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Selection and Analysis of Galactose Metabolic Pathway Variants of a Mouse Liver Cell Line

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To study the genetic expression and regulation of galactose-metabolizing enzymes, we mutagenized the mouse liver H2.35 cell line and selected for cell clones resistant to the toxic galactose analog, 2-deoxy-D-galactose (2-DOG). One cloned line, designated H12.10, was stably resistant to high levels of 2-DOG and was completely deficient in galactokinase activity. Galactokinase activity and growth sensitivity to 2-DOG could be restored by transfecting H12.10 cells with a plasmid containing the *Escherichia coli* galactokinase (*galK*) gene fused to a eucaryotic promoter; thus, the 2-DOG selection could be directed against transfected recombinant constructs in a liver cell line. We also found that H2.35 cells could not utilize galactose as a primary carbon source because of a deficiency in galactose-1-phosphate uridyltransferase; a variant line of H2.35 cells selected in galactose medium expressed higher levels of uridyltransferase activity. Finally, we found that in all mammalian cell lines tested, galactokinase expression was the same whether the medium contained glucose, galactose, or both sugars. These studies demonstrate differences between mammalian cells and yeast cells in the regulation of *gal* enzymes, and they define different schemes for obtaining altered expression of genes in the galactose metabolic pathway. The isogenic liver cell lines described here can also serve as model systems for studying galactosemias, which are inherited disorders of galactose metabolism in humans.

Human genetic deficiencies of galactose metabolism, known as galactosemias, can result in blindness, liver disease, and mental dysfunction in infants. Although little is known about the regulation of mammalian galactose metabolism, the relevant enzymology is defined. Upon entering a cell, galactokinase (EC 2.7.1.6) phosphorylates galactose at the C-1 position. Two pathways exist in the liver for metabolizing galactose-1-phosphate (gal-1-P). In the predominant pathway, gal-1-P uridyltransferase (EC 2.7.7.12) substitutes galactose in UDP-glucose to generate UDP-galactose and glucose-1-phosphate. In the minor pathway (1), a UDP moiety appears to be added directly from UTP by a UDP-glucose pyrophosphorylase (19). UDP-galactose is used generally for protein glycosylation and for lactose biosynthesis in the mammary gland. UDP-galactose can also be converted to UDP-glucose via an epimerase, incorporated into glycogen, and then released for glycolysis as needed. Galactosemia in infants, which is due to the accumulation of galactose by various organs, is ultimately the consequence of a hepatic deficiency in either galactokinase, or more often, the gal-1-P uridyltransferase (19).

Studies of the expression of the *gal* genes in bacteria and in yeast (*Saccharomyces cerevisiae*) cells (2, 12, 23) have revealed much about basic mechanisms of gene regulation (8, 14) in addition to the specific regulatory pathways that govern galactose metabolism (3, 15). The original goal of our work was to exploit the genetic manipulability of the galactose-metabolizing system, as established for unicellular organisms, to generate genetic selections for galactose pathway mutations in a mammalian cell line. Here, we present the isolation and characterization of hepatic cell lines deficient in either galactokinase or gal-1-P uridyltransferase. In the course of this work, we defined differences between mammalian cells and yeast cells in the repression and activation of galactokinase activity by different carbon sources. We also present an isogenic series of cell lines which may be useful for studying mammalian *gal* deficiencies and human galactosemias.

MATERIALS AND METHODS

Cell culture, mutagenesis, and genetic selection. Unless stated otherwise, H2.35 cells (28) and derivatives were cultured routinely at 33°C in Dulbecco modified Eagle medium (GIBCO) containing 1 mg of glucose per ml, 4% fetal bovine serum (Sterile Systems), and 0.2 μM dexamethasone (BM in reference 28; referred to here as glucose medium). For isolating 2-deoxygalactose resistant (2-DOG^r) cells, control H2.35 cells were first seeded at 10^5 cells per 100-mm (diameter) dish containing glucose medium and cultured at 33°C. H2.35 cells to be mutagenized were seeded at a 10-fold higher density to accomodate cell killing. After 4 h, medium was aspirated, the plates (without lids) were placed on a rotating platform, and cells were exposed for 3 or 6 s to UV light from a germicidal lamp in a tissue culture hood. The UV fluence was approximately 1.5 J/m^2 per s, as determined with an IL 1700 research radiometer (International Light). Glucose medium was then added to the plates. Alternatively, 20 or 30 μl of ethyl methanesulfonate (EMS) (Sigma Chemical Co.) was added to plates of cells in 15 ml of glucose medium. After 4 h, the EMS plates were aspirated, rinsed once with phosphate-buffered saline (PBS), and refed with glucose medium. Mutagenized and control cells were cultured in glucose medium for 48 h (approximately two cell doublings), then switched to glucose medium containing 1.5 mg of 2-DOG (referred to as 2-DOG medium) per ml. 2-DOG was from Sigma, and each lot was tested prior to use. The cells were fed fresh 2-DOG medium every 3 to 4 days. By 5 to 6 weeks, most of the cells died in 2-DOG medium and resistant colonies appeared. 2-DOG-resistant colonies were picked with filter papers soaked in a trypsin solution and subcloned in 2-DOG medium. Secondary colonies were picked, expanded in 2-DOG medium to establish clonal cell lines, and frozen under liquid nitrogen at about the sixth passage. In a

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parallel experiment, serial dilutions of H2.35 cells were plated in duplicate, mutagenized as above, and cultured for 2 weeks in glucose medium. The resulting colonies were fixed, stained, and counted to quantitate the fraction of viable cells after mutagen treatment.

