure 7. Northern blot hybridization analysis. The left lane contains mRNA from human fibroblast cell line (Hs 27) grown in the presence of galactose. The right lane contains mRNA from Hs27 grown in the presence of glucose.


2 Kb
GK2
Actin

cell line (Hs27) was grown in two different conditions: in the presence of galactose and glucose. The 2 kb mRNA transcript was expressed in equal amounts both in the fibroblasts grown in the presence of galactose and glucose.

Human Chromosome Mapping

Because a human galactokinase gene (GK1) has previously been mapped to human chromosome 17q23-25, we tested whether our galactokinase gene also resides on this chromosome by PCR amplification analysis of a GK2-specific segment. A set of PCR primers (hG5'-25 and hG3'-26) complementary to the 3' translated and untranslated regions of GK 2 was used to amplify a 205-bp region of the pJJGK cDNA insert; a segment of similar size was amplified also from total human genomic DNA (Figure 8a). Genomic DNAs from a panel of well-characterized human-mouse somatic cell hybrids (Table 1) retaining subsets of human chromosomes was used as a template for PCR (53-58). The primers did not yield a specific PCR product using mouse genomic DNA. The presence of an amplified PCR fragment was perfectly correlated with the presence of chromosome 15 in the human-mouse hybrid lines; it clearly does not correlate with the presence of chromosome 17. The collection of human-mouse hybrids includes seven hybrids that lack chromosome 15 but contain chromosome 17; none yielded an amplified fragment. In contrast, the one strain with chromosome 15 but not chromosome 17 (A59-3AaZ10a; lane 23, Figure 8a) yielded an amplified fragment. The human-mouse hybrids that yielded a PCR fragment that contained the fewest human chromosomes were strains NA 09940 (five human chromosomes) and A59-3Aaz10a (seven

Table 1. Mapping of a human galactokinase gene in a panel of human-rodent somatic cell hybrids. A collection of human-rodent cell lines was analyzed for the presence of sequences corresponding to the GK2 gene was determined by PCR amplification analysis. Presence of a known chromosome 15 gene (B2M) and a known chromosome 17 gene (GAS) was similarly analyzed. Presence or absence of characteristic bands is indicated by + or -- (see Figure 7).

* (-) indicates less than 9% of the cells contain a particular human chromosome.

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PCR Lane Assignments	DNA	Human Chromosomes*																								Amplified PCR Fragment		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	GK2	β2M	GAS
1	φX174 RF DNA/HaeIII																											
2	pJJGK																									+		
3	Human Fibroblast	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	B-82 mouse L cell	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	NA 09925	+	+	–	+	+	+	+	+	–	–	–	+	–	+	+	+	+	+	+	+	–	+	–	–	+	+	+
6	NA 09926	+	+	+	+	–	+	+	+	–	+	–	–	+	+	+	–	+	+	+	+	–	–	–	–	+	+	+
7	NA 09927	+	+	+	+	–	+	+	+	–	+	–	–	+	+	+	–	+	+	+	+	–	–	–	–	+	+	+
8	NA 09928	–	+	+	–	+	+	–	+	–	–	–	–	–	+	+	–	+	–	+	–	+	+	–	+	+	+	+
9	NA 09929	–	–	+	+	–	+	–	+	–	–	+	+	–	+	–	–	+	–	–	+	–	–	–	–	–	–	+
10	NA 09930A	–	+	+	–	+	–	+	–	–	–	–	+	+	+	+	–	+	+	–	+	+	+	–	–	+	+	+
11	NA 09931	–	–	–	–	+	–	+	–	–	+	–	+	–	+	–	–	+	–	–	+	+	–	–	+	–	–	+
12	NA 09932	–	–	–	+	+	+	–	+	–	–	+	+	–	–	–	–	+	–	–	–	+	–	–	–	–	–	+
13	NA 09933	+	–	+	+	+	+	+	+	–	–	–	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+
14	NA 09934	–	+	–	–	+	+	–	+	–	–	+	+	–	–	+	–	+	+	–	+	+	–	–	–	+	+	+
15	NA 09935A	–	–	+	+	+	+	–	–	–	–	–	+	+	+	–	–	+	+	–	–	+	+	–	–	–	–	+
16	NA 09936	–	–	–	+	–	+	+	+	–	+	+	–	–	+	–	–	+	–	+	+	–	+	–	–	–	–	+
17	NA 09937	–	–	+	+	–	+	+	+	–	–	–	+	–	+	+	–	+	+	–	–	–	–	–	–	+	+	+
18	NA 09938	–	–	–	+	+	+	+	–	–	–	+	+	–	+	–	–	+	–	–	+	+	+	–	–	–	–	+
19	NA 09940	–	–	+	–	–	–	+	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	+	+	+
20	NA 10324	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
21	NA 10567	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
22	NA 10611	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23	A59-3Aaz10a	+	–	–	–	–	–	–	–	–	+	–	–	+	–	+	–	–	+	–	+	–	–	+	–	+	+	–
24	MH-22.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+

