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Hepatic Gene Transfer in Animals Using Retroviruses Containing the Promoter from the Gene for Phosphoenolpyruvate Carboxykinase*

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Two methods are described for directing the expression of genes to the livers of animals using retroviral vectors containing the predominantly liver-specific promoter from the gene for phosphoenolpyruvate carboxykinase (PEPCK)-linked to the structural gene for either amino 3′-glycosyl phosphotransferase (*neo*) or bovine growth hormone (bGH). Replication-incompetent retrovirus was used to infect the livers of fetal rats by intraperitoneal injection of animals *in utero* or to infect adult rats by direct injection into the portal vein after partial hepatectomy. The proviruses were integrated into the hepatic DNA, and the chimeric genes were expressed from the PEPCK promoter for as long as 8 months after infection. The expression of the PEPCK-bGH gene was regulated by diet and hormones in a manner similar to the regulation of the endogenous PEPCK gene in the liver. The potential of this method for targeting genes to the liver is discussed.

The successful introduction of genes into the liver of intact animals is an important first step in the correction of liver-specific genetic diseases. Genes have been transiently introduced and expressed in the livers of animals by the injection of DNA precipitates either directly into the liver (Dubensky *et al.*, 1984) or into the peritoneal cavity (Benvenisty and Reshef, 1986). In addition, genes can be introduced and expressed transiently in the livers of animals following either injection into the portal vein of rats using a liposome carrier containing DNA (Kaneda *et al.*, 1989) or by a receptor-mediated soluble DNA carrier system (Wu *et al.*, 1988, 1989). Except for the recent report of Wu *et al.* (1989) using the soluble DNA carrier system, most attempts have not resulted in the stable incorporation of DNA into the genome of the liver, a requirement for permanent genetic modification.

Retroviruses are an alternative method for the stable introduction of genes into animals, using either replication-incompetent retrovirus or co-infection with replication competent virus. Jaenisch and co-workers have used both methods to infect tissues of preimplantation (Jaenisch *et al.*, 1975; Jaenisch, 1976; Harbers *et al.*, 1982; Soriano and Jaenisch, 1986) and midgestation (Jaenisch, 1980; Jahner *et al.*, 1982; Compere *et al.*, 1989; Stuhlmann *et al.*, 1984) mouse embryos. The use of replication-incompetent retroviruses to infect animals *in vivo* is limited by the titer of the virus, and the detection of proviral integration usually requires the use of a selectable marker gene. For example, midgestation mouse embryos were infected with replication-defective retrovirus that transduced the *ras* and *myc* oncogene, but proviral integration was noted only in tumors (Compere *et al.*, 1989). However, the liver was not the primary target for tumor formation in these animals since relatively few hepatic tumors were noted. Additionally, a replication-competent retrovirus was used to transfer a mutant dihydrofolate reductase (DHFR)[1] gene, linked to the SV40 promoter into midgestation embryos. RNA from the livers of animals infected as fetuses contained barely detectable amounts of RNA transcribed from this chimeric SV40-DHFR gene (Stuhlmann *et al.*, 1989).

Retroviral infection of primary hepatocytes followed by transplantation into animals (Demetriou *et al.*, 1986) is another method for the stable modification of the hepatic genome. Hepatocytes from fetal rats are easily infected with retroviruses, but infection of hepatocytes from adult animals is limited (Wolff *et al.*, 1987). This indicates that factors other than cell division (Varmus *et al.*, 1977) are required for efficient retroviral infection; these may include the absence of receptors for the viral envelope protein on the surface of adult hepatocytes (Delarco *et al.*, 1978). Transduced genes have been expressed successfully in rat hepatocytes (Wolff *et al.*, 1987; Ledley *et al.*, 1986, 1987; Wilson *et al.*, 1987, 1988; Peng *et al.*, 1988), but to date these hepatocytes are not viable for more than several weeks after they are reintroduced into animals.

In order to ensure the integration of a gene into the genome of the liver cell it is necessary that the cells are dividing. There are two situations in which hepatocytes undergo mitosis and DNA synthesis: the fetal liver and the regenerating liver. Although there is considerable species variation, at day 18 of gestation (of a 22-day term gestation), rat liver is divided equally between hepatic and hematopoietic cells (Greengard *et al.*, 1972). The proportion of hepatic tissue increases greatly during the remainder of fetal life so that by birth 85–90% of

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[1] The abbreviations used are: DHFR gene, dihydrofolate reductase gene; PEPCK gene, phosphoenolpyruvate carboxykinase gene; bp, base pair(s); kb, kilobase(s); bGH, bovine growth hormone; cfu, colony-forming units; Bt_2cAMP, dibutyryl cAMP; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; LTR, long terminal repeat.

the liver is composed of hepatocytes. The hematopoietic cells appear to migrate out of the liver and populate the bone marrow. This remodeling is accompanied by the overall growth of the liver and bone marrow. The active division of cells in the fetal liver during late fetal life makes them a potential target for retroviral transduction.

Hepatocytes also divide in the regenerating rat liver following the removal of up to 70% of the liver (Higgins and Anderson, 1931). Within the first 24 h following partial hepatectomy, the onset of DNA synthesis occurs in the hepatic remnant followed closely by mitosis (Fabrikant, 1968; Grisham, 1962). For example, the percentage of hepatocytes undergoing DNA synthesis increases from less than 1% to about 50% by 24 h. The cells hypertrophy, and by 1 month, the liver has returned to its prehepatectomy mass (Higgins and Anderson, 1931). Recently Wu *et al.* (1989) showed that genes can be stably introduced into the hepatic genome by injection of suitably packaged DNA into the regenerating liver.

In the present study we use retroviral vectors that contain marker genes linked to the promoter-regulatory region of the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) from the rat. This promoter contains regulatory elements that can direct expression of linked genes to the liver and kidney of transgenic animals (McGrane *et al.*, 1988) as well as hormone regulatory elements that respond to cAMP (Wynshaw-Boris *et al.*, 1984, 1986; Short *et al.*, 1986), glucocorticoids (Wynshaw-Boris *et al.*, 1986; Short *et al.*, 1986; Magnuson *et al.*, 1987; Peterson *et al.*, 1988), and insulin (Hatzoglou *et al.*, 1988; Magnuson *et al.*, 1987) in cultured cell lines. Here we report our attempt to introduce genes stably into rat liver by the injection of replication-incompetent retrovirus either into the peritoneal cavity of fetuses *in utero* or directly into the portal vein of adult rats immediately following partial hepatectomy.

