Somatic Cell and Molecular Genetics, Vol. 18, No. 1, 1992, pp. 89–96

Reconstitution of Enzymatic Activity in Hepatocytes of Phenylalanine Hydroxylase-Deficient Mice

Ta-Jen Liu,[1,2] Mark A. Kay,[2,3] Gretchen J. Darlington,[4] and Savio L.C. Woo[1-3]

[1]*Howard Hughes Medical Institute, Departments of* [2]*Cell Biology,* [3]*Molecular Genetics, and* [4]*Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030*

Received 20 November 1991

Abstract—*Phenylketonuria (PKU) is a metabolic disorder secondary to a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH). The recent creation of a mouse strain for PAH deficiency has provided an excellent model system to explore the possibility of its phenotypic correction by hepatic gene therapy. A recombinant retrovirus containing the mouse PAH cDNA under the transcriptional control of the human CMV promoter was constructed and used to transduce hepatocytes isolated from PAH-deficient mice. Viral-transduced hepatocytes produced dramatically higher levels of mouse PAH mRNA as compared to control mock-infected hepatocytes. The PAH mRNA was translated efficiently into PAH protein that is capable of converting phenylalanine to tyrosine in vitro. These results demonstrate that the PAH-deficient mouse hepatocytes can be readily reconstituted by retroviral-mediated gene transduction, which is a crucial step towards somatic gene therapy for PKU.*

INTRODUCTION

Phenylalanine hydroxylase (PAH, EC 1.14.16.1) is a liver-specific enzyme (1) that converts phenylalanine to tyrosine using tetrahydrobiopterin as a cofactor (2). Deficiency of PAH results in the disease phenylketonuria (PKU), in which the failure of normal phenylalanine catabolism in blood and other tissues leads to an accumulation of phenylalanine and its abnormal metabolites. This in turn causes severe mental retardation (3, 4) unless the patient is maintained on a low-phenylalanine diet. Although dietary therapy is successful in treating most of the symptoms of PKU patients, it suffers from some limitations: (1) This therapy is difficult to implement in certain patients who have poor compliance. (2) There are cumulative cognitive defects following diet discontinuation. (3) Successful correction of PKU phenotype by early dietary treatment has contributed to the development of a new syndrome termed "maternal PKU" (5), which is manifested as a birth defect in fetuses born to PKU mothers who have hyperphenylalaninemia due to diet discontinuation.

Somatic gene therapy, in which a recombinant phenylalanine hydroxylase gene would be introduced into cells of the patient in order to restore PAH enzyme activity, may be considered as an alternative treatment for PKU. Retroviral-mediated gene transfer is a powerful method of introducing genes into a variety of primary mammalian cells (6, 7). We have recently reported that the human PAH gene can be introduced into primary mouse hepatocytes and expressed normally in these cells (8) and that mouse hepatocytes

0740-7750/92/0100-0089$06.50/0 © 1992 Plenum Publishing Corporation

can be successfully transplanted into mice, where they survived and continued to function as liver cells for as long as the recipient animals lived (9). These studies provide strong support for the concept of gene therapy for hepatic deficiencies such as PKU.

Recently, a PAH-deficient mouse model ($Pah^{hph\text{-}5}$) was created by ethylnitrosourea mutagenesis (9). The homozygus mutants exhibit 3% of normal PAH activity while other biochemical components of phenylalanine catabolism are normal. The PAH-deficient mouse provides an excellent model to explore various techniques for somatic gene therapy of PKU. We report here the mouse PAH gene can be efficiently transfered into PAH-deficient mouse hepatocytes by a recombinant retrovirus. The transduced PAH gene is expressed at high levels in the hepatocytes and is translated into a functional PAH protein.

