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Hormonal Regulation of the Gene for the Type C Ecotropic Retrovirus Receptor in Rat Liver Cells

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The infectibility of the regenerating rat liver by ecotropic retroviruses was studied relative to the expression of the gene coding for the ecotropic retrovirus receptor (Ecor) that functions as a cationic amino acid transporter. It is known that the gene for the receptor is expressed in primary hepatocytes and hepatoma cells but is absent in adult liver cells. Isolation of a 2.85-kb cDNA for the rat Ecor suggested that the rat viral receptor is 97% homologous to the mouse viral receptor and that it contains the envelope-binding domain that determines the host range of ecotropic murine retroviruses. This explains the efficient infection of rat cells by ecotropic retroviruses. Since cell division is required for liver cells to be infected, we determined the susceptibility of the regenerating rat liver to infection at different time points after partial hepatectomy (0 to 24 h) in relation to the presence of receptor mRNA. Infection of the liver occurred only when the liver was exposed to virus 4 h after partial hepatectomy. This time course of infection paralleled expression of the gene for the Ecor, which was rapidly induced between 2 and 6 h during liver regeneration. However, expression of the dormant receptor gene in quiescent liver cells can be induced by insulin, dexamethasone, and arginine, indicating that cell division is not required for expression of the receptor gene in liver cells. A diet high in carbohydrate (low in protein) significantly increased the concentration of receptor mRNA in liver cells, indicating that hormones play a role in the regulation of expression of this gene in vivo. We conclude that the gene for the viral receptor is expressed in the regenerating and quiescent liver when the urea cycle enzymes are down regulated. The infection of the regenerating rat liver by ecotropic retroviruses at the time point of expression of the receptor gene supports the requirement of expression of this transporter for infection.

Gene therapy is a promising approach for the treatment of congenital metabolic diseases. Applying gene therapy to treat a significant number of these disorders will require the transfer and expression of the correct genes in a large number of hepatocytes in the liver. Replication-incompetent Moloney murine leukemia virus-based retroviruses are among the most commonly used vectors for the transfer of genes into somatic cells (20, 40, 42). However, very little is known about the factors that control the infectibility of cells (36, 52). Controlled experiments attempting to transfer genes into somatic cells using retroviruses may increase our understanding of the factors involved in efficient infection of specific tissues.

Gene transfer in the adult rat liver using retroviruses is possible only after partial hepatectomy (18, 25, 29, 30). Although fetal liver and regenerating liver cells are susceptible to infection (9, 12, 18, 25, 28–30, 46), quiescent liver cells cannot be infected with retroviruses. The isolation and characterization of a cDNA coding for the ecotropic retrovirus receptor (Ecor) by Albritton et al. (2) was an important step in our understanding of the role that viral receptors may have on cell infectibility. They demonstrated that the gene coding for the Ecor is not expressed in the livers of adult mice (31). However, the expression of this gene is enhanced in rapidly proliferating cells of hematopoietic origin (54), which suggests that Ecor plays a role in cellular proliferation. Since retroviruses infect only dividing cells, it is possible that cell cycle-dependent expression of the genes that code for viral receptors might be a significant factor for infectibility of host cells.

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It has been demonstrated that infection of the regenerating rat liver by replication-incompetent amphotropic retroviruses is most efficient 24 h after partial hepatectomy (18). Liver regeneration is a unique example of synchronized cell growth in vivo (15, 21, 35). In the first 12 h after 70% partial hepatectomy, the cells leave the G_1 phase and enter the S phase and by 24 h, mitosis and DNA synthesis peak (35). In this study, we examined the correlation of expression of the gene coding for the viral receptor in the regenerating rat liver with the efficiency of infection by replication-incompetent ecotropic retroviruses. Since this receptor functions as a cationic amino acid transporter (31, 47, 48) and it is known that transport of amino acids is regulated by hormones (6), we determined the hormonal regulation of expression of the dormant receptor or transporter gene in the rat liver. Identifying the mechanisms of regulation of expression of the genes that code for viral receptors will significantly improve gene transfer methods with retroviruses.