Figure 8a. PCR analysis of human-mouse hybrid cell lines for presence of the GK2 gene. A pair of PCR primers complementary to the 3'translated and untranslated regions of pJJGK was used to amplify 205 bp fragment. Lane 1-24 contained products of PCR reaction using the following DNA samples for amplifications: lane 1, Hae III fragments of jX174 (size markers); lane 2, pJJGK (containing GK2); lane 3, DNA from a normal human fibroblast ; lane 4, DNA from mouse line B-82, DNA samples from 20 human-rodent somatic cell hybrids (as indicated in Table 1). Contents of DNA samples are described in Table 1. The 205-bp band is the amplification product of the GK2 gene.

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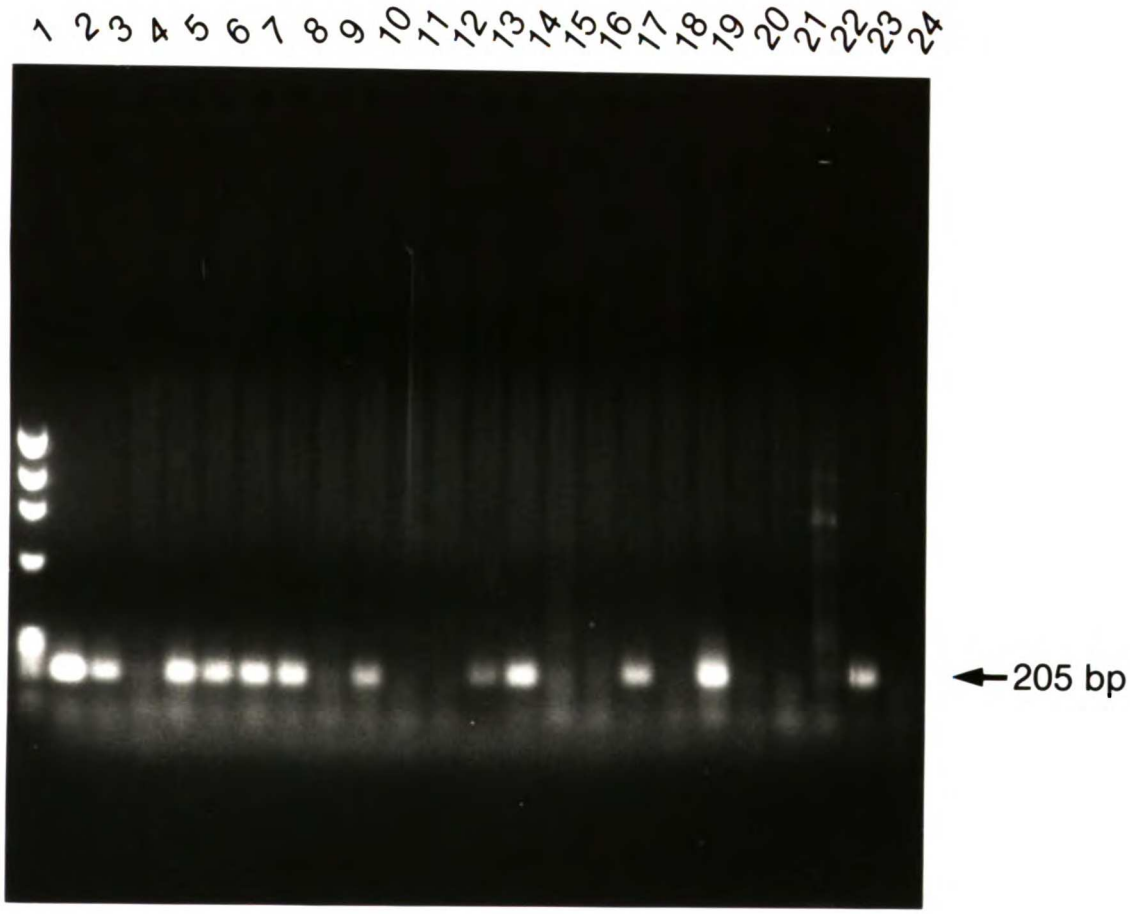
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
205 bp