MATERIALS AND METHODS

Construction and Production of Recombinant PAH Retrovirus. The LNCX retroviral vector (10) was kindly provided by Dusty Miller. The blunt-ended BamHI–HincII fragment encoding the mouse PAH cDNA (11) was inserted into the HpaI site of the LNCX vector. The resulting LNC-mPAH (Fig. 3A below) plasmid was transfected into the amphotropic packaging cell line GP+envAM12 (12) by overnight precipitation with calcium phosphate (13). Forty-eight hours posttransfection, the media was harvested, filtered through a 45-μm filtering unit, and used to infect the ecotropic packaging cell line GP+E-86 (14). G418-resistant colonies were picked, expanded, and analyzed for viral titer. The highest titer ecotropic producer [approximately 3×10^6 colony-forming units (CFU)/ml] was used for hepatocyte infection.

Isolation of Hepatocytes. Hepatocytes were isolated from a PAH-deficient mouse by a collagenase perfusion technique (15) using 0.3 mg/ml type B collagenase (Boehringer Mannheim, Indianapolis, Indiana; 0.197 units/mg), which yielded $2–4 \times 10^7$ hepatocytes per 6- to 8-week-old mouse. Isolated hepatocytes were plated onto Primaria tissue culture plates (Falcon) at a density of $2–5 \times 10^5$ cells/60-mm dish in 2 ml of tyrosine-free media (TFM) supplemented with 10% fetal bovine serum and antibiotics (penicillin at 100 units/ml, streptomycin sulfate at 100 μg/ml). After more than 80% of cells attached on the plate (around 1–2 h), the medium was changed to hormonally defined SUM-3 medium (16), which was subsequently changed every 24 h.

Infection of Hepatocytes. Viruses were harvested in high-glucose DMEM (Gibco) media with 10% fetal bovine serum and antibiotics. The conditioned medium containing either recombinant PAH or β-galactosidase (BAG) retrovirus was then filtered through 0.45-μm Millipore filters. Primary hepatocytes were infected for 2 h with or without (mock) recombinant retroviruses in the presence of 4 μg/ml of polybrene (Aldrich). The medium was then replaced with fresh SUM-3 medium and changed every 24 h. After 48 h cells were harvested by trypsinization. RNA and cell extracts were then prepared for analysis.

Cytochemical Stains. Two days after viral infection, cells were rinsed twice with PBS and then fixed for 5 min with 0.5% glutaraldehyde in cold PBS. After rinsing with PBS, the fixed cells were incubated with solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Stratagene; La Jolla, California) (17).

RNA Analysis. RNA was prepared by the hot phenol method and northern blotting was performed after formaldehyde–agarose gel electrophoresis as described (8). Blotted RNA was hybridized with the mouse PAH cDNA (11) probe prepared from random priming to a specific activity of 3×10^8 cpm/μg with [^{32}P]dCTP.

Protein Analysis. Cells were harvested by trypsinization from the plates two days after viral infection and lysed in 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecylsulfate, 50 mM Tris HCl (pH 7.5), and 0.1 M NaCl, 2 mM DTT; the cell lysate was spun down and the supernatant was collected for further analysis. Protein concentrations were determined using the Bradford protein assay (18) with a kit obtained from Bio-Rad (Richmond, California). The proteins were subjected to electrophoresis on SDS–10% polyacrylamide gels and blotted onto Immobilon membranes (Millipore) (19). Blots were blocked with 5% w/v nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in PBS (Blotto/Tween) (20) for 3 h at room temperature and incubated with a monospecific goat anti-rat phenylalanine hydroxylase primary antibody (21) in Blotto/Tween for 12 h at 4°C. The membrane was washed five times in PBS, then incubated with 10 μCi of ^{125}I-labeled streptococcal protein G (ICN) for 1 h at room temperature. After an additional five washes, the filter was dried and the specific bands were then visualized by autoradiography.