MATERIALS AND METHODS

Materials. All DNA-modifying enzymes and nucleotides were purchased from Boehringer Mannheim Biochemicals and Pharmacia-LKB and Biotechnology Inc. [α-^{32}P]dCTP (3,000 Ci/mmol) and [α-^{35}S]dCTP (1,000 Ci/mmol) were purchased from Du Pont-New England Nuclear. Restriction enzymes were used according to the specifications of the manufacturer. The pLJ vector and the Ψ2 cells were gifts from R. Mulligan, Massachusetts Institute of Technology, Boston. The pJET vector, which contains the cDNA for the mouse Ecor, was kindly provided by J. M. Cunningham, Massachusetts Institute of Technology.

Cloning and sequencing of the cDNA for the rat Ecor. A

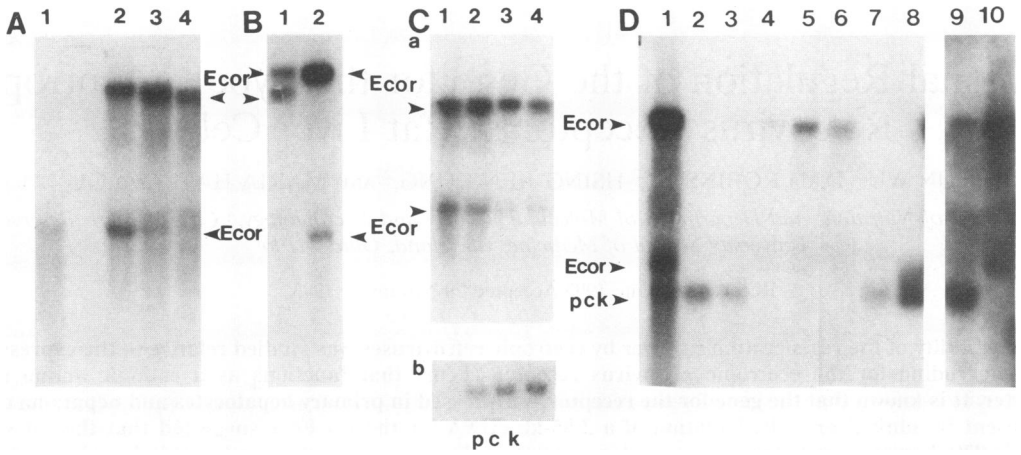

FIG. 1. Expression of the gene coding for the Ecor in rat cells. Northern blot analyses of RNA isolated from cells in culture (A, B, and C) or rat tissues (D) are shown. (A) 70% confluent FTO2B cells maintained for 24 h in serum-free medium (lane 1) or in serum-containing medium (lane 2), C6 glioma cells (lane 3), and NRK kidney cells (lane 4) were used. (B) NIH 3T3 cells (lane 1) or confluent FTO2B cells (lane 2) were used. The hybridization probe for panels A and B was the mouse Ecor cDNA. (C) FTO2B cells were plated at four different densities 2×10^4 (lane 1), 4×10^4 (lane 2), 8×10^4 (lane 3), and 1.6×10^5 (lane 4) cells per cm^2 and harvested 48 h later. The hybridization probes were the mouse Ecor cDNA (a) or PEPCK cDNA (*pck*) (b). (D) RNA was isolated from FTO2B cells (lane 1) or from tissues of male 80-g rats (livers [lanes 2 and 8], kidneys [lane 3 and 9], spleens [lane 4], and brains [lanes 5 and 10]). Lanes 8, 9, and 10 contain poly(A)$^+$ RNA. RNA from fetal liver at 18 days (lane 6) or liver 2 days after birth (lane 7) was used. The hybridization probes were the Ecor cDNA and the PEPCK cDNA. The arrowheads indicate the mRNA(s) for Ecor or PEPCK (*pck*).

λ-zap rat hepatoma cDNA library (Stratagene) was screened by using the mouse Ecor cDNA contained in the pJET plasmid (2) as a hybridization probe. The pBluescript SK$^-$ that contained the positive cDNA clones were excised from the hybridizing phage as described by the manufacturer. Sequencing of subclones of the isolated cDNAs was performed by using synthetic oligonucleotides within the T3 and T7 promoters of the pBluescript.

Generation of virus and infection of animals. DNA transfection, rescue of the recombinant retrovirus, and infection of cells were done as described previously (24). The β-galactosidase virus (8) used to infect animals was concentrated as follows. Viral supernatants from virus-producing Ψ2 cells were centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatants were centrifuged for an additional 12 h at the same speed and temperature. The pellet contained the virus which was dissolved in 1% of the original volume in tissue culture medium (Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum). Aliquots of the virus-containing medium were stored at −80°C. The titer of the virus produced by Ψ2 cells was measured by infecting NIH 3T3 cells and was approximately 5×10^5 CFU/ml.