Cell extract preparation. Confluent cells were released from plates, washed twice with PBS, and suspended at a concentration of $10^5/\mu l$ in a solution containing 10 mM Tris hydrochloride (pH 8), 1 mM dithiothreitol, 0.1% Triton X-100, and 10% glycerol. Cells were disrupted by three cycles of freezing in a dry ice-acetone bath and thawing at 37°C. Cell debris was removed by centrifugation in a microfuge for 15 min at 4°C, and the supernatant (extract) was stored at −20°C. Alternatively, cytoplasmic S-100 extracts were prepared from BALB/c mouse livers by the method of Dignam et al. (10). H2.35 cell extracts prepared by either the freeze-thaw or Dignam method had comparable enzymatic activities (data not shown). Protein concentrations were determined with the Bradford protein assay (Bio-Rad Laboratories).

Galactokinase enzyme assays. Fifty-microgram amounts of protein were used in duplicate 30-min filter assays of galactokinase, with [^{14}C]galactose and ATP as substrates, as described previously (5). Titration experiments indicated that the assay was linear over the 30-min period. Blank reactions included all assay reagents except cell extract.

We found a small degree of variation in the absolute level of galactokinase activity in a given cell line when extracts were prepared and assayed at different times and different passage numbers. In a systematic study of this variation, we found that H2.35 and L cells harvested at confluence had about 13% more galactokinase activity than cells harvested at one-fifth the cell density. Although the absolute enzyme activities for a cell line could differ in different experiments, the relative levels of activities for different cell lines plated at the same time and at the same density were consistent from experiment to experiment.

Uridyltransferase enzyme assays. Uridyltransferase assays were set up in duplicate and were similar to galactokinase assays, except that all reactions lacked NaF and some included UDP-glucose (Sigma) with a final concentration of 5 mM; control reactions contained no UDP-glucose. Protein was added, and the reactions proceeded at 37°C for 45 min. The reaction mixes were then boiled for 2 min and cooled to room temperature for 5 min. Calf intestinal alkaline phosphatase (1.5 U, 2,000 U/mg; Boehringer Mannheim) was added to each, and the mixtures were incubated at 37°C for 45 min. Precipitates were removed by centrifugation at 12,000 × *g* for 2 min. The supernatants were mixed with 0.1 μmol of UDP-galactose, spotted onto prechromatographed polyethyleneimine-cellulose plates (Macherey-Nagel), chromatographed in two dimensions with the Tris hydrochloride (Ta) and $(NH_4)_2SO_4$-EDTA (Sb) solvents exactly as described previously (6), and exposed to X-ray film overnight. One-dimensional chromatography was in the Tris hydrochloride solvent, and a ^{14}C-labeled product was seen comigrating with UDP-galactose only when the UDP-glucose substrate was present in the reaction mix. Alternatively, the reaction supernatants were spotted onto DE81 filters and washed five times with 5 ml of distilled water in a vacuum filtration device, and the filters were counted in 4 ml of Optifluor (Packard Instrument Co., Inc.). Filters from control reactions retained less than severalfold the counts on filters from blank reactions containing no extract. The galactokinase assays shown in Table 5 were performed in a way similar to the control uridyltransferase reaction, except that phosphatase was not added. The uridyltransferase assay exhibited a delay in accumulation of the UDP-galactose product (data not shown), demonstrating a requirement for synthesis of gal-1-P in the reaction mix.

Media for testing galactose utilization. Serum was depleted of glucose by dialyzing 1 volume of serum against two changes of 100 volumes of PBS for 24 h each at 4°C. Media contained Dulbecco modified Eagle medium (glucose-free; GIBCO) with dialyzed fetal bovine serum at 4% for H2.35 and H34.2 cells or at 10% for L and HepG2 cells, 0.2 mM dexamethasone (only for H2.35 and H34.2 cells), and either glucose (Sigma) or galactose (Pfanstiehl Laboratories or J. T. Baker) at a final concentration of 1 mg/ml. Cells cultured in galactose at 5 mg/ml exhibited the same growth phenotypes as cells in 1 mg/ml (data not shown). We tested the glucose levels in dialyzed serum and in galactose solutions with a Dextrostix assay (Miles Laboratories, Inc.). Growth of H34.2 cells in the various media was quantitated by counting cells within defined areas of the plates shown in Fig. 3.

DNA transfections and nucleic acid analysis. The RSV-*galK* plasmid (pRDZB.JT; kindly provided by Mitchell Reff) contains the Rous sarcoma virus (RSV) long terminal repeat (20) fused to the *Escherichia coli galK* gene, with the bovine growth hormone gene polyadenylation signal downstream. The plasmid conferring G418 resistance, pSAN2, contains the simian virus 40 enhancer linked 5′ to a fusion of the mouse serum albumin promoter region and the Tn*5* neomycin-resistance gene (*neo* [9]), with the simian virus 40 early poly(A) site downstream. Portions (2 μg) of a 1:1 mixture of the plasmids were introduced into plates (10^6 H12.10 cells per plate) by the standard calcium phosphate precipitation procedure (27). After 1 day of nonselective growth (glucose medium) in the presence of DNA, the cells were rinsed with Dulbecco modified Eagle medium and cultured nonselectively for an additional 24 h; after this interval, the cells were cultured in glucose medium containing 0.3 mg of G418 per ml. Colonies that formed after several weeks of feeding with G418 were picked, subcloned nonselectively, and expanded for analysis. Southern blots, Northern (RNA) blots, and primer extension assays were performed as described previously (28). The sequence of the *galK* primer was 5′-GGTGT GAGTGGCAGGGTAGCC-3′.