EXPERIMENTAL PROCEDURES

Construction of Retroviral Vectors, DNA Transfection, and Rescue of Recombinant Retrovirus—The parent vector, pLJ (Korman *et al.*, 1987), which was a generous gift from Dr. Richard Mulligan, M. I. T., Boston, MA, was used for the construction of retroviral vectors. pLJ is a Moloney murine leukemia virus-based vector that was designed to produce replication-incompetent retrovirus. Replication-incompetent retrovirus was generated by transfection of vector DNA into Ψ2 cells (Mann *et al.*, 1983). Details on the construction of the retroviral vectors used in this study as well as the methods for characterization and identification of the virus have been described previously by Hatzoglou *et al.* (1988). The titer for individual viruses was measured by determining the G418 resistance of NIH3T3 cells that had been infected with the virus. NIH3T3 cells were plated at a density of 10^5 cells/dish and were infected with serial dilutions of virus that had been filtered through membranes with a pore size of 0.45 μm in the presence of 8 μg/ml Polybrene. After 2 days, infected cells were passaged into media containing G418, and colonies of surviving cells were counted 2 weeks later. The presence of wild-type virus in the viral supernatants was determined by titering media from the mass cultures of NIH3T3 cells infected with the virus and selected with G418. No G418-resistant colonies were observed. The blood of animals infected with the retrovirus did not contain recombinant infectious retrovirus after analysis using the method described by Stuhlmann *et al.* (1989).

DNA Probes—The following probes were used for DNA/DNA or DNA/RNA hybridization experiments: PEPCK, a 620-bp *Bam*HI/*Bgl*II fragment from the 5′ end of the PEPCK gene (Yoo-Warren *et al.*, 1983); *neo*, a 1-kb *Bgl*II/*Eco*RI fragment from the 3′ end of the *neo* gene (Fig. 1); bGH, a 1-kb *Bam*HI/*Eco*RI fragment containing the entire bGH cDNA (Hampson *et al.*, 1989); PbGH, a 2.8-kb *Eco*RI fragment containing the entire PEPCK-bGH chimeric gene (see Fig. 1). All DNA probes were labeled using [α-^{32}P]dCTP by the method of random oligo priming as described by the manufacturer. The specific activity of the DNA probes labled in this manner was 10^9 cpm/μg of DNA.

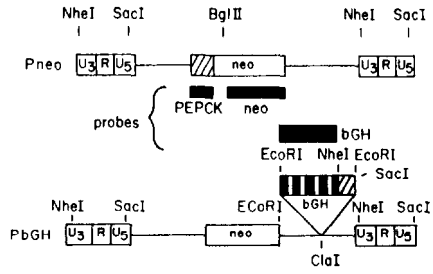

FIG. 1. **Structure of the retroviral vectors P*neo* and PbGh.** The genes for bGH and the bacterial amino 3′-glycosyl phosphotransferase (*neo*), which renders cells resistant to G418, and the bovine growth hormone structural gene (bGH), were linked to the promoter-regulatory region of the PEPCK gene (*hatched box*, −550/+73 for P*neo* virus and −450/+73 for PbGH virus). The *neo* gene (*open box*) in both vectors was in the same transcriptional orientation as the 5′ LTR and terminated in the 3′ LTR. The PEPCK-bGH gene, containing five exons (*solid boxes*) and four introns (*open boxes*) as well as the bGH polyadenylation signal (Woychik *et al.*, 1982), was inserted into the unique *ClaI* restriction site of pLJ(−SV40) (Hatzoglou *et al.*, 1988) in the orientation opposite to the 5′ LTR. Ecotropic, helper-free virus was produced by transfecting the Ψ2 packaging cell line (Mann *et al.*, 1983) with each of these retroviral vectors. Stable, virus-producing lines of Ψ2 cells were selected in G418, and the titers of the virus secreted from these lines were determined in NIH3T3 fibroblasts. DNA probes are indicated with *solid boxes* contained between the vectors.

For S1 nuclease mapping of *neo* or bGH RNA the following probes were used: (*a*) a ^{32}P 5′ end-labeled 80-mer oligonucleotide (5′GGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCCGATTACACACCACAAAACACACCCAAAACCCCAA-3′), having at the 5′ end 50 nucleotides that are identical with the sequence inside the *neo* gene and at the 3′ end 30 nucleotides of random sequence; (*b*) a *PvuI*/*Bgl*II fragment containing 530 bp (−457 to +73) of the PEPCK promoter, 302 bp at the 5′ end of the *neo* gene, and 620 bp of pBR322 was 5′ end labeled at the *Bgl*II site using [γ-^{32}P]ATP; (*c*) a 782-bp CfoI fragment containing the third, fourth, and part of the fifth exon and the entire intron D of the bGH gene. This fragment of DNA was 3′ end labeled with T4 polymerase and [α-^{32}P]dCTP (see Hampson *et al.*, 1989). Details of the labeling procedure have been published elsewhere (Maniatis *et al.*, 1982).

Isolation and Analysis of Cellular DNA and RNA—Isolation of genomic DNA and total cellular RNA was carried out by standard procedures that have been described in detail previously (Maniatis *et al.*, 1982). Southern blotting analysis and agarose gel electrophoresis were performed by standard methods (Southern, 1975; Davis *et al.*, 1986).

Amplification of DNA Using the Polymerase Chain Reaction—One μg of genomic DNA and 1 ng of plasmid DNA were amplified in 100 μl of buffer solution recommended by the manufacturer (United States Biochemical Corp.). After heating for 10 min at 94 °C to denature the genomic DNA, the DNA was amplified through 25 cycles. The conditions for each cycle were: denaturation at 94 °C for 2 min, hybridization at 65 °C for 7 min, and extension at 72 ° C for 15 min. Five μl of the amplified DNA was used for analysis on a 1% agarose gel.

Administration of Retrovirus to Animals—For retroviral infection of fetal rats, the uterus of pregnant animals (between 14 and 20 days of pregnancy) was delivered through a midline incision, and 100 μl of viral stock containing 10^4 cfu (PbGH) or 10^7 cfu (P*neo*) was injected directly into the peritoneal cavity of the fetus. The animals were then allowed to develop to term. Of more than 200 animals injected in these studies, approximately 90% survived the procedure.

Partial hepatectomy was performed on male Sprague-Dawley (180-g) rats by removal of the median and left lateral lobes (approximately 70% of the liver was removed), and 1 ml of viral stock, containing 10^5 cfu of PbGH, was injected directly into the portal vein immediately after hepatectomy.

Hormonal Treatment of Cells and Animals—FTO-2B hepatoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 5% calf serum. The cells were changed to media without serum and incubated for 4 h in the presence

of 0.5 mM Bt_2cAMP and 1.0 mM theophylline or 50 nM porcine insulin. Animals were given Bt_2cAMP and theophylline (30 mg/kg of body weight) interperitoneally three times every 20 min. Dexamethasone at 1 mg/kg of body weight was given interperitoneally as indicated in the figures.

Measurement of bGH—The concentration of bGH in the blood of the animals was determined by an enzyme-linked immunosorbent assay (ELISA) (McGrane *et al.*, 1988).