Hepatectomy (70%) was performed on male Sprague-Dawley (50-g) rats by removal of the median and left lateral lobes. At different time points after partial hepatectomy, the portal vein was cannulated and virus (1 ml) was slowly (2 min) infused into the liver.

Hormonal treatment of cells and animals. NIH 3T3 and FTO2B rat hepatoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% calf serum and 5% fetal calf serum. The cells were transferred to medium without serum and incubated for the times indicated in the figures in the presence of 0.5 mM dibutyryl cyclic AMP, 50 nM porcine insulin, 0.84 mM L-arginine, or 1 μM dexamethasone. The effect of cell density on expression of the Ecor gene was studied by plating the cells at different densities in Corning 12-well tissue culture plates. Cells were seeded at 2×10^4, 4×10^4, 8×10^4, and 1.6×10^5/cm^2 and were harvested for analysis 48 h later. Rats treated with dexamethasone were injected intraperitoneally with 1 mg/kg of body weight. L-Arginine (1 g/kg) was given to rats intraperitoneally.

DNA probes. The following DNA probes were used in this study: (i) *pck*, a 1,100-bp *Pst*I fragment from the 3′ end of the phosphoenolpyruvate carboxykinase (PEPCK) cDNA (3); (ii) *alb*, a 1,000-bp DNA insert from the pALB cDNA (23); (iii) *otc*, an *Eco*RI fragment of the ornithine transcarbamylase cDNA (22); (iv) *tat*, the cDNA for the tyrosine aminotransferase gene (43); (v) *rpL32*, the cDNA for the ribosomal protein L32 (14); and (vi) *Ecor*, the cDNA from pJET (2) that contains the mouse Ecor cDNA. Probes for Northern (RNA) blot analysis were labeled with [α-^{32}P]dCTP for 1 h at 37°C, as previously described (34). The specific activities of the probes were 10^8 to 10^9 cpm/μg of DNA.

X-Gal histochemical staining. Liver samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 4 h at 4°C, slowly frozen in liquid Freon 22 that was cooled with liquid nitrogen, and stored at −80°C until use. Liver sections 7 μm thick were prepared with a cryostat and mounted onto 3-aminopropyltriethoxysilane-coated microscope slides. The sections were then immersed in 4% paraformaldehyde in PBS for 5 min at 4°C, washed in PBS, immersed in a solution containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and incubated for 2 h at 37°C and then for 12 h at 25°C. Experimental details of this method have been published previously (32).

RESULTS

Expression of the gene coding for the receptor in rat cells. Mouse Ecor cDNA (2) was used to determine the pattern of expression of the gene coding for the receptor in rat tissues and transformed rat cell lines. Northern blot analysis demonstrated the presence of two mRNAs (7.9 and 3.4 kb) in FTO2B rat hepatoma cells, C6 glioma cells, and NRK kidney cells (Fig. 1A, lanes 2 to 4, respectively). The pattern of the receptor mRNAs in the rat cells was different from that in the mouse

cells, which contained two mRNAs at 7.9 and 7.0 kb (Fig. 1B, lane 1). Interestingly, there was a significant difference in the intensities of the two rat mRNA bands, the 7.9-kb band being five times more intense than the 3.4-kb band (Fig. 1B, lane 2). However, the relative intensities of the two mRNAs were dependent on the density of cultured cells. In order to demonstrate that cell density regulates the expression of the gene for the viral receptor, we analyzed RNA isolated from FTO2B cells plated at different densities (Fig. 1C). The concentration of the receptor mRNA decreased with increasing cell density. In contrast, as expected (4), the concentration of mRNA coding for the liver enzyme PEPCK increased with increasing density (Fig. 1C, bottom).

The distribution of the receptor mRNA in rat tissues was identical with that in mouse tissues. All the tissues tested expressed the receptor gene, except the liver (Fig. 1D, lane 2). The factors that influence the expression of the receptor gene in the rat or mouse liver are not known. However, this gene is expressed in the late-gestation fetal rat liver but is silent immediately after birth (Fig. 1D, lanes 7 and 8). As a control, the expression of the PEPCK gene was analyzed in parallel, since the gene is not expressed in the fetal liver but is expressed immediately after birth (Fig. 1D, pck). The 7.9-kb mRNA was present in all tissues, but the 3.4-kb mRNA was detected only in brain tissue (Fig. 1D, lane 10). The nature of the two mRNA transcripts in the rat cells is unknown.