RESULTS

Reduced galactokinase activity in 2-DOG-resistant cells. Genetic mutants of *gal* enzymes can be selected directly by culturing bacteria (4), yeast cells (16), or mammalian cells (24) in the growth-inhibiting drug 2-DOG. Although the exact mechanism of 2-DOG toxicity is unknown, the toxic substance is probably 2-deoxygalactose-1-phosphate or an unidentified derivative, because 2-DOG-resistant (2-DOG^r) cells frequently were mutant for galactokinase (4, 16, 24), whereas uridyltransferase mutants of yeast cells were not resistant to the drug (16). We performed 2-DOG selections with the H2.35 cell line, which was clonally derived from mouse hepatocytes infected with a temperature-sensitive simian virus 40 (28).

H2.35 cells were mutagenized with UV light or EMS and then cultured in medium containing 1.5 mg of 2-DOG per ml. We selected for 2-DOG^r colonies, subcloned them in selective medium, and expanded them to establish clonal cell lines. We also expanded colonies arising from nonmutagenized cells cultured under identical selective conditions. The nonmutagenized cells gave rise to 2-DOG^r colonies at a higher frequency than the mutagenized cells (Table 1); thus,

TABLE 1. Recovery of 2-DOG-resistant H2.35 cells

Treatment	% Survival[a]	2-DOG^r colonies/ survivor[b]
Control (none)	100	8×10^{-4}
UV light		
4.5 J/m^2	38	5×10^{-5}
9 J/m^2	15	1.3×10^{-4}
EMS		
20 ml	35	1.1×10^{-5}
30 ml	10	2×10^{-5}

[a] Values represent the average of duplicate plates at each of two different densities of plated cells.

[b] Values were calculated by dividing the average number of 2-DOG^r colonies by the number of viable cells present on day 1 of exposure to 2-DOG.

there was a significant number of resistant cells preexisting in the population. The lower frequency of resistant cells in the mutagenized populations may have been due to cell loss from overcrowding, since the mutagenized cells were plated at a 10-fold higher density and 2-DOG^r colonies took 4 to 5 weeks to resolve.

H2.35 cells and various 2-DOG^r clones at about their sixth passage were cultured in glucose or in 2-DOG medium, respectively, and galactokinase activity was measured in whole cell lysates. The 2-DOG^r cell lines had no more than half of the parental H2.35 level of galactokinase activity (Table 2). Because galactokinase levels varied significantly between different clones, some of clones probably were of independent origin. Seven randomly chosen clones with intermediate levels of galactokinase activity were cultured nonselectively for 23 to 35 cell divisions and then tested for enzyme activity. Five of the clones continued to express low levels of galactokinase activity, whereas two clones, H12.20 and H12.24, expressed higher levels (Table 2). Hence, the presence of 2-DOG is unnecessary for the lower galactokinase activity seen in resistant cells.

One cell clone, designated H12.10, arose from UV-treated cells and had no detectable galactokinase activity at the fifth passage. Indeed, after 34 passages that included an estimated 130 cell doublings in 2-DOG medium, followed by 210 cell doublings in nonselective glucose medium, galactokinase activity was still undetectable. Therefore, the galactokinase-negative phenotype is not an adaptive response to the drug. To determine whether H12.10 cells retained their resistance to 2-DOG following the period of nonselective growth, the cells were cultured in various concentrations of 2-DOG for 8 days and then fixed and stained in situ. H12.10 cells were resistant to 6 mg of 2-DOG per ml, a concentration fourfold higher than that used in the original selection (Fig. 1, row H12.10). By contrast, the parental H2.35 cells were highly sensitive to 1.5 mg of 2-DOG per ml, as expected. Because the galactokinase deficiency in H12.10 cells was complete and extremely stable, they were chosen for further study.

Bacterial *galk* gene restores galactokinase activity in H12.10 cells. To test whether *Escherichia coli* galactokinase could function in H12.10 cells, we transfected the cells with a plasmid containing the RSV promoter (20) fused to the *E. coli galK* gene; a plasmid conferring resistance to the antibiotic G418 was cotransfected. In this experiment, stable G418-resistant clones were selected and pooled, and lysates were prepared from the mixed cell population. Galactokinase activity in the pooled, *galK*-transfected H12.10 cells was 1.2-fold higher than in the parental H2.35 cells, suggesting that the *E. coli* gene was functional.

TABLE 2. Galactokinase levels in H2.35 cell derivatives

Cell line	Mutagen[a]	Galactokinase activity[b]
H2.35		40.60 Δ 3.19
H12.25	None	1.42 Δ 0.49
H12.26	None	15.80 Δ 0.51
		17.14 Δ 1.35[c]
H12.27	None	5.76 Δ 0.36
H12.28	None	8.74 Δ 0.16
H12.1	UV 9	22.66 Δ 0.15
H12.2	UV 9	7.99 Δ 0.53
H12.4	UV 9	11.5 Δ 0.46
H12.6	UV 9	16.45 Δ 1.13
		17.11 Δ 0.33[c]
H12.7	UV 4.5	12.27 Δ 0.78
H12.8	UV 4.5	19.75 Δ 1.29
		11.56 Δ 0.73[c]
H12.9	UV 4.5	8.7 Δ 0.24
H12.10	UV 4.5	0.09 Δ 0.04
H12.11	UV 4.5	13.70 Δ 1.62
H12.12	UV 4.5	13.85 Δ 3.24
H12.13	EMS 30	10.03 Δ 1.6
H12.15	EMS 30	21.04 Δ 2.07
		23.55 Δ 3.59[c]
H12.18	EMS 30	3.93 Δ 0.02
H12.20	EMS 20	10.68
		34.02 Δ 5.71[c]
H12.21	EMS 20	22.17 Δ 1.46
		12.29 Δ 1.47[c]
H12.22	EMS 20	20.69 Δ 0.15
H12.23	EMS 20	10.32 Δ 0.71
H12.24	EMS 20	12.05 Δ 0.58
		27.92 Δ 0.38[c]

[a] UV 4.5 and UV 9, Exposure to 4.5 and 9 J/m^2, respectively, of UV light; EMS 20 and EMS 30, exposure to 20 and 30 ml of EMS, respectively; None, clones that arose on nonmutagenized control plates.