human chromosomes); their only common chromosome is chromosome 15. These observations unequivocally show that the GK2 gene does not reside on chromosome 17 and indicate that it is instead located on chromosome 15.

To verify the chromosome composition of our somatic cell hybrid panel, we have carried out a similar PCR analysis using primers for genes known to reside on either chromosome 15 or 17 (59). As expected PCR primers for the human b2-microglobulin gene (assigned to human chromosome 15q21-22) amplified a 357-bp fragment only from DNA of cell lines reported to contain chromosome 15 (Table 1 and Figure 8b). Amplification data for this gene are identical to that obtained for GK2. Likewise, PCR primers for the human gastrin gene (assigned to chromosome 17q) amplified a 297-bp fragment from DNA of cell lines reported to contain chromosome 17 (Table 1 and Figure 8c). These results confirm the chromosome composition of our hybrid cell panel with respect to chromosome 15 and 17 and support our contention that GK2 is not located on chromosome 17 but rather on chromosome 15.

Figure 8b. Identifying somatic cell hybrid cell lines that contain human chromosome 15. PCR primers for the human b2-microglobulin gene were used to amplify a 357 bp fragment as indicated. Lane assignments are described in Table 1 and are identical to those described in Figure 3a except for lane 1, which contains a 1-kb DNA ladder for nucleotide size markers; pJJGK (lane 2 in Figure 3a) was not analyzed.