Isolation of a cDNA for the rat Ecor and comparison with the mouse Ecor. In order to understand better the regulation of expression of the rat Ecor gene relative to the two mRNA transcripts (7.9 and 3.4 kb) present in rat cells, we isolated the cDNA for the rat receptor from a rat hepatoma cDNA library.

Although rat cells can be efficiently infected with ecotropic retroviruses, it is not known whether mouse and rat cells express the same viral receptor(s). In addition, rat cells contain an mRNA transcript (3.4 kb) that hybridized with the mouse receptor cDNA but is absent from mouse cells. We isolated 10 cDNA clones after screening a rat hepatoma cDNA library, using the mouse Ecor cDNA-specific probe (2). An entire open reading frame that was very similar to the mouse cDNA (2) was found in three overlapping clones (Fig. 2A), although the rat cDNA is longer at the 3′ end by 420 bases. A composite structure of these three cDNA clones (Fig. 2A, λR1, λR4, and λR5) gives a sequence of 2.85 kb. If a poly(A) sequence of approximately 100 nucleotides is included, our cDNA clone can account for 3.0 kb of the 3.4-kb mRNA transcript. The 2.85-kb rat cDNA appeared to contain the entire 3′ untranslated region, as evidenced by a short poly(A) tail of 16 bases positioned 16 nucleotides downstream of a putative polyadenylation signal AATAAT (Fig. 2B). The CA bases commonly used as cleavage-polyadenylation sites were also present. The additional 0.4 kb of the 3.4-kb transcript could be provided by sequences at the 5′ end. The nature of the 7.9-kb mRNA is unclear at this time. In an attempt to more precisely determine the nature of these two transcripts (Fig. 1A), we used fragments of the rat cDNA to perform Northern blot analysis. Both bands hybridized with all the cDNA probes used for this analysis (data not shown). Additionally, using PCR with primers positioned at the extreme ends of our cDNA clones, we were unable to identify novel cDNAs corresponding to alternative splicing. Therefore, it appears that both transcripts encompass the entire sequences represented by our cDNA clones. The identities of the extra sequences that make up the 7.5- and 3.4-kb transcripts remain unknown.

The 2.85-kb rat cDNA which we isolated encodes a 624-amino-acid receptor protein that is highly homologous (97% homology) to the 622-amino-acid mouse receptor protein (Fig. 2C). Single amino acid differences are found along the entire length of the two proteins, with a cluster of nonhomology in the 3d extracellular domain. This domain also contains the two additional amino acids of the rat receptor at positions 222 and 227 (Fig. 2C). Albritton et al. (14) elegantly demonstrated that the amino acid sequence NVKYGE of the extracellular domain of the mouse receptor is a determinant for viral binding and infectivity by ecotropic retroviruses. The identical sequence was present in the rat receptor at positions 234 to 241 (Fig. 2C). However, the sequence flanking the virus binding site 221-ENKSSPLCGNND-232 was divergent from the mouse 221-EKNFSCNNND-232.

Expression of the gene for the rat Ecor in the regenerating liver and infection by a retrovirus containing the β-Gal gene. In an earlier study (25), we demonstrated that the regenerating rat liver was infected with recombinant ecotropic retroviruses when the virus was injected immediately after partial hepatectomy. Infecting rats immediately after partial hepatectomies was done for reasons of convenience. We decided to examine the expression of the gene for the Ecor during the first 24 h of regeneration and to determine the ability of the liver to be infected by ecotropic retroviruses. The expression of the gene for the receptor peaks at 3 to 4 h after partial hepatectomy (70%) in the regenerating liver and is undetectable 24 h later (Fig. 3A). Therefore, the livers were exposed to virus at three time points after 70% partial hepatectomy, i.e., immediately and 4 and 24 h after partial hepatectomy. We used an ecotropic retrovirus which contained the β-galactosidase (β-Gal) gene under the transcriptional control of the Moloney murine leukemia virus long terminal repeat, since it has been shown that the regenerating rat liver was infected efficiently by a similar amphotropic retrovirus 24 h after partial hepatectomy (18). Virus (1 ml) was injected into the portal veins of six rats (two rats at each time point) at the time points mentioned above. The rats were sacrificed 3 weeks later. At this time point, the liver had fully regenerated (15). Frozen liver sections were used to determine expression of the β-Gal gene by the X-Gal histochemical assay (32). If hepatocytes were infected, we would expect cells expressing the β-Gal gene to stain blue. Hepatocytes stained blue only in the liver sections of rats infected 4 h after partial hepatectomy (Fig. 3B). However, the efficiency of infection was low. The low titer of the virus (5×10^5 CFU/ml) cannot explain the inefficiency of infection of the regenerating liver. In an earlier study (25), we demonstrated that the regenerating liver can be infected immediately after partial hepatectomy. This is possibly due to the low level of expression of the receptor in the first hour following hepatectomy (Fig. 3A).