PAH Enzymatic Assays. PAH activity was assayed in vitro by measuring the production of [^{14}C]tyrosine from [^{14}C]phenylalanine in presence of either liver extract or cellular extract from cultured hepatocytes, 50 mM Tris (pH, 7.5), 1 mM phenylalanine, 4 × 10^5 cpm [^{14}C]phenylalanine, 0.15 M KCl, 20 units catalase, 200 μM 6-MPH_4, and 2 mM DTT (22). The reaction mixture was incubated at 37°C for 60 min, stopped by boiling for 5 min, and chilled for 5 min. Marker amino acids (50 mM Phe, 50 mM Tyr) were added and the protein was pelleted in an Eppendorf microfuge (22). [^{14}C]Phenylalanine and [^{14}C]tyrosine were separated on silica gel 60 TLC plates (EM) in chloroform–methanol–ammonia 55:35:10 and quantified by scintillation counting.

RESULTS

Retroviral Infection of Mouse Hepatocytes. The conditions for optimal viral transduction of primary mouse hepatocytes were determined by infecting these cells with a high titer (2 × 10^6 CFU/ml) ecotropic retrovirus expressing the *E. coli* β-galactosidase gene under transcriptional control of the PGK promoter (Fig. 1A) (23). The transduction efficiency was assessed by counting the number of blue hepatocytes after X-gal

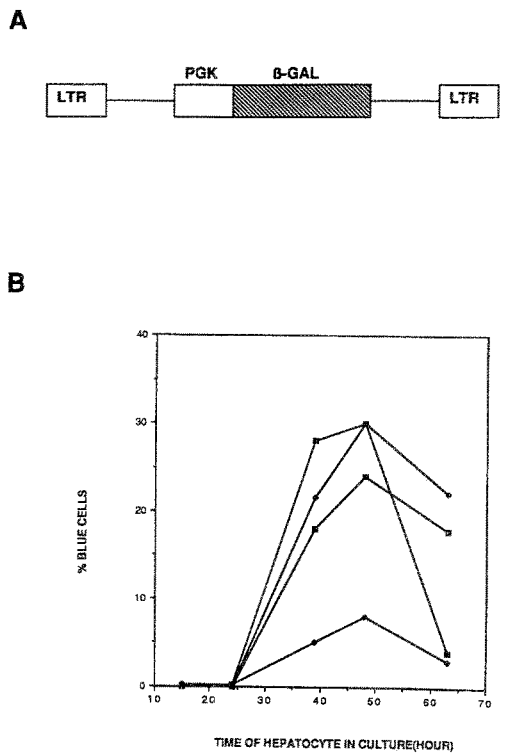

Fig. 1. Conditions for retroviral infection. (A) Structure of β-galactosidase retroviral (BAG virus) vector. The expression of *E. coli* β-galactosidase is under the transcriptional control of phosphoglycerol kinase (PGK) ubiquitous cellular promoter (23). (B) Optimization of viral infection of hepatocytes. Primary mouse hepatocytes plated at different densities: —⊡—, 1 × 10^5 cells; —◆—, 2 × 10^5 cells; —□—, 5 × 10^5 cells; —◇—, 1 × 10^6 cells/60-mm dish were infected with BAG virus, as described in the Materials and Methods section. The percentage of β-galactosidase-positive hepatocytes values were obtained from scoring 500 hepatocytes each on replicate dishes.

staining. There were two parameters that affected the efficiency of viral transduction (Fig. 1B). The optimal density of plated hepatocytes required for retroviral infection was determined to be 2–5 $\times$ 10^5/60-mm culture dish (49 cm^2 surface area). The second important parameter was the length of time the hepatocytes were grown in culture prior to retroviral infection. Optimal retroviral infection was obtained between 39 and 48 h after plating. Under these conditions, about 30% of hepatocytes were stained blue after viral transduction (Fig. 2). These optimal conditions established were then used in subsequent experiments.