RESULTS

Retroviral Vectors Used to Infect Animals

Two recombinant retroviral vectors, containing the G418 resistance gene (P*neo*) alone or in combination with the bovine growth hormone gene (PbGH), were used to generate ecotropic retrovirus (see Fig. 1). In both retroviral vectors a segment of the PEPCK promoter-regulatory region was linked to the marker genes and the vector transfected into Ψ2 cells. Single transfected clones of Ψ2 cells were assayed for viral production, and Ψ2 clones producing high titer virus were selected. Retroviral stocks with titers of approximately 8×10^7–1×10^8 cfu/ml for P*neo* virus and 8×10^4–2×10^5 cfu/ml for PbGH virus were used to infect rat fetuses at various times of gestation or adult rats via the portal vein following partial hepatectomy. All viral stocks were free of helper virus since the culture medium from NIH3T3 cells, which had been infected with the virus, did not confer G418 resistance when added to noninfected NIH3T3 cells. In a previous report (Hatzoglou *et al.*, 1988) we demonstrated that the P*neo* virus could infect hepatoma cells and that the level of expression of the PEPCK-*neo* gene was rapidly induced by Bt_2cAMP and glucocorticoids and inhibited by insulin. In contrast, transcription of the PEPCK-bGH gene, contained in the PbGH virus, was induced 30-fold by glucocorticoids but not altered by Bt_2cAMP.

Infection of Late Gestation Fetuses

Transduction of Pneo Virus and Its Integration into the Hepatic Genome—P*neo* retrovirus was injected into the peritoneal cavity of rat fetuses late in gestation. Fetuses from different mothers were injected with 100 μl (10^7 viral particles) of P*neo* virus at 14, 16, 18, 19, and 20 days of gestation. DNA was isolated from the livers on the 1st day after birth or when the animals were 3 months old, digested with *Sac*I (see Fig. 1), and analyzed by Southern blotting analysis using *neo* as a probe. Although the expected proviral fragment was 4.1 kb, a predominant DNA band of 3.7 kb and two less prominent bands of 5.1 and 2.6 kb were found to hybridize with the *neo* probe (Fig. 2*A*). When DNA from the livers of 3-month-old rats that had been injected with the P*neo* virus late in gestation was analyzed, only the 3.7-kb proviral DNA band hybridized with a *neo* probe after *Sac*I digestion (Fig. 2*B*). The less prominent bands of 5.1 and 2.6 kb may be extrachromosomal DNA fragments since they could only be detected within the 1st week after infection. We also analyzed in parallel genomic DNA isolated from FTO-2B cells infected with P*neo* virus (*FTO-PCK*, Fig. 2*B*) in order to estimate the percentage of liver cells that contained the provirus. Since the FTO-PCK cells contained at least a single copy of the integrated provirus, we used 20 μg of DNA from infected liver cells and only 1 μg from FTO-PCK cells. A comparison of the relative intensities of the appropriate two bands in Fig. 2*B* (4.1 and 3.7 kb) indicates that approximately 2–5% of the liver cells contains the provirus. This is only a rough approximation since the distribution of the provirus throughout the liver may not be homogeneous.

As noted above, the P*neo* provirus that integrated into the hepatic genome contained a deletion of approximately 400 bp. This deletion was not detected in FTO-2B cells infected with the same virus and grown in G418 (Fig. 2*B*). Furthermore, the deletion was not present in DNA isolated from FTO-2B cells infected with the same virus, which had not been selected in G418 (data not shown). This means that the deletion originated in the liver before proviral integration. We analyzed the modified provirus further to determine the position of the deletion and whether it affected the expression of the PEPCK-*neo* gene.

Hepatic DNA containing the deleted provirus was digested simultaneously with *Sac*I and *Bgl*II and analyzed by Southern blotting using *neo* as a probe. A deletion of 400 bp was noted between the 5′ LTR and the *Bgl*II site in the provirus (data not shown). To analyze this deletion further, we performed PCR analysis of the 5′ end of the provirus containing the deletion. Two oligonucleotides, 30 nucleotides in length, were synthesized; one was at the 3′ end of the *neo* gene, 690 bp downstream from the *Bgl*II site, and the other was at the 5′ end of the provirus including the *Sac*I site in the LTR (Fig. 3, *bottom*). DNA from the livers of infected rats was amplified by PCR (Fig. 3*A*, *left side, lanes 1–4* and *lanes 7–9*). We noted a 1.8-kb fragment that was 400 bp smaller than the amplified retroviral DNA from the retroviral vector (Fig. 3*A*, *left side, lane 6*, and Fig. 3*B*, *left side, lane 2*). One animal contained an additional larger deletion (Fig. 3*A*, *left side, lane 2*). When the amplified fragment of DNA from the liver of infected animals was digested with *Bgl*II and hybridized with a 3′-specific probe (*neo* (*α*)), a 690-kb fragment was obtained, suggesting the absence of any deletion in this region.

The presence of four restriction sites in this fragment of DNA (*Eco*RI, *Pvu*I, *Ava*II, and *Bgl*II) allowed us to localize further the site of the deletion in the provirus. The amplified DNA from either the vector (Fig. 3*A*, *right side, V*) or the liver of infected animals (Fig. 3*A*, *right side, L*) was digested with the appropriate restriction enzyme and compared on an agarose gel (Fig. 3*A*, *right side*). In the DNA from the liver, the *Eco*RI site was absent, indicating that this site was deleted. However, the *Pvu*I site, which is 200 bp 5′ from the *Eco*RI site, and the *Ava*II site, which is 390 bp 3′ of the *Eco*RI site, were retained. The same gel was transferred to nitrocellulose and was hybridized with a *neo* (*b*) probe (Fig. 3*B*, *right side*). These results place the proviral deletion between the 5′ end of the PEPCK promoter and the *Sac*I site of the LTR. The deletion did not affect the start site of transcription from either the LTR or the PEPCK promoter as will be shown in Fig. 4. No provirus was detected in kidney, lung, spleen, or brain from animals that contained provirus in the liver (data not shown).

Expression of the Pneo Provirus in the Liver—Despite the deletion in the 5′ end of the provirus, there was expression of the PEPCK-*neo* gene in the livers of adult rats injected with retrovirus *in utero*, indicating that the deletion did not interfere with transcription from the PEPCK promoter. Expression of the provirus was determined by isolating RNA from the livers of 2- or 3-month-old rats that had been injected with the P*neo* virus late in fetal life. Infected animals were treated with Bt_2cAMP and theophylline 2 h prior to isolation of tissues (Fig. 4*A*, *lanes 1–3* and *11–15*) or were kept as controls without treatment (Fig. 4*A*, *lanes 4–7* and *8–10*). S1 nuclease mapping was used to detect *neo* mRNA, using a single-stranded oligonucleotide containing, at the 5′ end, 50 nucleotides corresponding to the *neo* gene and 30 nucleotides of random sequence. This *neo* DNA was 5′ end labeled and used as a hybridization probe. The expected size DNA fragment (50 nucleotides) was protected in RNA isolated from