Regulation of expression of the gene for the receptor in liver cells. Ecor functions as a cationic amino acid transporter (31, 47, 48) of the y+ system. Transport of arginine by the adult rat liver is marginal (50), which is in agreement with the lack of expression of the gene for the transporter. A reasonable hypothesis for the lack of expression of the gene for the receptor in the adult liver is that it protects the circulating arginine against the high levels of hepatic arginase. This explanation suggests that the expression of the genes for the receptor and the urea cycle enzyme arginase may be inversely regulated. Since the gene for the transporter is expressed in the regenerating liver (Fig. 3) only when the activities of the urea cycle enzymes are marginal (5), we looked at the effects of hormones that regulate the concentration of urea cycle enzymes on the pattern of expression of the receptor gene in the adult rat liver.

Insulin and low-protein diet are the major negative regulators of urea cycle enzymes (39). Injection of dexamethasone,

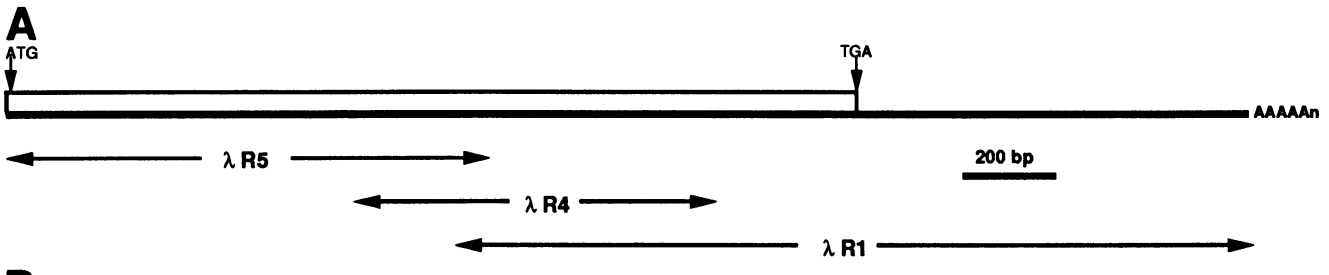

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FIG. 2. Isolation of a rat Ecor cDNA. (A) Diagram of the 2.85-kb rat cDNA composed of the three overlapping clones (λR5, λR4, and λR1), isolated from a rat hepatoma cDNA library. Sites of initiation and termination of protein synthesis are indicated. (B) Nucleotide sequence and deduced amino acid sequence of the rat cDNA. The asterisk indicates the termination codon. (C) Alignment of the 624-amino-acid rat receptor protein (R) with the 622-amino-acid mouse receptor protein (M) and the 629-amino-acid human receptor protein (H). The solid triangles indicate the putative N glycosylation sites suggested for the mouse protein (1). The region that has been suggested to serve as the viral binding domain (1) is underlined. Lowercase letters indicate amino acid sequences of the rat and and human proteins which are not homologous to the mouse protein.