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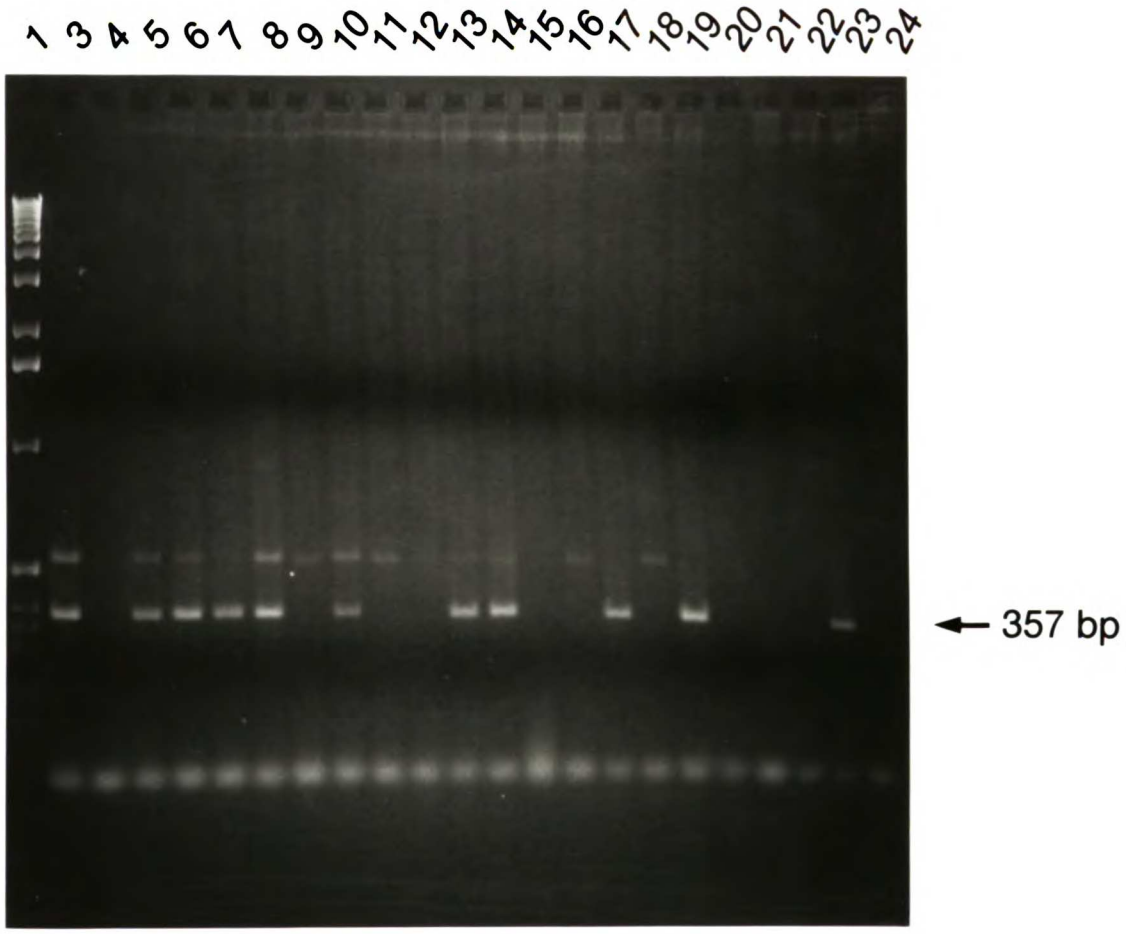
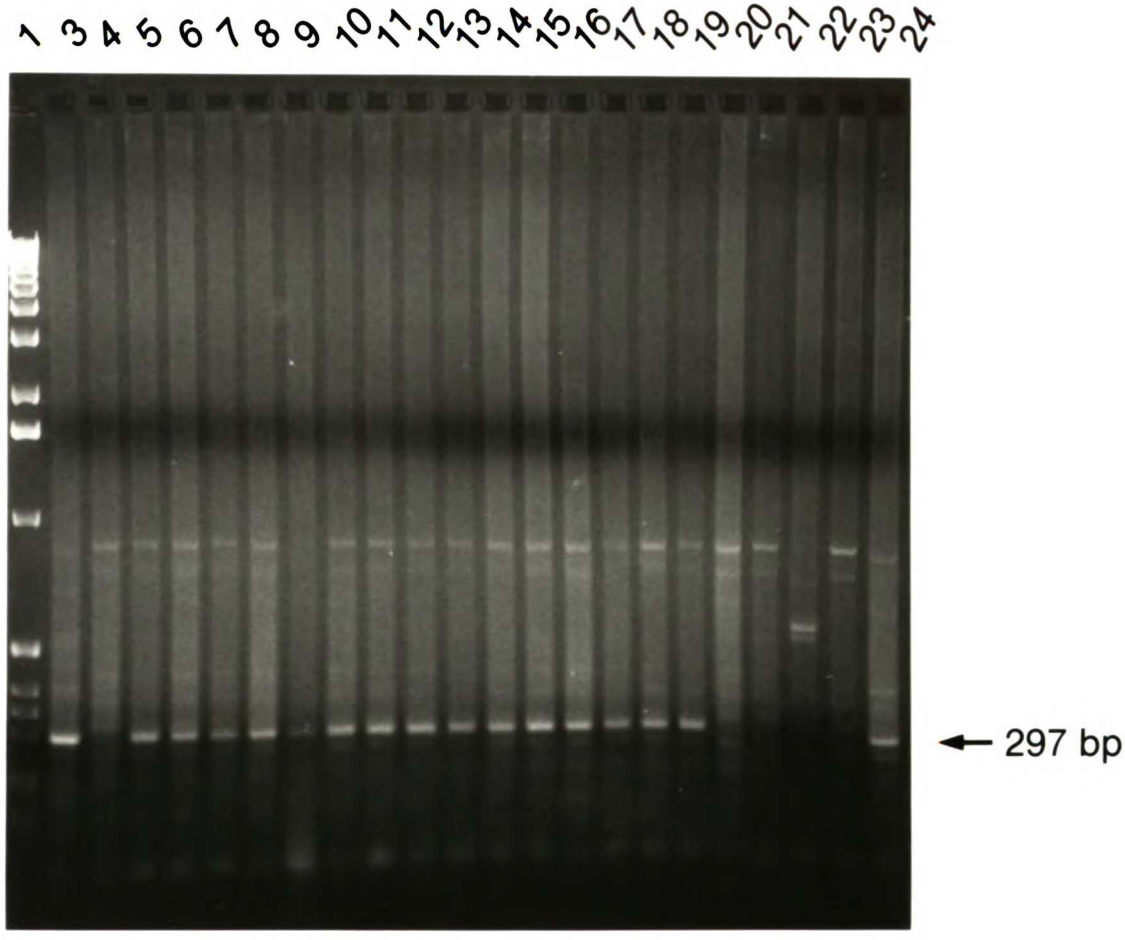