Recombinant Mouse PAH mRNA Expression in Viral-Transduced Hepatocytes. A recombinant retroviral vector LNC-mPAH was constructed (Fig. 3A) and a high titer (3×10^6 CFU/ml) recombinant virus was isolated and used to infect PAH-deficient hepatocytes under the conditions described above. Two days postinfection, total RNA was isolated from the virus-producing cell line, and from viral- and mock-infected hepatocytes from PAH-deficient mice, and was analyzed by northern blot hybridization using the mouse PAH cDNA as a probe. There is a 2.1-kb endogenous PAH mRNA detected in both a control RNA isolated from control normal mouse liver (Fig. 3B, lane 1) and in freshly plated hepatocytes from a PAH-deficient mouse (Fig. 3B, lane 2). Surprisingly, no hybridizable endogenous PAH mRNA is detected in mock-infected deficient hepatocytes (Fig. 3B, lane 3), suggesting that the steady-state level of the endogenous PAH mRNA is down-regulated in hepatocytes under the culture conditions. The virus-producing cell line (Fig. 3B, lane 5)

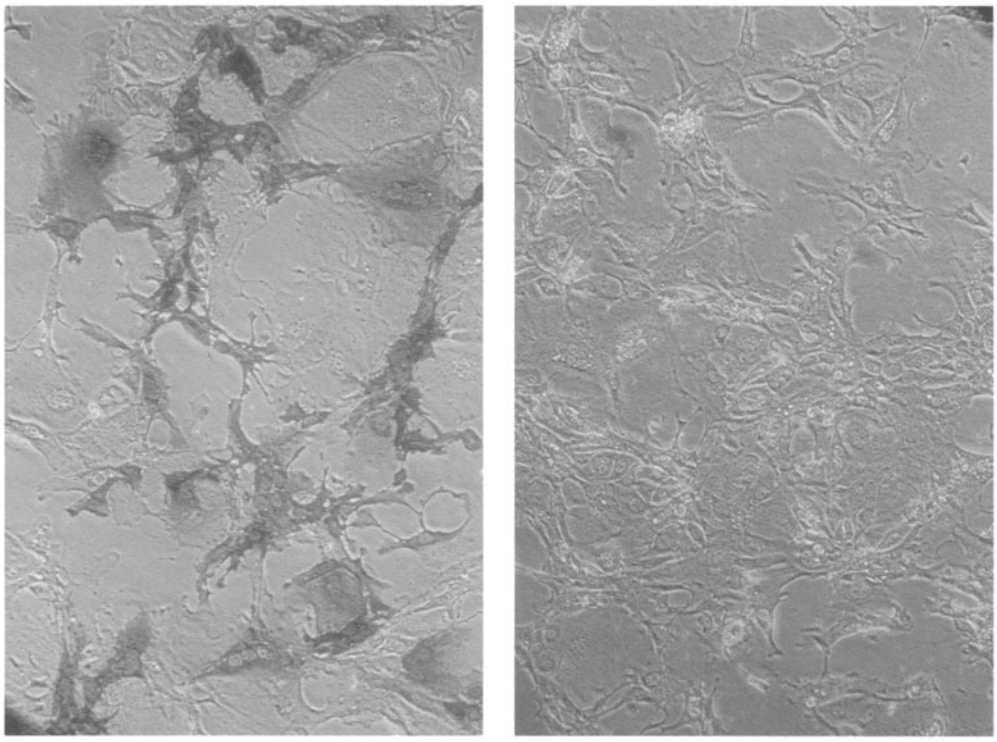

Fig. 2. Histochemical expression of β-galactosidase activity in viral-transduced and mock-infected hepatocytes. There are about 30% of hepatocytes stained blue after retroviral infection (A). As a control, the mock-infected hepatocytes show no cells stained blue (B).