insulin, or arginine induced expression of the gene for the receptor, as indicated by the accumulation of both mRNA transcripts in the liver 2 h after injection (Fig. 4A, lanes 1 to 3). A diet low in protein and high in carbohydrates also induced expression of the gene (Fig. 4A, lane 5), indicating that the gene for the receptor is regulated in a manner opposite that of the urea cycle enzyme arginase (39). The cDNAs for the urea cycle enzyme ornithine transcarbamylase and gluconeogenic enzyme PEPCK were used as hybridization probes to demonstrate the negative regulation of gene expression in the liver exerted by insulin and low-protein high-carbohydrate diet (Fig. 4A, bottom). Since injection of arginine or dexamethasone induces insulin secretion in rats (19), we assume that insulin induces expression of the receptor gene in quiescent liver cells. However, we tested the effects of glucocorticoids arginine and insulin on the expression of the receptor gene in rat hepatoma tissue culture cells. FTO2B cells, which are a cloned derivative of H35 Reuber hepatoma cells (37), have been used extensively to study liver-specific gene expression. To ensure that the FTO2B cells express the gene for the viral receptor, we performed the hormonal treatments using both FTO2B and FTO2B cells infected with an ecotropic retrovirus containing the selectable marker *neo* gene. The pattern of expression of the receptor gene in the hormone-treated cells was the same in infected and noninfected cells. A time course of induction by dexamethasone is presented in Fig. 4C. The expression of the tyrosine aminotransferase (*tat*) gene was also determined (Fig. 4C, bottom), since dexamethasone induces expression of this gene (26). The concentrations of both mRNAs increased rapidly after treatment with dexamethasone. However, the 7.9-kb mRNA was also induced in nontreated cells (Fig. 4C, lanes 1, 3, 5, 7, and 9). This mRNA induction is probably caused by components of the growth medium such as arginine. Increasing the concentration of arginine in the medium causes a twofold induction in the concentration of the receptor mRNAs (Fig. 4B). Insulin also significantly increased the level of 7.9-kb Ecor mRNA in cells which were maintained in serum-free medium for 6 h before treatment (Fig. 4D, lanes 1