1 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
357 bp

Figure 8c. Identifying hybrid cell lines that contain human chromosome 17. Primers for the human gastrin gene amplified a 297 bp fragments as indicated. Assignments were the same as in Figure 3b.


1 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
297 bp

DISCUSSION

Cloning of a Human cDNA Encoding a Human Galactokinase

The main goal of the work described in this thesis was to isolate a human galactokinase gene. Two lines of evidence clearly indicate that we have cloned a human cDNA encoding a human galactokinase. First, expression of the human cDNA in Saccharomyces cerevisiae complements a yeast strain with galactokinase deficiency and allows growth on a synthetic medium containing galactose as the sole carbon source. Secondly, the amino-acid sequence of the cloned cDNA shares a high degree of similarity with galactokinases from Saccharomyces carlsbergensis as well as from other species of yeast and from E. coli. The fact that we have isolated a functional cDNA, prepared from hepatoma RNA, indicates that the gene corresponding to this cDNA is not a pseudogene but rather a gene that is expressed at least in hepatoma cells. Northern RNA analysis demonstrates the presence of a transcript of 2.0 kb from human fibroblast cell line Hs27.

The method of cloning a human gene by complementing yeast mutations provides an excellent alternative to using reduced stringency DNA hybridization, PCR amplification or antibodies with a bacterial expression library. For this complementation approach to be successful, however, we are relying on yeast to translate a human cDNA in order to synthesize a functional protein. A useful feature of this scheme is that complementation usually selects for complete coding regions during the initial cloning step. The potential for isolating other human homologous of known yeast gene is immense. Further, studies that examine the

structure-function relationship of a gene are simplified, since complementation in yeast can be used as an assay for testing protein activity mutagenized in vitro cDNA.

Structural similarities between GK2 and other genes. The GK2 open reading frame contains a high degree of similarity to galactokinases from several other species. Alignment of all known galactokinase sequences (61) reveals six distinct regions with identities (A-F; Figure 5). These matches provide strong evidence that GK2 encodes galactokinase. The greatest sequence variability occurs between regions C and D, making it very unlikely that this region contains the catalytic site or binding site for ATP.

The GK2 open reading frame also shows amino acid similarities to the Saccharomyces cerevisiae GAL3 gene-- 23% overall identity, with substantial identities in regions A-D. (Regions corresponding to E and F are apparently missing from GAL3; 62) In yeast, it has been suggested that GAL1 encodes a bifunctional protein (63, 64) that performs a function redundant to the GAL3 gene product in addition to galactokinase activity. It is intriguing to speculate that human galactokinase GK2 may also retain this redundant function.