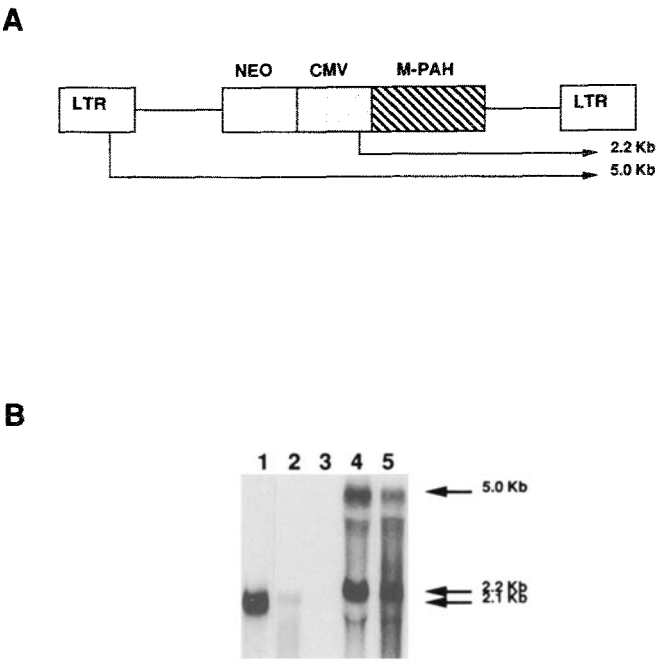

Fig. 3. Expression of PAH mRNA in viral-transduced hepatocytes. (A) Schematic of LNC-mPAH vector and RNA transcripts from the vector. Transcription from the 5′ LTR promoter in provirus results in a full-length proviral transcript of 5.1 kb. Transcription from the internal CMV promoter results in a 2.2-kb mRNA. (B) Total cytoplasmic RNA isolated from virus-producing cell lines and from hepatocytes infected with the LNC-mPAH virus were subjected to northern blot analysis. RNA were fractionated on formaldehyde–agarose gel, transfered on to a nylon membrane (Hybond-N^+), and hybridized to a 1.4-kb mouse PAH cDNA probe label by random priming. Lane 1, 10 μg of RNA from freshly plated normal mouse hepatocytes; lane 2, 10 μg of RNA from freshly plated PAH-deficient hepatocytes; lane 3, 10 μg of RNA from mock-infected PAH-deficient hepatocytes; lane 4, 10 μg of RNA from PAH-deficient hepatocytes infected with LNC-mPAH virus; lane 5, 10 μg of RNA from a virus-producing cell line.

and the PAH-deficient mouse hepatocytes infected with LNC-mPAH recombinant retrovirus (Fig. 3B, lane 4) exhibit two PAH mRNA species. The 5.0-kb transcript is the full-length proviral transcript initiated from the 5′ LTR promoter (24). The 2.2-kb mRNA band corresponds to the transcript originating from the internal CMV promoter. There appears to be twofold higher transcript expressed from the CMV promoter than from the LTR promoter in the infected hepatocytes (Fig. 3B, lane 4). This result indicates that the CMV promoter is stronger than the retroviral LTR promoter, similar to the result observed in primary rabbit hepatocytes (25). In the viral-transduced hepatocytes (Fig. 3B, lane 4), the steady-state level of recombinant PAH mRNA is about 20-fold higher than that of freshly plated hepatocytes isolated from a PAH-deficient mouse (Fig. 3B, compare lanes 2 and 4). The high level of PAH mRNA in viral-transduced hepatocytes indicates that the mouse PAH cDNA was successfully transduced into hepatocytes and efficiently transcribed.

Expression of Mouse PAH Protein in Viral-Transduced Hepatocytes. Western blot analysis was performed to correlate the levels of PAH mRNA to the amount of PAH protein production. A 52-kDa band recognized by a monospecific anti-PAH antibody (21) was observed in cellular extracts isolated from freshly plated normal mouse hepatocytes (Fig. 4A, lane 1). The level was much reduced in PAH-deficient mice (Fig. 4A,