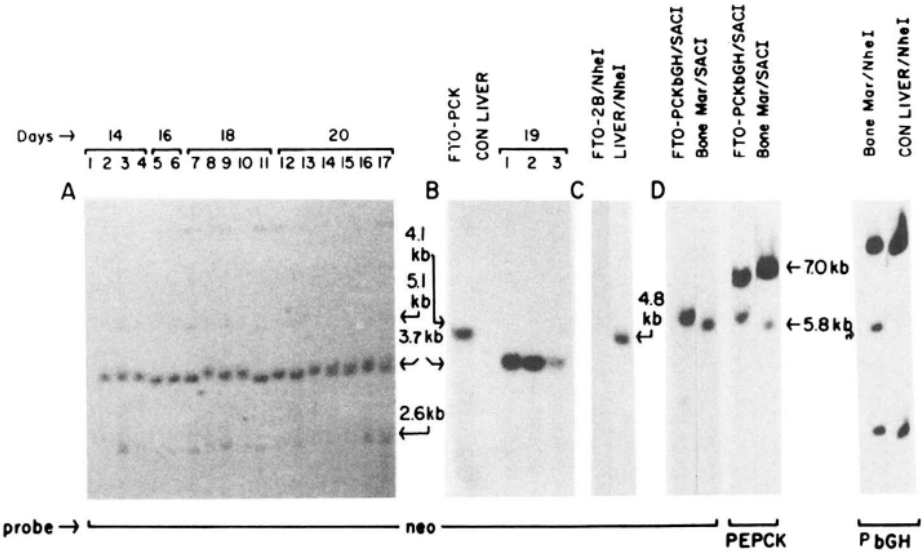

FIG. 2. **Southern analysis of DNA isolated from livers of rats infected with retrovirus as fetuses.** Newborn rats (*A*) or animals 3 months of age (*B*) were infected *in utero* with the P*neo* virus at the day of gestation indicated at the *top* of the figure. The DNA was digested with *Sac*I and hybridized with a *neo* DNA probe (see Fig. 1). DNA from FTO-2B cells infected with the virus, which were selected for expression of the *neo* gene in medium containing G418 and DNA from the livers of noninfected animals, was analyzed as a control (*B*). In order to compare directly the proviral sequences in the hepatic genome with that of infected FTO-2B cells we used 20 μg of hepatic DNA and 1 μg of DNA from FTO-2B cells. *C*, Southern analysis was performed on DNA from the livers of rats infected *in utero* with PbGH virus at 19 days of fetal life. In this analysis the DNA was digested with *NheI* and hybridized with the *neo* DNA probe (see Fig. 1). The 4.8-kb band is indicated. DNA from uninfected FTO-2B cells was included as a control. *D*, bone marrow cells were isolated from 2-month-old rats that had been infected with PbGH virus at 19 days of fetal life. DNA was isolated, digested with *Sac*I, and analyzed with either the PEPCK or *neo* DNA probes. DNA digested with *Nhe*I was analyzed with the PbGH probe. *Panels B, C,* and *D* represent individual Southern blots in which several lanes were rearranged for economy of presentation.

the livers of infected animals (Fig. 4*A*) and in FTO-2B cells infected with the same virus (FTO-PCK). *Neo* RNA was detected at varying levels in the livers of all animals containing the provirus. No *neo* RNA was detected in noninfected FTO-2B cells (Fig. 4*A*, *last lane* on the *right*) or RNA isolated from the livers of noninfected control animals (data not shown).

Transcription of the PEPCK-*neo* gene initiated within the PEPCK promoter contained in the provirus. S1 nuclease mapping was performed using hepatic mRNA isolated from the livers of 3-month-old animals infected with the P*neo* virus at day 19 of fetal life. The animals were given Bt_2cAMP and theophylline for 2 h, and the RNA was isolated from the liver and analyzed using a 5′ end labeled PEPCK-*neo* hybridization probe. A protected DNA fragment of 375 nucleotides was observed in RNA from each of these animals (Fig. 4*B*, *lanes 1–5*), indicating that this *neo* mRNA was transcribed from the PEPCK promoter. The 710-nucleotide fragment represents RNA initiating upstream from the PEPCK promoter, presumably within the 5′ LTR. FTO-2B cells infected with the same virus were analyzed in parallel and had the expected pattern of hormonal regulation of gene expression from the PEPCK promoter (induction by cAMP and inhibition by insulin; Fig. 4*B*, *left side*). RNA from both the liver and hepatoma cells infected with P*neo* virus was chromatographically separated on the same gel to allow comparison of the start site of gene transcription (Fig. 4*B*).

Introduction of PbGH Virus into the Livers of Fetal Rats—We also introduced PbGH virus into the livers of fetal rats. This retrovirus has the advantage that the level of PEPCK-bGH gene expression could be determined by measuring the concentration of serum bGH by ELISA (McGrane *et al.*, 1988). In a set of experiments parallel to those described with P*neo* virus, animals were infected with PbGH virus at 19 days of fetal life and the levels of bGH in the serum determined at 2 months of age. Of the 79 animals analyzed, 18 had concentrations of serum bGH between 3.5 and 30 ng/ml (see Table I), which had no detectable effect on the growth of the rats. Since transcription from the PEPCK promoter is induced by hormones, we determined the effect of the administration of dexamethasone to rats expressing bGH. Dexamethasone induced the concentration of bGH in the serum of 6 of the 18 animals that were positive for expression of bGH. In Fig. 5 we present the results of a time course of induction of the PEPCK-bGH gene by dexamethasone. Since the basal levels of bGH in the animals varied widely, a direct comparison of the effect of hormones on gene expression between animals was not possible. Therefore, serum bGH was measured in the same animal before and after glucocorticoid administration. Rats were treated at 2 months of age with dexamethasone every 24 h for 3 days. Three of the four animals had an increased level of serum bGH (Fig. 5), indicating that the expression of the transduced PEPCK-bGH gene could be regulated by glucocorticoids. In addition, several animals that did not have detectable levels of bGH in the serum were also injected with dexamethasone and analyzed in parallel. There was no detectable bGH in the serum of these animals 24 h after hormone treatment (data not shown).

The level of bGH mRNA in the livers of the three animals that responded to dexamethasone administration was determined by S1 nuclease mapping, using a 3′ end-labeled bGH hybridization probe. The predicted 274-nucleotide fragment was protected (Fig. 4*B*, *lane 6*), indicating that transcription of the bGH mRNA initiated within the PEPCK promoter.