[b] Values are the average numbers of nanomoles of gal-1-P synthesized per hour per milligram of protein, minus the average blank value. Δ, Difference between the two samples assayed.

[c] Galactokinase activity in the same cell clones as on the previous line but cultured in the absence of 2-DOG for 23 to 35 days prior to the assay.

***galK* expression in H12.10 cells confers sensitivity to 2-DOG.** Stable resistance to 2-DOG in H12.10 cells could be due solely to a deficiency in galactokinase activity, or it could be due to a deficient regulatory mechanism that prevented the expression of one or more genes in the galactose metabolic pathway, such as galactose permease. Only if the former case was true would a transfected *E. coli galK* gene restore sensitivity to 2-DOG in H12.10 cells. The ability of *galK* to complement the galactokinase insufficiency could then be used to drive negative genetic selections in mammalian cells.

To test these possibilities, we needed to see how different levels of *galK* expression affected cell growth in 2-DOG. We therefore repeated the stable transfection of H12.10 cells, but this time, we subcloned individual G418-resistant colonies and expanded them into separate cloned lines. Five different transfectant clones were chosen to verify the presence of *galK* genes and to quantitate the expression of *galK* mRNA and enzyme. Cultures of each clone were harvested and divided into portions for preparing total cellular DNA, RNA, and protein.

Transfectant H12.10.5 neither took up the *galK* gene (Fig. 2A, lane 3) nor expressed any *galK* mRNA (Fig. 2B), and it exhibited the same background level of galactokinase activity as the parent, nontransfected H12.10 cell line (Table 3). Thus, the galactokinase-negative phenotype was stable to

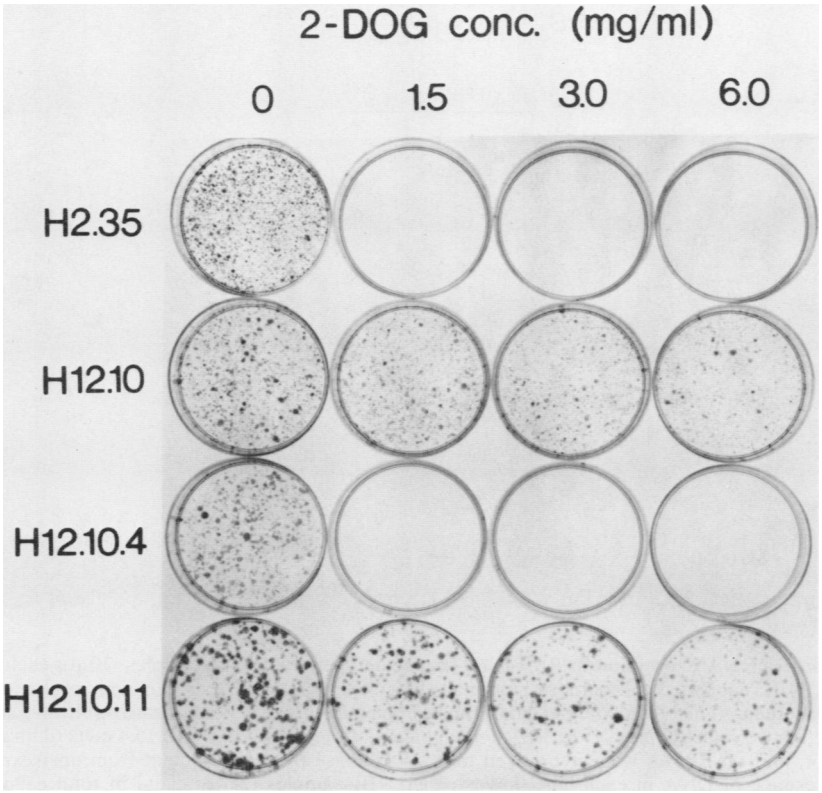

FIG. 1. Growth sensitivity to 2-DOG conferred by the endogenous mammalian or transfected bacterial galactokinase genes. Cells (10^4) of each line were plated onto 60-mm diameter plates in glucose medium containing the designated concentrations (conc.) of 2-DOG and fed every 3 days thereafter. After 9 days in culture, cells were fixed to the plates with Formalin, stained with methylene blue, and photographed.

repeated clonal selection and analysis. Four transfectants contained the *galK* gene: H12.10.4, H12.10.6, H12.10.7, and H12.10.11 (Fig. 2A). The expected 1.5-kilobase RSV-*galK* mRNA was detectable by Northern (RNA) blot analysis in three of the four cell lines (H12.10.4, H12.10.6, and H12.10.7) (Fig. 2B). Using a more sensitive primer-extension assay, we detected *galK* transcripts that initiated at the appropriate site of the RSV promoter (20) in all of the *galK*-positive cells, including the H12.10.11 line (Fig. 2C); the latter cells expressed about a 10-fold lower amount of *galK* mRNA than the other lines. In general, galactokinase levels correlated well with the relative levels of *galK* mRNA in the transfectants (results are summarized in Table 3). We chose two transfectants for further study; H12.10.11 cells, which had one-third the galactokinase activity of wild-type H2.35 cells, and H12.10.4 cells, which had 2.5-fold the galactokinase activity of H2.35 cells.

When we cultured H12.10.4 cells in various concentrations of 2-DOG, it was clear that high-level expression of *galK* caused the cells to become as sensitive to 2-DOG as the parent H2.35 cells (Fig. 1). By contrast, the H12.10.11 transfectant remained resistant to 2-DOG (Fig. 1). We conclude that strong expression of the *E. coli galK* gene can completely complement the mammalian defect causing resistance to 2-DOG.