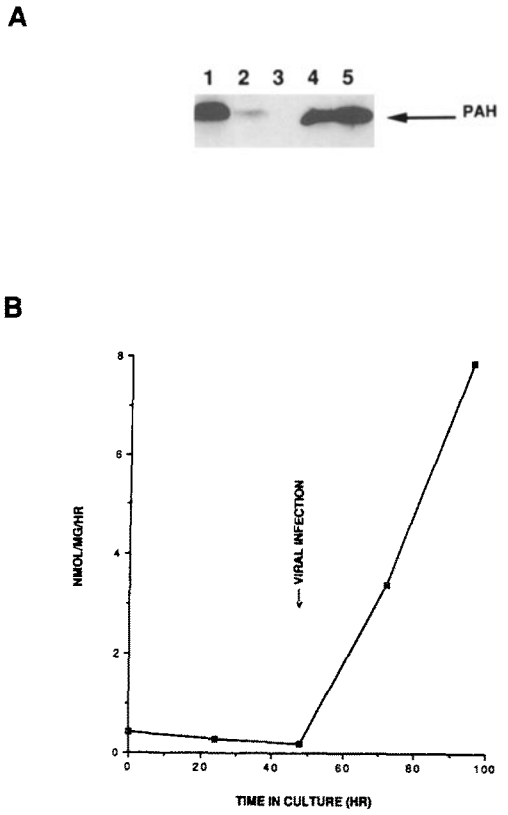

Fig. 4. Western blot analysis and enzymatic assay of PAH protein produced by infected hepatocytes. (A) Cell extracts isolated from either liver or hepatocytes were subjected to SDS–polyacrylamide gels, blotted onto nitrocellulose, and probed with monospecific anti-rat PAH antibody. Lane 1, 100 μg of cell extract from normal mouse hepatocytes; lane 2, 100 μg of cell extract from PAH-deficient mouse hepatocytes; lane 3, 100 μg of cell extract from mock-infected deficient hepatocytes; lane 4, 100 μg of cell extract from viral-transduced hepatocytes; lane 5, 100 μg of cell extract from virus-producing cell line. (B) Cell extracts isolated from hepatocytes at different times in culture were subjected to in vitro PAH enzymatic assay as described in Materials and Methods. The PAH specific activity at 0 h was 0.42 nmol/mg/h and increased to 8 nmol/mg/h at 48 h postinfection.

lane 2). The same 52-kDa band also appears in extracts from the virus-producing cell line (Fig. 4A, lane 5). There is no detectable PAH protein in extracts of mock-infected hepatocytes from PAH-deficient mice (Fig. 4A, lane 3). The amount of immunoreactive protein increased dramatically in PAH-deficient hepatocytes after viral transduction (Fig. 4A, lane 4). The amount of immunoreactive protein produced in viral-transduced hepatocytes correlates with the amount of PAH mRNA present after viral transduction (see Fig. 3B). This result indicates that the mouse PAH mRNA produced from hepatocytes after viral transduction is translated efficiently into immunoreactive PAH protein.

Immunoreactive PAH Protein is Functional. In order to verify that the immunoreactive PAH protein produced from viral-transduced hepatocytes was functional, cellular extracts from viral-transduced hepatocytes were used in an in vitro PAH enzymatic assay. In hepatocytes isolated from PAH-deficient mice, the specific activity of PAH protein at 0 h after plating is 0.42 nmol/mg/h and drops to almost zero at 48 h after plating (Fig. 4B). After viral transduction, the PAH activity increases to 8 nmol/mg/h at 48 h postinfection (Fig. 4B). This change represents a 20-fold increase in PAH activity. These experiments demonstrate that the immunoreactive PAH protein is indeed functional. Furthermore, the enzymatic activity correlates well with the amount of mRNA and PAH protein produced in viral-transduced hepatocytes isolated from PAH-deficient mice.

DISCUSSION

The liver plays an essential role in somatic homeostasis as the major site of intermediary metabolism and the source for most serum proteins. Many human metabolic disorders involve genes that are normally expressed specifically in the liver. Thus, in considering somatic gene therapy for disorders such as PKU, it would be desirable to introduce the normal PAH gene into hepatocytes that constitutively synthesize and reduce the biopterin cofactor.