Dietary Regulation of the PEPCK-bGH Gene—Expression of the endogenous PEPCK gene in rat liver is inhibited markedly by feeding carbohydrate because of the strong negative effect of insulin on gene transcription (Shrago *et al.*,

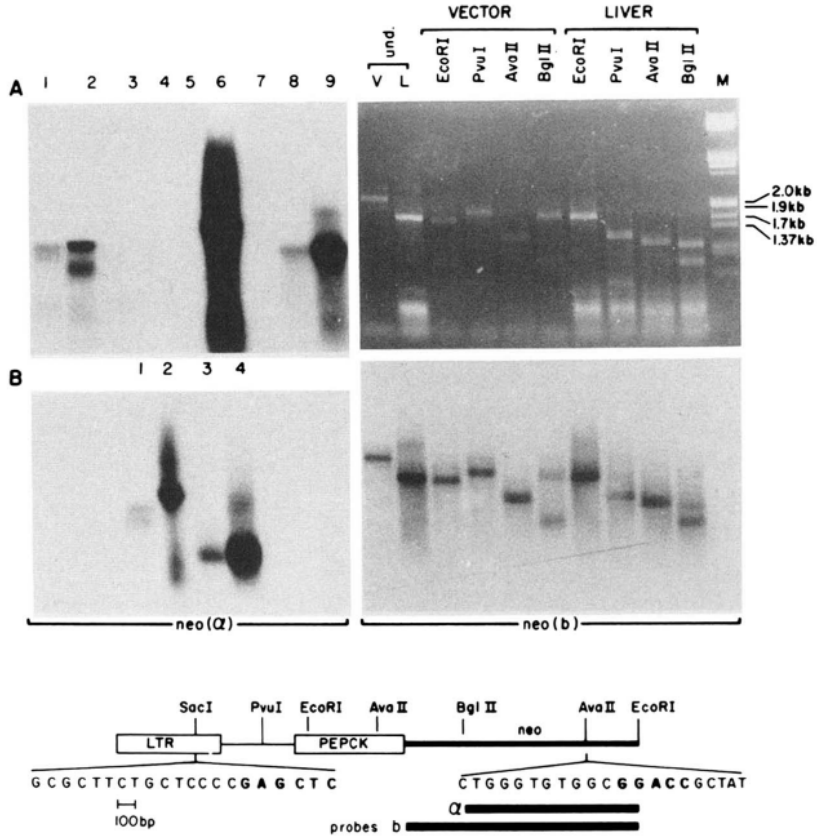

FIG. 3. **PCR analysis of the deletion in the P*neo* provirus.** *Panel A (left side),* Southern analysis of DNA generated by PCR from genomic DNA from the livers of 3-month-old rats that were infected *in utero* at day 19 of fetal life (*lanes 1–4* and *7–9*), using the oligonucleotides primers shown at the *bottom* of the figure. DNA from the liver of a control noninfected animal (*lane 5*) and plasmid DNA from the retroviral vector (*lane 6*) were analyzed in parallel. Each *lane* contains 5 μl of the reaction (100 μl) after amplification of 1 ug of genomic DNA or 1 ng of plasmid DNA. The DNA probe was *neo* (α), as indicated at the *bottom* of the figure. *Panel B (left side),* Southern analysis of DNA generated by PCR and digested with *Bgl*II. *Lane 1,* undigested DNA as in *lane 2* of *panel A; lane 2,* undigested, plasmid DNA as in *lane 6* of *panel A; lane 3,* the DNA in *lane 1* digested with *Bgl*II; *lane 4,* the DNA in *lane 2* digested with *Bgl*II. The hybridization probe was *neo* (α). *Panel A (right side),* restriction enzyme analysis of DNA generated by PCR of DNA isolated from the liver (*L*) of an infected animal (*panel A, lane 9*). DNA generated by PCR from retroviral vector DNA (*V*) was also analyzed. *Lanes V* and *L* contained undigested amplified DNA from the retroviral vector and the liver. Analysis of amplified vector and liver DNA digested with the enzymes indicated at the *top* of the figure is also shown. Phage λ DNA (*M*) was digested with *Eco*RI and *Hin*dIII and included as a size marker. *Panel B (right side),* Southern analysis was performed on the DNA analyzed in *panel A* (*right side*) using the *neo* (b) probe shown at the *bottom* of the figure.

1963; Andreone *et al.,* 1982). Conversely, starvation (Foster *et al.,* 1966; Tilghman *et al.,* 1974) or feeding a diet high in protein (Peret and Chaney, 1976) induces the expression of the PEPCK gene, presumably by increasing cAMP and lowering the serum insulin concentration. The expression of a PEPCK-bGH gene (containing −450 to +73 of the PEPCK promoter-regulatory region) was also regulated by diet in a similar manner in the livers of transgenic animals containing this gene (McGrane *et al.,* 1988). To test whether expression of the PEPCK-bGH gene in the livers of animals infected late in fetal life was regulated by diet, 10 of the 18 rats that were expressing bGH were fed alternating high carbohydrate and high protein diets. The concentration of bGH in the blood of four of the rats was decreased after a week of the diet high in carbohydrate and increased markedly after the same time on the high protein carbohydrate-free diet (Fig. 6*B*). There was a steady decrease in serum bGH during the course of the experiment in two of the rats (only one is shown), and one animal had both an increase and decrease in serum bGH during carbohydrate feeding. In addition, 3 of the 10 animals did not respond to diet and are not included in Fig. 6.

Integration of the PbGH Virus into the Hepatic Genome—Proviral DNA was detected in the liver of infected animals (Fig. 2C), but the provirus was deleted or rearranged. We were unable to detect the expected size fragments after digestion of hepatic DNA with enzymes that cut within the provirus. For example, we noted a 4.8-kb DNA fragment hybridizing with the *neo* probe instead of the expected size fragment, 5.8 kb (Fig. 2*C*) when hepatic DNA from an animal producing 40 ng/ml bGH after administration of dexamethasone (see Figure 5) was digested with *NheI*.The same DNA band hybridized with the PEPCK probe (data not shown), indicating that the *NheI* site contained in the PEPCK promoter (see Fig. 1) was lost, perhaps due to a rearrangement within the provirus.

Introduction of Genes into the Livers of Adult Animals

Expression and Regulation by Diet and Hormones—Another approach for introducing genes into animals was the infection of adult animals with PbGH retrovirus during liver regeneration. Of the 40 animals undergoing the procedure, four rats expressed between 15 and 83 ng/ml bGH within 3 weeks after partial hepatectomy. One of these animals was identified as part of an experiment that measured the time course of expression of the PEPCK-bGH gene (Fig. 7). A peak concen-