Constitutive expression of galactokinase activity in various mammalian cell lines. To examine the regulation of galactokinase expression, we tested the levels of galactokinase activity in H2.35 cells, in fibroblastic mouse L cells (26), and in the hepatocarcinoma cell line HepG2 (13), cultured in glucose, galactose, or both sugars as primary carbon sources. Strikingly, galactokinase activities were remarkably constant in all cell lines regardless of the carbon source (Table 4). Thus, unlike the situation in unicellular eucaryotes such as yeast cells (15) and *Tetrahymena thermophila* (17), galactokinase appears to be expressed constitutively in mammalian cells.

Next we asked whether galactokinase activity and hepatocyte differentiation were independent of one another in the temperature-sensitive H2.35 line. We cultured H2.35 cells and *galK*-transfected H12.10.7 cells under conditions that partially induce hepatocyte differentiation; specifically, the cells were cultured at the restrictive temperature for simian virus 40 large T antigen, in a hormonally defined serum-free medium and on a collagen gel substratum (see reference 28 for details). The galactokinase activities were within 15% of the levels seen in the same cells cultured under standard (dedifferentiating) conditions, indicating that the enzyme was expressed independently of the differentiated state.

Galactokinase-positive H2.35 cells cannot utilize galactose as a primary carbon source. We anticipated that *galK* expression could also be used to cause H12.10 cells to grow in medium containing galactose as a primary carbon source. However, we discovered that neither the parent H2.35 cells (Fig. 3, row H.235) nor *galK*-transfected H12.10.4 cells (data not shown) could utilize galactose (Fig. 3) efficiently as a primary carbon source. In galactose medium, only very small numbers of H2.35 and H12.10.4 cells remained on the plate after 11 days, and the galactokinase-negative H12.10 cells died off completely (data not shown). We reproducibly found that the presence of galactose was slightly inhibitory to the growth of H2.35 cells in glucose medium (Fig. 3, row

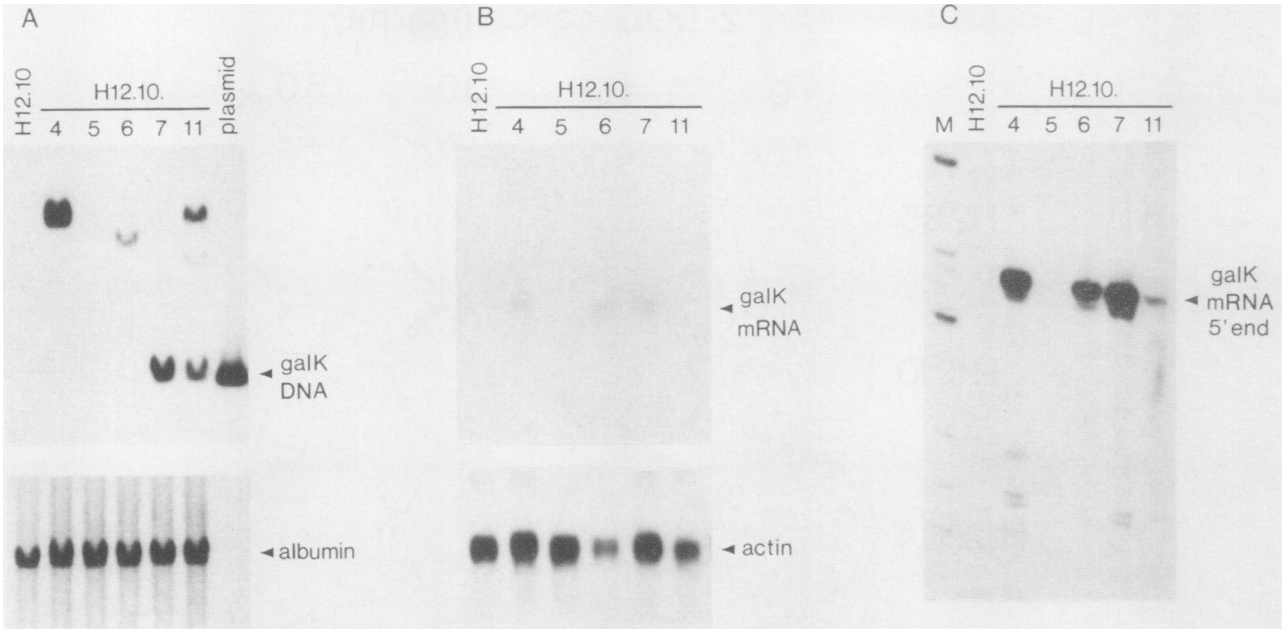

FIG. 2. Recombinant *galK* mRNA expression in RSV-*galK*-transfected cell lines. (A) Samples (10 μg each) of the designated genomic DNAs were digested with *Bgl*II, subjected to Southern blot analysis with a nick-translated *galK* gene probe (upper panel) and exposed to X-ray film with intensifying screens. The blot also included a 10-pg sample of *Bgl*II-digested (linearized) RSV-*galK* plasmid. Further analysis demonstrated the presence of two intact copies of the RSV-*galK* portion of the plasmid in H12.10.4 cells (data not shown). The *galK* probe was removed from the blot, and the DNAs were hybridized to a 1.6-kilobase *Bam*HI fragment from upstream of the albumin gene, as a positive control for the presence of DNA in each lane (lower panel). (B) Samples (25 μg each) of total cellular RNA were subjected to Northern blot analysis with a nick-translated *galK* gene probe and exposed to X-ray film with intensifying screens (top panel). The apparent molecular size of the *galK* mRNA, 1.5 kilobases, is consistent with the size predicted from the positions of initiation and polyadenylation signals in the RSV-*galK* plasmid (M. Reff, personal communication). Subsequently, the *galK* probe was removed and the RNAs were hybridized to a β-actin cDNA probe as a positive control for the presence of RNA in each lane (bottom panel). (C) Samples (50 μg) of total cellular RNA were hybridized to an end-labeled, *galK*-specific DNA primer, and the primer was extended with reverse transcriptase. The primer extension products were fractionated on a sequencing gel and exposed to X-ray film with an intensifying screen. Lane M, DNA sequencing ladder standard. The longest primer extension product (shown by the arrow) maps to the initiation site predicted by the RSV long terminal repeat sequence (20).