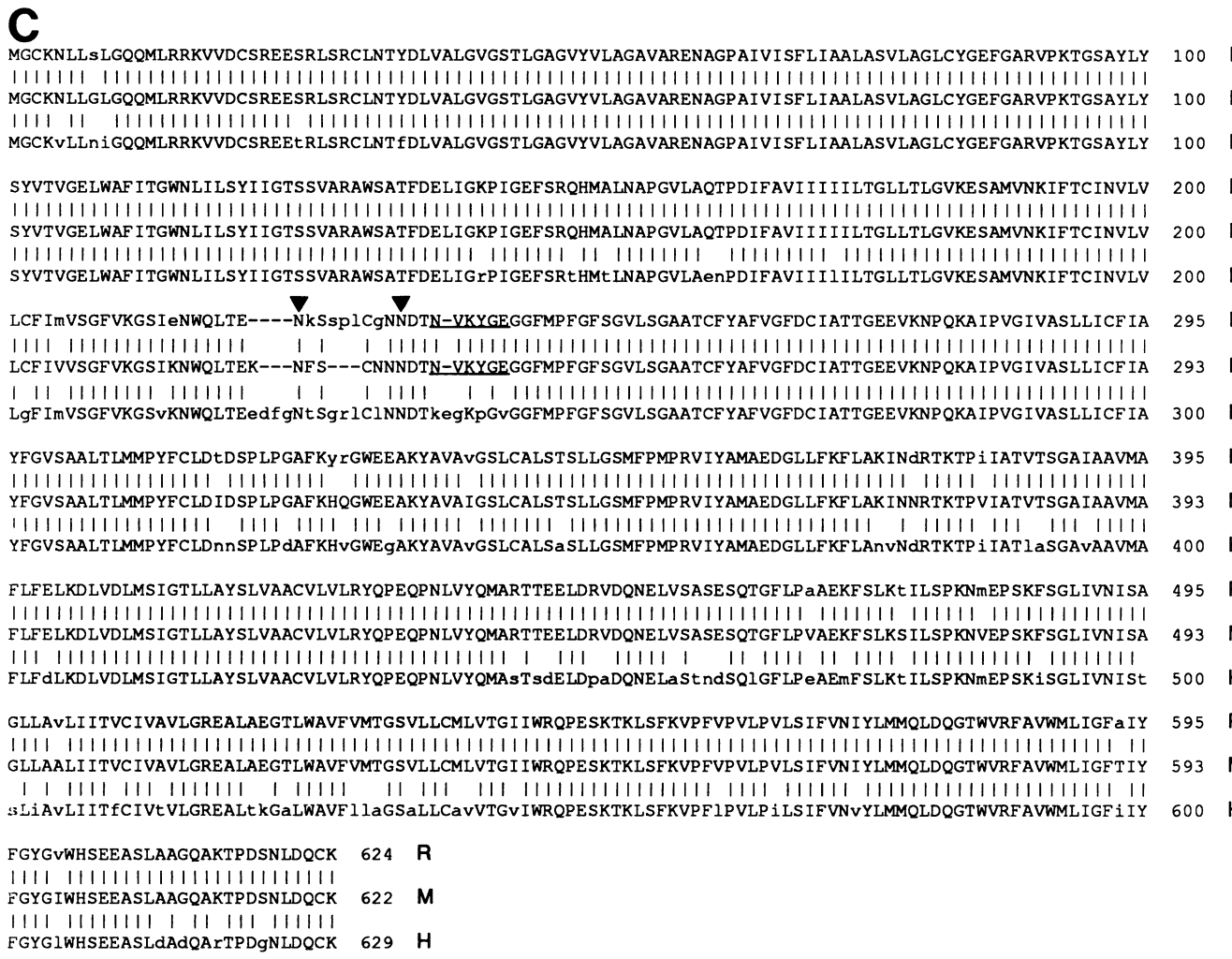

FIG. 2 *Continued.*

to 3). In addition to our data, positive regulation by glucocorticoids and insulin has been described for the gene encoding the critical enzyme of polyamine biosynthesis, ornithine decarboxylase gene (44).

Infection of the regenerating liver 24 h after partial hepatectomy. The time course of expression of the gene for the receptor in the regenerating liver (Fig. 3) indicates that there is no detectable receptor mRNA in the regenerating liver 24 h after partial hepatectomy. However, dexamethasone induced expression of the gene transiently in the regenerating liver, 24 h after hepatectomy. Ecor mRNA accumulated 1 h after injection (Fig. 4A, lane 4) and returned to undetectable levels 2 h later (data not shown). Infection of the regenerating liver 24 h after hepatectomy and 2 h after injection with dexamethasone resulted in the presence of cells in the liver which expressed the recombinant provirus. Expression of the provirus was determined 3 weeks after infection (Fig. 3C and D). The cells expressing the provirus were distributed unevenly throughout the liver, as we observed groups of cells expressing the β-Gal gene (Fig. 3C and D). The expression of the proviral β-Gal gene was localized around the portal triads, suggesting a position-dependent expression of the gene for the receptor in the liver acinus. Although hepatocytes stained blue (Fig. 3D, a and c), other types of cells in the liver have probably been infected. Further studies are needed to determine the cell populations in the liver acinus that express the provirus (and therefore can become infected). We conclude that the expression of the gene for the receptor at this time point of the cell cycle results in infection of the liver cells, since the nondexamethasone-treated regenerating liver 24 h after hepatectomy cannot become infected.

DISCUSSION

The isolation (2) and characterization (1, 31, 47, 48) of the cDNA coding for the Ecor by Albritton et al. was a significant contribution to further studies on the efficiency of gene transfer with retroviruses. In this study we have demonstrated that the expression of the gene for the Ecor that functions as an amino acid transporter in the rat liver is subject to positive and negative regulation. The identification of factors involved in the regulation of expression of genes for viral receptors is an important step in understanding the mechanisms of cell infectibility. Transient expression of the Ecor gene during liver regeneration suggests that the receptor may be involved in cell growth. Since Ecor functions as an amino acid transporter, we