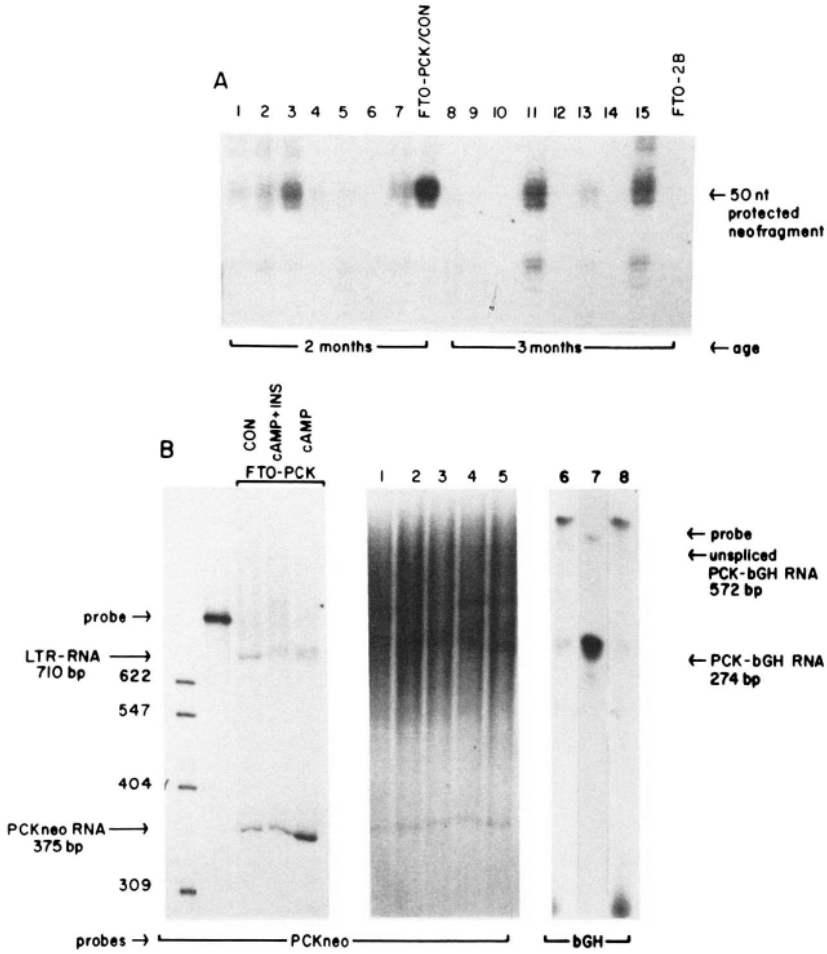

FIG. 4. **Expression of P*neo* RNA and PbGH RNA in the livers of animals infected with virus during fetal life and after partial hepatectomy.** *Panel A,* RNA was isolated from the livers of 2- and 3-month-old rats infected with P*neo* virus at 19 days of fetal life and analyzed by S1 nuclease mapping. *Lanes 1–3* and *11–15* contain RNA from the livers of animals treated with Bt_2cAMP and theophylline for 2 h. Infected animals, not treated with hormones (*lanes 4–10*), were injected with saline. A 50-nucleotide (*nt*) fragment of DNA was protected using an 80-nucleotide synthetic oligonucleotide corresponding to a segment of the *neo* gene. RNA from FTO-2B cells infected with P*neo* virus but not treated with hormones (*FTO-PCK/CON*), was included for comparison. *Panel B,* the start site of gene transcription for PEPCK-*neo* (*lanes 1–5*) and PEPCK-bGH (*lanes 6* and *8*) was determined by S1 nuclease analysis of hepatic RNA. For the determination of the PEPCK-*neo* RNA from the livers of animals treated as described in *panel A,* a hybridization probe, labeled at the *Bgl*II site of the *neo* gene (*second lane*) was used. FTO-2B cells, infected with P*neo* virus, were analyzed on the same gel for comparison of proviral gene transcripts. The hormonal treatments are indicated at the *top* of the gel. The protected fragment from the transcription start site within the PEPCK promoter is indicated on the *left side* of the figure. Hepatic RNA from rats infected with the PbGH virus *in utero* (*lane 6*) or via the portal vein after partial hepatectomy (*lane 8*) and given 1 mg/kg dexamethasone daily for 3 days prior to collection of RNA, were analyzed by S1 nuclease mapping using a 3′ end-labeled DNA fragment. RNA from the liver of a transgenic mouse (McGrane *et al.,* 1988) bearing the same PEPCK-bGH chimeric gene was also analyzed for comparison (*lane 7*). *Lanes 6* and *8* were exposed to x-ray film for 3 days, and *lane 7* was exposed for 30 min. Molecular size markers, generated by the digestion of pBR322 with *Hpa*II, are included in the *left lane.*

TABLE I

Integration and expression of chimeric genes introduced into the liver of animals by viral infection

Virus	Animals analyzed	Provirus	Expression	Regulation	
				Diet	Dexamethasone
Infected *in utero*					
P*neo*	70 (7)[a]	35	20	ND[b]	ND
PbGH	79 (8)	30[c]	18	4/25	6/64
Infected after partial hepatectomy					
PbGH	40	4	4	2/20	ND

[a] The number of litters of fetal rats analyzed is shown in parentheses.

[b] ND, not determined.

[c] Provirus was detectable only by PCR analysis of hepatic DNA.

tration of 24 ng/ml bGH was noted by day 12 after infection, and concentrations of between 14 and 24 ng/ml were maintained over a period of 25 days. However, 2 months later, the same animal had only marginal levels of serum bGH (2 ng/ml). An uninfected rat, subjected to partial hepatectomy and injected with tissue culture media, contained background levels of material reacting with the ELISA. Other animals included in this time course had undetectable levels of serum bGH (data not shown).

The effect of diet on the expression of the PEPCK-bGH gene was also determined using three of the four animals noted above. Three weeks after partial hepatectomy, these animals were fed alternating high carbohydrate or high protein diets. Two of the three rats responded to the high carbohydrate diet with a decrease in serum bGH whereas refeed-

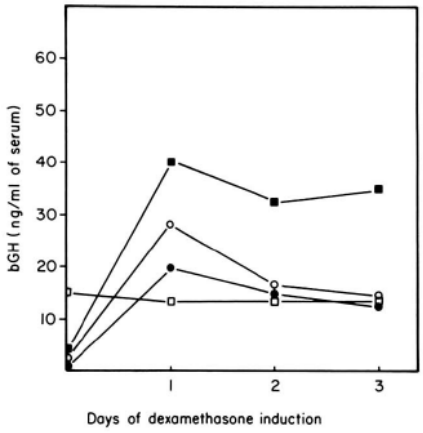

FIG. 5. **Effect of dexamethasone on the production of serum bGH in rats infected with PbGH at 19 days of fetal life.** Two months after birth four animals expressing low levels of bGH were given 1 mg/kg of body weight of dexamethasone daily for 3 days, and the concentration of serum bGH was determined by ELISA (McGrane *et al.*, 1988).