H.235), whereas the presence of 2-DOG in glucose medium caused a very strong growth inhibition (Fig. 1). Because H2.35 cells are galactokinase positive and are sensitive to 2-DOG, their inability to utilize galactose appears not to be due to the lack of either cellular uptake or phosphorylation by galactokinase. The fact that mouse L cells had comparable galactokinase levels (Tables 4 and 5) and grew nearly as well in galactose medium as in glucose medium (Fig. 3, row L cell) demonstrates that there are no general repressors of galactose metabolism in the Dulbecco modified Eagle medium or dialyzed serum. HepG2 cells also utilized galactose efficiently as a primary carbon source (data not shown).

TABLE 3. Characteristics of transfected H12.10 cells

Transfectant	Galactokinase activity[a]	RSV-*galK* mRNA[b]
H12.10	0.05 Δ <0.02	0
H12.10.4	103.5 Δ 3.55	120
H12.10.5	0.15 Δ 0.07	0
H12.10.6	72.93 Δ 3.64	81
H12.10.7	107.5 Δ 2.35	176
H12.10.11	13.07 Δ 0.02	12

[a] Values are determined as described in Table 2, footnote *b*. Δ, Difference between the two samples assayed.

[b] Data are relative optical density units from soft laser densitometry scans of different autoradiographic exposures of the primer extension analysis shown in Fig. 2C.

Uridyltransferase deficiency in H2.35 cells. We considered it possible that in H2.35 cells, gal-1-P might not be metabolized due to a deficiency in the gal-1-P uridyltransferase. We devised a coupled galactokinase-uridyltransferase reaction, in which synthesis of gal-1-P in the extract provided a substrate for the transferase. When an adult mouse liver cell extract was provided with UDP-glucose, ^{14}C-galactose, and ATP, two-dimensional thin-layer chromatography of the reaction products demonstrated conclusively that UDP-galactose was synthesized (Fig. 4). One-dimensional thin-layer chromatography analysis showed that a product comigrating with UDP-galactose was also produced with an extract from HepG2 cells, whereas no comigrating product was observed in reactions that contained all substrates except UDP-glucose (data not shown). Filter quantitation of the assay showed that liver extracts contained only 29% of the galactokinase activity of H2.35 cell extracts (Table 5), but when UDP-glucose was added, 20% of the gal-1-P was converted to phosphatase-resistant UDP-galactose in 45 min (Table 5). By contrast, in H2.35 cell extracts, only 1.0% of the gal-1-P was utilized by uridyltransferase (Table 5); L and HepG2 cells utilized 2.2 and 2.9%, respectively. The higher levels of production of UDP-galactose by L and HepG2 cells, compared with H2.35 cells, apparently permitted growth on galactose as a primary carbon source (Fig. 3).

A galactose-metabolizing variant of H2.35 cells. The lack of growth of H2.35 cells in galactose medium provided the

TABLE 4. Galactokinase activities in cell lines cultured with different primary sources of carbon

Cell line	Primary carbon source[a]	Galactokinase activity[b]
HepG2	Glucose	44.68 Δ 4.84
	Galactose	45.05 Δ 1.15
	Glucose plus galactose	39.59 Δ 1.07
L cells	Glucose	29.72 Δ 3.08
	Galactose	27.97 Δ 0.15
	Glucose plus galactose	26.75 Δ 0.35
H2.35	Glucose	42.39 Δ 4.99
	Galactose	45.52 Δ 0.02
	Glucose plus galactose	48.76 Δ 0.67
H34.2	Glucose	52.56 Δ 2.22
	Galactose	45.92 Δ 0.62
	Glucose plus galactose	44.41 Δ 1.42

[a] All carbon sources were present at 1 mg of each per ml in medium containing dialyzed fetal bovine serum. Cells were plated in glucose medium, washed twice in situ with PBS, and then cultured in the designated media for 48 h before the cell extracts were prepared.

[b] Values are determined as described in Table 2, footnote *b*. Δ, Difference between the two samples assayed.

TABLE 5. Uridyltransferase activities in mouse liver and H2.35 cell derivatives

Extract	Control reaction[a]	Gal-1-P synthesis[a]	UDP-galactose synthesis[a]	Uridyl-transferase activity[b] (%)
Liver	0.22 ± 0.01	11.82 ± 0.49	2.37 ± 0.52	20
L cells	0.38 ± 0.01	29.48 ± 2.16	0.66 ± 0.07	2.2
HepG2	0.67 ± 0.07	49.65 ± 1.9	1.44 ± 0.29	2.9
H2.35	0.14 ± 0.09	40.68 ± 3.9	0.39 ± 0.08	1.0
H34.2	0.25 ± 0.06	49.45 ± 5.23	0.91 ± 0.26	1.8

[a] Average values (means ± standard errors) of duplicate reactions from two separate experiments (except HepG2; one experiment) expressed as nanomoles synthesized per hour per milligram of protein minus the average control reaction values (for gal-1-P and UDP-galactose synthesis) (controls lacked UDP-glucose but were treated with phosphatase).