The GAL3 mutation in S. cerevisiae delays galactose inductions of GAL/MEL gene transcription (62). Multiple copies of the intact GAL1 gene partially suppress the phenotype of GAL3 mutant cells. Using different GAL1 mutants, the GAL1 protein has been shown to consist of both galactokinase and a GAL3-like inducer-forming activity(63). Interestingly, GK2 and GAL3 of Saccharomyces cerevisiae share similarity in regions A-

E, except for the carboxyl terminal region F. Furthermore, the fact that GAL3 has been proven to lack galactokinase activity in yeast (63) suggests that this glycine-enriched region F is one of the essential components of the catalytic site for the phosphorylation of galactose. A close phylogenetic tree relationship between GAL3 and GAL1 proteins in yeast brings up the intriguing possibility that these proteins may have diverged from a common ancestry (Figure 6). It is also conceivable that GAL3 may be unique to the yeast system, without homologous genes in bacteria or human cells.

Regulation of GAL gene transcription. The regulatory pathway of GAL gene transcription is known in the yeast system. Galactose induces transcription of the GAL1 gene in a process that requires the transcriptional activator GAL4. This process is inhibited by the regulator GAL80. GAL4 binds to short conserved DNA sequences upstream of the GAL1 gene, activating transcription(65). GAL80, in the absence of galactose, binds to GAL4, preventing it from activating transcription(66). Whether the synthesis of galactokinase is induced by galactose in human cells, as it is in yeast, is not known. We were able to carry out a preliminary analysis of this question from our results of the Northern hybridization blot experiment (Figure 7), which indicated that the 2 kb mRNA transcript hybridized specifically to our putative human galactokinase cDNA probe. The transcript was identified both in the presence of galactose and glucose in equal amounts, supporting the notion that the same regulatory system for galactokinase does not govern expression of GK2 in human fibroblast (Hs 27) cells. One explanation may be that GK2 is constitutively turned on regardless of whether galactose or

glucose is present. Another possibility is that the regulation of GK2 expression in human cells is much more complicated or responds to different environmental cues than those of yeast galactokinase gene expression. Another interpretation of this result is that GK1 is the gene that is inducible in the presence of galactose and that GK2 is constitutively expressed or that expression of GK2 gene in its galactose inducibility may be tissue specific.

Mapping of a Human Galactokinase Gene to Chromosome 15

Several prior studies (32-35) have mapped a human galactokinase gene to chromosome 17 q23-25, closely linked to the thymidine kinase gene. It seems beyond question that chromosome 17 contains a structural gene for a galactokinase since transfer of an individual human chromosome into a mammalian cell confers galactokinase activity (35). We were therefore surprised to find that GK2 did not map to chromosome 17 but appeared to map elsewhere. The chromosome transfer experiments with chromosome 17 (35) and earlier data (33) argue strongly that the galactokinase activity conferred by this chromosome does not require other chromosomes, notably 15. These observations rule out the possibility that the locus on chromosome 17 is a positive regulator of the gene on chromosome 15.

It seems plausible to imagine that there are at least two galactokinase genes, GK1 on chromosome 17 and GK2 on chromosome 15. The ability of the GK2 cDNA to provide galctokinase activity in yeast and the presence of its transcript in HS 27 cells indicate that this gene is functional. There remains, however, a possibility that the GK2 gene is non-functional

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in wild-type cells. If humans contain two different galactokinase genes, one might expect to observe isozymes for galactokinase. Enzymological studies of human galactokinase do not give a clear answer to the question of the number of isozymes. The apparent identification of a structural gene for galactokinase should provide impetus for such studies. If chromosome 17 contains a structural gene for a galactokinase, we might expect this gene to have similarity to GK2, based on the extensive conservation of sequence observed in bacteria, yeasts, and humans. This is under study. We plan to carry out PCR reactions using primers from conserved segments of the GK2 gene and attempt to identify a chromosome 17-specific band. A low stringency hybridization screening using the GK2 gene as the probe could also be used to try to identify any cross reaction to a gene located on chromosome 17.