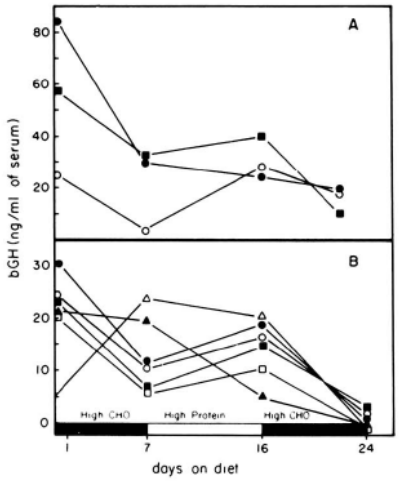

FIG. 6. **The effect of diet on the production of serum bGH from animals infected with PbGH virus.** *Panel A,* rats were infected via the portal vein following partial hepatectomy. Three weeks after viral infection and partial hepatectomy the rats were fed a high carbohydrate (*CHO*) diet (81.5% sucrose, 12.2% casein, 0.3% DL-methionine, 4% cotton seed oil, 2% brewers' yeast, and 1% mineral mix plus vitamins) for 7 days followed by a high protein diet (64% casein, 22% α-cell nutritive fiber, 11% vegetable oil, 2% brewers' yeast and 1% mineral mix with vitamins). *Panel B,* rats were infected *in utero* at day 19 of fetal life. Animals at 2 months of age were fed the same alternating diets as described above. Serum bGH was determined by ELISA (McGrane *et al.*, 1988).

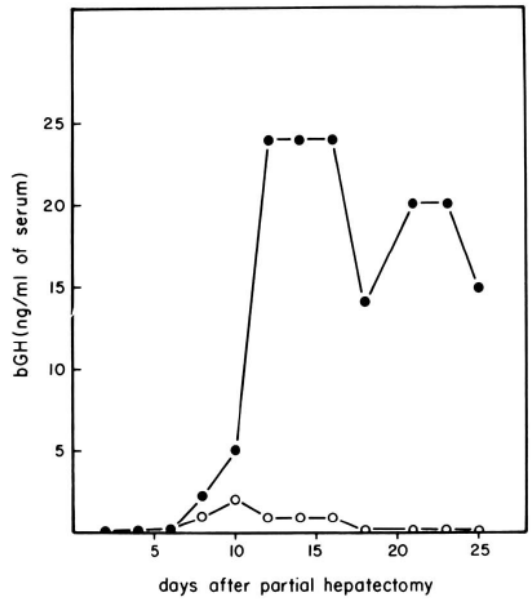

FIG. 7. **Production of serum bGH by a rat infected with PbGH virus via the portal vein immediately following partial hepatectomy.** ●——●, animals infected with virus; ○——○, non-infected control.

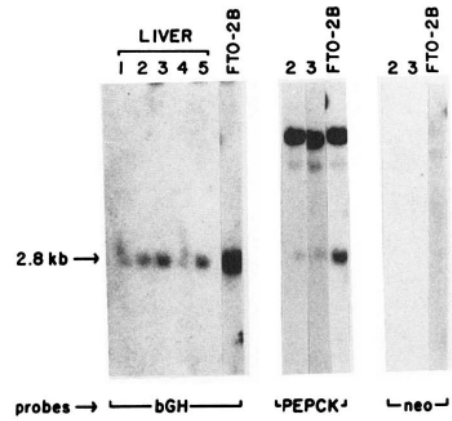

FIG. 8. **Analysis of DNA isolated from the regenerated liver of a rat infected with the PbGH virus via the portal vein after partial hepatectomy.** DNA was extracted from five pieces of the regenerated liver from the same animal and Southern analysis performed after digestion of the DNA with *Eco*RI. DNA fragments of the predicted size were detected, using the bGH or PEPCK DNA probes (see Fig. 1). FTO-2B cells infected with the same virus and selected in G418 for expression of the *neo* gene served as a control. As expected, there was no hybridization with the *neo* DNA probe. The endogenous PEPCK gene is present in FTO-2B cells and rat liver hybridized with the PEPCK DNA probe yielding the predicted size fragments (*upper two bands*). The *numbers* on the *top* of the figure indicate the different pieces of the liver used for DNA analysis.

ing a diet high in protein caused a slight increase in serum bGH (Fig. 6*A*). The high carbohydrate diet decreased the concentration of bGH in the blood of the third animal but subsequent refeeding of a high protein diet did not cause the expected induction. Noninfected rats subjected to partial hepatectomy had undetectable levels of serum bGH.

Finally, we analyzed for both the presence of provirus in the livers of animals infected with PbGH after partial hepatectomy and its expression. The animals were killed 2 months later, and DNA was isolated from pieces of the regenerated liver. Southern blotting of this genomic DNA indicated that the PEPCK-bGH gene was transferred intact (Fig. 8) since a 2.8-kb fragment was detected after *Eco*RI digestion (see Fig. 1). The same 2.8-kb fragment also hybridized with the PEPCK probe (Fig. 8), indicating the presence of the intact PEPCK-bGH gene. The PEPCK probe also hybridized with the endogenous PEPCK gene (*upper two bands* in Fig. 8). Proviral DNA was not detected in kidney, spleen, brain, and heart of infected animals (data not shown).

RNA from the livers of animals infected after partial hepatectomy, which contained the PbGH provirus, was also analyzed by S1 nuclease mapping. We noted the expected 274 nucleotide fragment of DNA protected from S1 nuclease digestion (Fig. 4*B, lane 8*), indicating that the stably transduced PEPCK-bGH gene was expressed in the regenerated livers of rats infected with the retrovirus as adults. *Lane 7* in Fig. 4*B* is a control that contains RNA from the liver of a transgenic animal containing the PEPCK-bGH chimeric gene. Liver RNA from uninfected, control animals did not

have a fragment corresponding to the bGH transcript (data not shown).

Retroviral Targeting of Bone Marrow Cells—Since the liver contains approximately 40% hematopoietic cells (Greengard *et al.*, 1972) at day 19 of gestation, infection of fetal liver with retrovirus could result in proviral integration into both parenchymal and hematopoietic cells, leading to the presence of the provirus in bone marrow cells. To test this possibility we digested DNA isolated from bone marrow cells with *Sac*I, which produced the expected 5.8-kb fragment (Fig. 2*D*) hybridizing with the *neo* probe, providing proof that the intact provirus could find its way into genomic DNA in bone marrow cells. Digestion of the same DNA with *NheI* produced a 5.8-kb fragment that also hybridized with the PbGH probe (Fig. 2*D, right panel*) and the *neo* probe (data not shown). The PbGH probe also hybridized with a 500-bp fragment (3′ end of the provirus) that is not included in Fig. 2*D*. FTO-2B cells infected with the same virus (FTO-PCKbGH) were also analyzed in parallel for comparison. Deletions or rearrangements of the provirus did not occur in all tissues since the same animal contained an intact provirus in bone marrow cells and a rearranged form in the liver. Bone marrow cells were also isolated from animals which contained serum bGH and cultured in the presence of G418. No surviving cells were detected, indicating that the proviral *neo* gene was not expressed in these cells.