[b] Uridyltransferase activities are expressed as the percentage of UDP-galactose product relative to the total amount of gal-1-P substrate synthesized in the reaction.

opportunity to select for spontaneously occurring variants that could utilize galactose. Accordingly, we plated 10^5 H2.35 cells per 100-mm plate in glucose medium, changed to galactose medium after two days, and after 4 and one-half weeks in culture, observed, on average, a single tight colony of cells per plate. Only one colony that was picked survived subcloning in galactose medium; it was then expanded nonselectively for about 35 cell doublings to establish a cloned line. We then tested this variant cell line, H34.2, for growth in various media. H34.2 cells grew slowly in galactose medium to about 10% of the level achieved in glucose medium (Fig. 3, row H34.2). However, H34.2 cells grew moderately in pyruvate and galactose (each at 1 mg/ml), about 40% of the level in glucose medium. Apparently, when pyruvate is provided as an energy source for H34.2 cells, galactose can be used to supply nonglycolytic requirements for 6-carbon sugars.

To understand the change in H34.2 cells that permitted galactose utilization, we assayed galactose-metabolizing enzymes. In two separate experiments performed in duplicate, H34.2 cells exhibited a gal-1-P uridyltransferase activity that was 1.8-fold higher than for H2.35 cells (Table 5). One-dimensional thin-layer chromatography analysis of the reac-

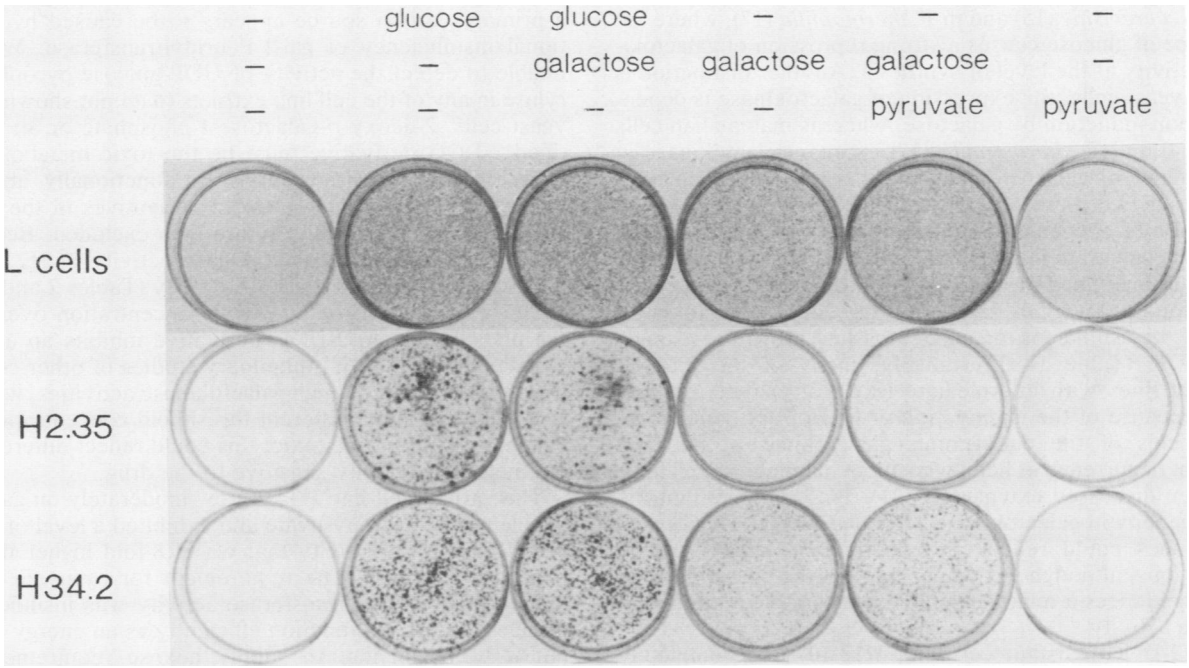

FIG. 3. Different growth patterns of mouse cell lines in galactose media. Cells (2×10^4) of each line were plated onto 60-mm diameter plates in glucose medium. After 1 day, the cells were rinsed in situ with PBS and changed to medium containing 1 mg of each of the carbon substrates shown per ml. Cells were cultured until the glucose plates attained confluence, which was 4 days for L cells and 11 days for H2.35 and H34.2 cells. Cells were fixed to the plates with Formalin, stained with methylene blue, and photographed.

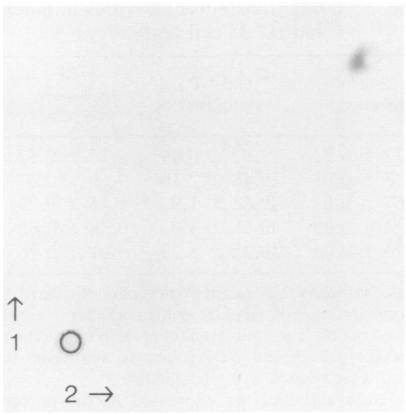

FIG. 4. Chromatographic analysis demonstrates the synthesis of [^{14}C]UDP-galactose in a UDP-glucose uridyltransferase assay. Reaction products were mixed with nonradioactive UDP-galactose and then chromatographed on a polyethyleneimine cellulose plate with a Tris hydrochloride solvent in the first dimension and then with a $(NH_4)_2SO_4$-EDTA solvent in the second dimension. The spot on the lower left is the origin. The position of the nonradioactive UDP-galactose was visualized with UV light and corresponded precisely to the position of the spot at the upper right.

tion products demonstrated the synthesis of UDP-galactose (data not shown). Taking together the results with H2.35, H34.2, and L cells, we conclude that gal-1-P uridyltransferase activity is rate limiting for growth of H2.35 cells on galactose as a primary carbon source.