The viral transduction efficiency of hepatocytes from PAH-deficient mice in this study is 30% at 48 h after plating. These

hepatocytes were cultured in hormonally defined media, which serves the dual function of sustaining hepatocyte proliferation and differentiation and preventing overgrowth of fibroblast and endothelial-like cells. We have reported recently that under similar culture conditions the transduction efficiency of primary rabbit hepatocytes (25) was 20%. In a larger animal such as the dog, 25% of the hepatocytes can be transduced by a β-galactosidase recombinant retrovirus (M. Kay and S.L.C. Woo, unpublished data). Maximal transduction efficiency of ≈25% was reported in adult rat hepatocytes cultured in extracellular matrix (ECM)-coated dishes (26). It also has been shown that 22–25% of rat hepatocytes cultured in ECM-coated dishes can be transduced with a different recombinant retrovirus, neomycin phosphotransferase (NPT) (27). Different culturing conditions from ours and others have little or no influence on the viral transduction efficiency. It is possible that better transduction efficiencies may be achieved by using younger animals for hepatocyte isolation. Because hepatocytes undergo a limited number of divisions in culture, inclusion of a more potent mitogen in the culture medium would increase the transduction efficiency of hepatocytes or even allow transduced hepatocytes to be expanded. Recently, there is a report of 70–90% transduction efficiency of fetal rat hepatocytes cultured in ECM with inclusion of chemical modulators of hepatocyte growth and differentiation such as gelatin and hydrocortisone (28). This study demonstrates the efficient retroviral-mediated gene transfer of phenylalanine hydroxylase into PAH-deficient hepatocytes, resulting in the complete reconstitution of the phenylalanine hydroxylating system. There was at least 20-fold more PAH mRNA produced in viral-transduced hepatocytes as compared to mock-infected hepatocytes. The amount of immunoreactive protein and the enzymatic activity present in the viral-transduced PAH-deficient hepatocytes are well correlated with the steady-state levels of PAH mRNA. Viral-transduced hepatocytes from PAH-deficient mice exhibit about 20-fold higher levels of PAH enzymatic activity in vitro as compared to mock-infected hepatocytes. These results indicate that the conditions for culturing hepatocytes do not affect their integrity with respect to viral infection and expression of the transferred gene, since the transduced mouse PAH gene can still be expressed and properly translated into functional immunoreactive PAH protein.

We and others have demonstrated recently that transgenic mouse hepatocytes can be transplanted into congenic recipients where they survived and continued to function as hepatocytes for the life of the recipient (29). Somatic gene therapy of the PAH-deficient mouse model would therefore involve obtaining hepatocytes from an affected animal, transducing a normal PAH gene into these cells by retroviral-mediated gene transfer, followed by heterologous transplantation of the transduced hepatocytes into congenic recipients. The present work represents a first step in this direction by demonstrating the successful enzymatic reconstitution in PAH-deficient hepatocytes in vitro. Techniques developed in the phenotypic correction of PAH deficiency in the mouse model will be critical for the treatment of human PKU patients in the future by hepatic gene therapy.

ACKNOWLEDGMENTS

We thank Drs. J. David McDonald, Alexandra Shedlovsky, and William Dove for the $Pah^{hph\text{-}5}$ mouse strain; Dr. Arthur Bank for providing the GP+E-86 and GP+AM12 packaging cell lines and Dr. Phil Soriano for providing the recombinant β-galactosidase retrovirus; Dr. Dusty Miller for supplying the LNCX vrial vector construct; and Drs. Randy Eisensmith and Anu Bhattacharya for constructive comments on the manuscript. This

work was partially supported by National Institutes of Health grant DK 44080. T.J.L. is an Associate and S.L.C.W. is an investigator of the Howard Hughes Medical Institute. M.A.K. is supported by NRSA GM 13894.