The disease locus for galactokinase deficiency galactosemia. Our observations raise anew the question of the location of the genetic determinant for galactokinase deficiency disease: does it reside on chromosome 15 or on chromosome 17? Having cloned a human galactokinase cDNA from a hepatoma cell line, we can determine whether patients with galactokinase deficiency have alterations in GK2. A cloned galactokinase gene should make it possible to determine definitively whether weak mutation on the GK2 gene is responsible for presenile cataracts. One approach would be to isolate GK2 genes from heterozygotes and homozygotes mRNA and attempt to complement gal1$^-$ mutants of S. cerevisiae. If a GK2 gene from a normal individual's mRNA is able to complement a gal1$^-$ mutant of S. cerevisiae, while the GK2 gene from

affected mRNA is not able to do so, this would strongly support the notion that GK2 alteration results in galactokinase deficiency galactosemia.

Another strategy would be to introduce a defective GK2 gene into a mouse and search for any clinical manifestations of galactokinase deficiency galactosemia, for example, cataract formation. In addition, the transgenic mouse that carries weak mutation on the GK2 gene can then be tested for presenile cataract formation. Therapeutic effects of aldose reductase inhibitor or other drugs may also be studied in these transgenic mice with defective GK2 genes.

Closing comments

Variations of clinical sequela between two galactosemia types. As discussed in the introduction, there is a great difference in the clinical manifestations of galactokinase and transferase deficiencies. Transferase deficiency galactosemia has a generalized systemic involvement, whereas galactokinase deficiency is limited to the lens. One explanation may be that the accumulation of galactitol in GK deficiency or galactose-1-phosphate in GALT deficiency have a tissue-dependent cytotoxic effect. For example, although the elevated levels of galactose-1-phosphate may cause irreversible damage to the hepatocytes or neurons, high galactitol level may have no cytotoxic impact on these same tissues.

It is also possible to imagine that galactokinase has a tissue-specific gene expression. Hence, GK1 might be specifically expressed in fibroblasts, GK2 in hepatoma cells, and yet another unidentified gene, GKr, in the lens.

Thus, patients who presents with cataract formation for galactokinase deficiency may possess a defective GKx gene. In contrast, for transferase deficiency galactosemia, the same GALT gene that is ubiquitously expressed in all tissues may be the one that is defective in the affected individual.

Transgenic yeast? I would like to reiterate the exciting prospect of using the method of complementing yeast mutants to determine whether a homologous human gene is functionally defective or not. As mentioned above in the case of GK2, we can attempt to complement a yeast gal1$^-$ mutant with GK2 cloned from a galactokinase-deficient individual and determine if this GK2 protein is functional or not *in vivo*. We may also selectively mutate different regions of GK2 *in vitro* and carry out the same experiment to find out which region is important for catalyzing the phosphorylation of galactose. This strategy can also be tried with other human diseases where a candidate gene has been identified and both yeast and human genes encode proteins with similar functions.

Regulatory protein deficiency? I would like to make a comment about the possibility that deleterious mutations of a gene encoding a regulatory protein may contribute to galactokinase deficiency. There have been a few patients that lack enzymatic activity of both galactokinase and transferase proteins (24). One possibility is a laboratory error in carrying out these enzymatic assays. It is extremely unlikely that a patient would have two separate mutations causing these deficiencies. Another possibility is that these individuals has a mutation of a trans-acting transcription factor, analogous to GAL4 of yeast.

Trans-acting factors in part govern the pattern of expression of specific genes by responding to a variety of extracellular and intracellular cues. In yeast, we know that GAL4 in the presence of galactose acts as a positive regulator for the transcription of GAL1 and GAL7, which encode galactokinase and transferase, respectively. In the event that the GAL4 protein no longer functions normally, galactokinase and transferase will not be synthesized in significant amounts. If a human homologue of GAL4 were to be defective, for instance, we would have affected individuals with both transferase and galactokinase deficiencies.

Galactokinase deficiency galactosemia is a biochemical description given to explain the cause for the clinical manifestion of this disorder. At the molecular level, it is conceivable that more than one type of galactokinase deficiency will be found. This idea stems mainly from our finding that at least two galactokinases exist in humans. Moreover, if a regulatory protein gene defect were also to occur in humans (i.e., a possible human GAL4 homologue defect), we will need to view galactosemia in a different light. The broader implication is that other inherited disorders often attributed to a single-gene defect may also turn out to have a molecular alteration of trans-acting transcriptional factors.

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