DISCUSSION

Our results demonstrate that galactokinase was expressed in mammalian cells grown in glucose while in the absence of galactose. This is a major difference from the situation in the yeast *S. cerevisiae* (15) and in *T. thermophila* (17), where the presence of glucose causes a strong repression of galactokinase activity at the level of synthesis. Another distinction is that in yeast cells, the expression of galactokinase is dependent upon induction by galactose, whereas mammalian cells (Table 4) and *T. thermophila* (17) express galactokinase in the absence of galactose. Clearly, there are significant differences between unicellular eucaryotes and mammals in the regulation of *gal* genes. The lack of direct regulation by the galactose substrate in mammals is presumably compensated by as yet undefined mechanisms of hormonal control.

We found that a 50% reduction in cellular galactokinase activity was sufficient to cause a marked growth resistance to 2-DOG (Table 1). Presumably, many of the 2-DOG-resistant lines with intermediate levels of enzyme activity arose because of the inactivation or loss of only one of the two alleles of the endogenous galactokinase gene. The frequent occurrence of hemizygosity in mammalian cell lines has been discussed extensively (21). We speculate that the heterogeneity in galactokinase activities in the 2-DOG-resistant clones could reflect chromosomal instability in the populations, although we cannot rule out the possibility that the drug induces a relatively stable but variable reduction in enzyme activity.

One 2-DOG-resistant cell line, H12.10, was completely deficient in functional galactokinase. Because a transfected *E. coli galK* gene could restore galactokinase activity, the galactokinase deficiency in H12.10 cells is probably not due to a dominant mutation that counteracts enzyme activity. Presumably, the defect is due to the inactivation of both chromosomal alleles of the endogenous galactokinase gene. Because the deficiency is stable in long-term, nonselective culture, we suggest that it occurred by severe inactivation of one allele, followed by either mitotic crossing over or loss of the wild-type chromosome (and perhaps duplication of the mutant one), to generate a hemizygous (or homozygous) mutant; two independent stable mutations would seem less likely. We also found that serum albumin mRNA levels were inducible in H12.10 cells cultured under conditions (28) that promote hepatocyte differentiation (data not shown), showing that the 2-DOG selection did not generally perturb hepatic function.

We showed that high-level expression of a transfected *E. coli galK* gene could restore 2-DOG sensitivity in the H12.10 cell line, making it possible to use the *galK* marker to employ negative genetic selections for studying liver gene regulation. For example, a liver-specific promoter could be fused to *galK*, and the construct could be used to select against the expression of a relevant hepatocyte-specific activator. The recovery of a desired "factor mutant" would be enhanced by performing multiple selections, i.e., by adding a second construct containing the same promoter fused to a different selectable marker, such as the herpes virus thymidine kinase gene (7), and selecting against the expression of *galK* and *tk* simultaneously. The fact that the 2-DOG selection is sensitive to small differences in galactokinase levels makes it particularly suited for such selections, because the loss of one regulatory factor might be compensated partially by the presence of a distinct factor with a similar DNA binding specificity (11, 22, 25).

A previous study showed that a transfected *galK* gene could complement the galactokinase deficiency of a variant Chinese hamster ovary cell line (24) by assaying growth in galactose medium lacking glucose (18); growth in 2-DOG medium was not tested. In contrast, the inability of H2.35 cells or *galK*-transfected H12.10 cells to utilize galactose as a primary carbon source appears to be caused by a functional insufficiency of gal-1-P uridyltransferase. We were unable to detect the activity of UDP-glucose pyrophosphorylase in any of the cell line extracts (data not shown). As in yeast cells, 2-deoxy-D-galactose-1-phosphate or an unidentified 2-DOG derivative must be the toxic metabolite, because uridyltransferase activity is functionally absent in H2.35 cells, yet 2-DOG is toxic; derivatives in the normal galactose metabolic pathway are thus excluded. Because a twofold difference in galactokinase activity in H2.35 cells had a marked effect on 2-DOG toxicity (Tables 2 and 3; Fig. 1), there appears to be a critical concentration over which the phosphorylated 2-DOG derivative inhibits an essential metabolic process. In preliminary studies of other cell lines that contain similarly high galactokinase activities, we found that each line had a different threshhold concentration over which 2-DOG became toxic; this could reflect differences in the metabolic activity sensitive to the drug.

The variant cell line H34.2 grew moderately on galactose supplemented with pyruvate and exhibited a level of gal-1-P uridyltransferase activity that was 1.8-fold higher than the parent H2.35 cells. The requirement for pyruvate implies that the increase in transferase activity was insufficient to permit galactose to function efficiently as an energy source, but it was sufficient to supply hexose requirements for protein glycosylation and ribose synthesis. Thus, H2.35 cells appear to be an appropriate system for selecting for *cis*- and *trans*-acting mutations affecting uridyltransferase levels. Perhaps the addition of pyruvate to galactose medium would

facilitate future selections for galactose-metabolizing variants.

Finally, we suggest that the isogenic cell lines described here should greatly facilitate the study of physiological consequences of galactosemic disorders. Galactosemia occurs when galactose accumulates in the blood and is converted to toxic substances by various organs. In this study, we found that galactose was slightly inhibitory to the growth of H2.35 cells in glucose medium (Fig. 3). Thus, H2.35 cells and their isogenic derivatives could be used to study abnormal fates of galactose and to assess the contributions of uridyltransferase to abnormal galactose metabolism. The deficient cells described here could also be used in transfection and complementation assays to study the function of cloned human galactokinase and uridyltransferase genes. Such studies could explore the challenges associated with gene therapy of galactosemic individuals.

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