LITERATURE CITED

1. Crawford, M., Gibbs, D., and Sheppard, D. (1981). *J. Inherited Metab. Dis.* **4**:191–195.
2. Kaufman, S. (1976). *Adv. Neurochem.* **2**:1–132.
3. Scriver, C.R., Kaufman, S., and Woo, S.L.C. (1989). The hyperphenylalaninemias. In *The Metabolic Basis of Inherited Disease, Vol. II, 6th ed.,* (eds.) Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D. (McGraw-Hill, New York).
4. Guttler, F. (1980). *Acta Pediatr. Scand. Suppl.* **280**:1–80.
5. Lenke, R.R., and Levy, H.L. (1980). *N. Engl. J. Med.* **303**:1202.
6. St. Louis, D., and Verma, I.M. (1988). *Proc. Natl. Acad. Sci. U.S.A.* **85**:3150–3154.
7. Palmer, T., Thompson, A., and Miller, D. (1989). *Blood* **73**:438–445.
8. Peng, H., Armentano, D., MacKenzie-Graham, L., Shen, R.-F., Darlington, G., Ledley, F., and Woo, S.L.C. (1988). *Proc. Natl. Acad. Sci. U.S.A.* **85**:8146–8150.
9. McDonald, D., Bode, V., Dove, W., and Shedlovsky, A. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87**:1965–1967.
10. Miller, A.D., and Rosman, G.J. (1989). *BioTechniques* **7**:980–990.
11. Ledley, F., Grenett, H., Dunbar, B., and Woo, S.L.C. (1990). *Biochem. J.* **267**:399–406.
12. Markowtiz, D., Goff, S., and Bank, A. (1988). *Virology* **167**:400–406.
13. Chen, C., and Okayama, H. (1987). *Mol. Cell. Biol.* **7**:2745–2752.
14. Markowtiz, D., Goff, S., and Bank, A. (1988). *J. Virol.* **62**:1120–1124.
15. Berry, G., and Friend, D. (1969). *J. Cell Biol.* **43**:506–520.
16. Darlington, G., Kelly, J., and Buffone, G. (1987). *In Vitro Cell Dev. Biol.* **23**:349–354.
17. Macgregor, G., Mogg, A., Burke, J., and Caskey, C. (1987). *Somat. Cell Mol. Genet.* **13**:253–265.
18. Bradford, M. (1976). *Anal. Biochem.* **72**:248–254.
19. Burnette, W. (1981). *Anal. Biochem.* **112**:195–203.
20. Harlow, E., and Lane, D. (1988). In *Antibodies,* (eds.) Harlow, E., and Lane, D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), pp. 497–503.
21. Robson, K., Chandra, T., MacGillivray, R., and Woo, S.L.C. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**:4701–4705.
22. Ledley, F., Grenett, H., McGinnis-Shelnutt, M., and Woo, S.L.C. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**:409–413.
23. Soriano, P., Friedrich, G., and Lawinger, P. (1991). *J. Virol.* **65**:2314–2319.
24. Armentano, D., Yu, S.F., Kantoff, P., von Ruden, T., Anderson, W.F., and Gilboa, E. (1987). *J. Virol.* **61**:1647–1650.
25. Armentano, D., Thompson, A., Darlington, G., and Woo, S.L.C. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87**:6141–6145.
26. Wilson, J., Jefferson, D., Chowdhury, J., Novikoff, P., Johnston, R., and Mulligan, R. (1988). *Proc. Natl. Acad. Sci. U.S.A.* **85**:3014–3018.
27. Anderson, K., Thompson, J., DiPietro, J., Montgomery, K., Reid, L., and Anderson, W.F. (1989). *Somat. Cell Mol. Genet.* **15**:215–227.
28. Koch, K., Brownlee, G., Goss, S., Martinez-Conde, A., and Leffert, H. (1991). *J. Cell Sci.* **99**:121–130.
29. Ponder, K., Gupta, S., Leland, F., Darlington, G., Finegold, M., DeMayo, J., Ledley, F., Chowdhury, J., and Woo, S.L.C. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88**:1217–1221.