DISCUSSION

We have used retroviral transduction to introduce chimeric genes into the livers of fetal and adult animals. The animals tolerated the procedures well, and no liver tumors were observed by either gross or microscopic examination. Although we have introduced functional genes into the livers of animals, there are facets of this system which clearly need to be delineated further. The overall level of infection of hepatocytes is low, and not all of the animals injected with the virus *in utero* or after partial hepatectomy contain the integrated provirus. A summary of the experimental results for the two methods used in this study is presented in Table I. About half of the animals infected with retrovirus *in utero* had provirus in the liver. This contrasts with the lower frequency of integration of the PbGH provirus into the liver (10%) after partial hepatectomy. Mouse hepatocytes can be infected during fetal life, but hepatocytes from adult mice have not been successfully infected with Moloney murine leukemia virus, even after partial hepatectomy (Jaenisch and Hoffman, 1979). This may be due to the presence of receptors for the viral envelope protein which are present in fetal hepatocytes but not in adult liver cells (Wolff *et al.*, 1987). After partial hepatectomy a small percentage of liver cells undergoes dedifferentiation, and these cells may be targets for retroviral infection. Since the adult liver contains 40% nonhepatic cells (Panduro *et al.*, 1986), it is possible that proviral integration into nonhepatocytes would result in an unpredictable pattern of expression of the PEPCK-bGH chimeric gene. McGrane[2] have demonstrated recently that the same segment of the PEPCK promoter used in the present work will direct the expression of a linked structural gene to the periportal (but not the pericentral) region of the liver. Thus, the extent of integration of the provirus in the liver may not correlate directly with the level of expression of proviral genes in that tissue. We are currently using a retrovirus containing the β-galactosidase gene in order to identify the cells containing the provirus in animals infected by the two methods outlined in the paper.

One unusual observation in this study was the specific deletion noted in the hepatic genome of animals infected with the P*neo* virus. Since the same deletion was present in the livers of all of the animals infected with P*neo* virus, it probably originated before integration of the provirus into the hepatic genome. The virus used to infect the animals did not contain this deletion since we could detect no deleted provirus in FTO-2B cells infected with P*neo* virus and selected in G418. We assume that the viral RNA was spliced in the liver cells before it was reverse transcribed or in the reverse transcribed DNA before integration. Since this proviral deletion was not noted in infected FTO-2B cells in culture but only in the livers of animals infected with the virus as fetuses, it could be due to the fact that this segment of the PEPCK promoter is inactive in the liver during fetal development.[2] Interference between viral enhancer elements and the PEPCK promoter might result in this specific deletion. A similar situation involving a proviral deletion has been described by Stuhlmann *et al.* (1989). The deletions and rearrangements of the PbGH provirus were also noted in the livers of animals, but these deletions were random. Compere *et al.* (1989) did not report this type of proviral rearrangements in the livers of animals infected as midgestation embryos with replication-defective retroviruses containing the LTR-linked oncogenes *myc* and *ras*. However, they analyzed only the tumors so it is possible that rearranged proviruses were present in the livers but were not expressed. Despite numerous deletions in both of the viruses noted in our study, the PEPCK-*neo* and PEPCK-bGH genes were expressed and regulated in the livers of infected animals.

Since the fetal liver late in gestation consists of hepatocytes and hematopoietic cells, infection with retroviruses has the potential to infect cells in both the liver and bone marrow. We have demonstrated that genes were transferred stably to bone marrow cells after retroviral infection of late gestation fetuses but were not expressed, due perhaps to the use of the PEPCK promoter which is not active in these cells. It is interesting to note that the same animal that contained an intact provirus in bone marrow cells had a rearranged provirus in the liver. This supports the idea that the deletions and rearrangements observed in the livers of these animals occurred before proviral integration. We are currently working with retroviruses containing several promoters linked to different structural genes to determine whether there is expression of transduced genes in the bone marrow cells.[3] This approach may also be useful in tracing the migration of hematopoietic progenitor cells from the liver to the bone marrow late in fetal development.

A number of factors may influence the transfer and expression of genes in animals by retroviruses. Since the retrovirus used in these studies is replication defective, the viral titer is a critical factor in determining the number of liver cells infected. The titer of the P*neo* virus was high (1×10^8 cfu/ml), which correlated with the much higher level of expression than the PbGH virus (10^5 cfu/ml) in animals infected as fetuses. The gestational age of the animals at the time of retroviral infection has also been shown to influence the tissue distribution of the provirus and its expression. Infection of late gestation fetuses resulted in proviral integration mainly in the liver and bone marrow. A wider tissue distribution of the integrated provirus occurred when younger fetuses were infected.[4] This is predictable based on the studies of Jaenisch and his co-workers on the integration and expression of retroviruses in preimplantation (Jaenisch *et al.*, 1975; Jaen-

[2] M. M. McGrane, unpublished results.

[3] W. Clapp and S. Gerson, unpublished results.

[4] D. W. Clapp, unpublished observations.

isch, 1976; Harbers *et al.*, 1982; Soriano and Jaenisch, 1986) and midgestation embryos (Jaenisch *et al.*, 1982; Compere *et al.*, 1989). The infection of fetuses at various ages of gestation may permit the efficient infection of other tissues of the animal.

It seemed reasonable that injection of virus at the time of partial hepatectomy would lead to integration in the liver since active cell division occurs within 24–48 h after partial hepatectomy (Fabrikant, 1968; Grisham, 1962). In contrast to our findings, Jaenisch and Hoffman (1979) reported that the regenerating mouse liver was not infected with wild-type endogenous virus. However, they examined the liver only 2–5 days after partial hepatectomy, at a time when animals infected with PbGH virus were not expressing the transduced PEPCK-bGH gene. After partial hepatectomy, liver cells dedifferentiate and express α-fetoprotein, a protein normally expressed only in fetal liver (Panduro *et al.*, 1986). Since adult hepatocytes are not susceptible to Moloney murine leukemia virus infection, this dedifferentiated population of liver cells may be targets for retroviral infection.

The selection of an internal promoter that permits the regulated expression of the transduced genes in the liver is a critical factor in determining the level of expression of the gene introduced into this tissue. The PEPCK promoter-regulatory region used in these studies is a strong promoter (Lamers *et al.*, 1982), which, when introduced into cells with retroviral vectors, responds appropriately to hormones (Hatzoglou *et al.*, 1988). Also, the PEPCK-bGH chimeric gene in the livers of transgenic mice can be regulated in a predictable fashion by dietary manipulation. It may ultimately be possible, using the techniques described in this report, to target the expression of chimeric genes to the bone marrow by infecting fetal rats with retrovirus containing a tissue-specific promoter that is linked to a structural gene of interest.

The ability to transfer genes stably into tissues by the infection of late fetuses or adult animals with transducing retroviruses suggests that this method may be useful for correcting genetic disorders. It may be possible to correct genetic defects in the liver by linking the gene responsible for the defect to a promoter such as PEPCK, which contains elements that direct high levels of expression in a particular tissue. We have shown that by devising a method to deliver infectious retrovirus to a particular tissue, genes can be transferred to that tissue in the whole animal. Animal models for tissue-specific genetic diseases can also be used to test the usefulness of this approach for gene therapy.

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