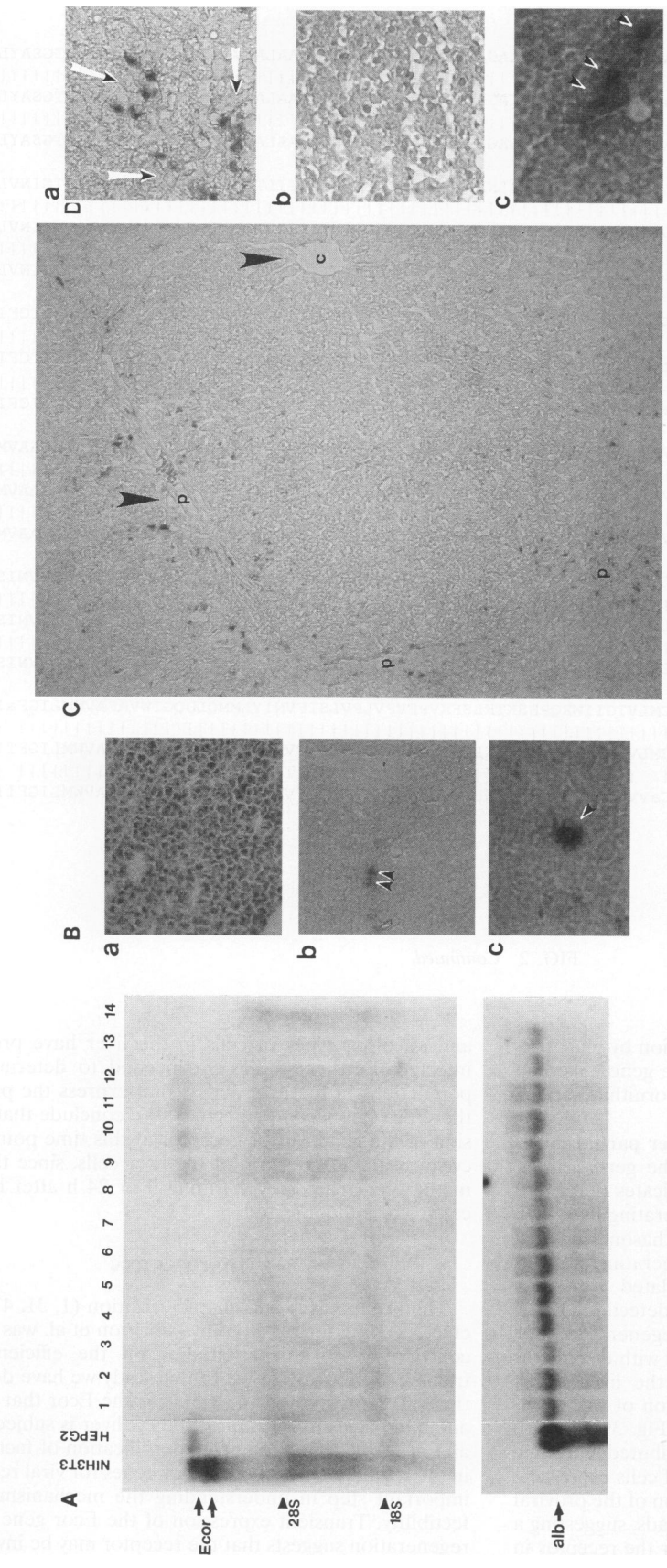

FIG. 3. (A) Expression of the gene coding for the Ecor in the regenerating rat liver. RNA was isolated from the livers of rats (80 g each) at different time points after 70% partial hepatectomies, and Northern blot analysis was performed by using the mouse Ecor cDNA as a hybridization probe. Northern blot analysis of the RNA at different time points (0 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 3 h, 4 h, 6 h, 8 h, and 24 h) is presented in lanes 1 to 14, respectively. RNA isolated from mouse NIH 3T3 cells and from human hepatoma HepG2 cells was analyzed in parallel for comparison. The bands representing the RNA for Ecor are indicated at the left side of the gel. The same blot was also hybridized with a cDNA for human albumin and is shown in the gel at the bottom of the figure (*alb*). Expression of the gene for the rat Ecor is evident only between 2 and 6 h (lanes 9 to 12) after partial hepatectomy. (B) Histochemical staining of liver sections from a rat infected with retrovirus 4 h after partial hepatectomy. Virus carrying the β-Gal gene was injected into the portal vein of a rat 4 h after partial hepatectomy. The liver was examined for expression of the β-Gal gene 3 weeks later. Liver sections (7 μm thick) were prepared from two different pieces of liver (b and c), mounted on coated glass plates, stained with X-Gal, and observed with a binocular microscope. A serial section of liver from this rat was stained with pluidine blue (1%) (a) or with X-Gal (b and c). The arrowheads in panels b and c indicate the cells which stained blue with X-Gal. A small number of blue cells were distributed unevenly throughout the liver. Magnifications, ×10 for panel b and ×40 for panel c. (C and D) Histochemical staining of liver sections from a rat infected with retrovirus 24 h after partial hepatectomy after injection of dexamethasone for 2 h. The portal triad (p) and central vein (c) are indicated. X-Gal-stained blue cells were found only around the portal triad. Panel D shows higher magnifications of the serial liver section shown in panel C stained with X-Gal (a and c) and hematoxylin-eosin (b). Groups of X-Gal-stained blue cells are indicated with arrows in panels a and c. Magnifications, ×20 for panels a and b and ×40 for panel c.

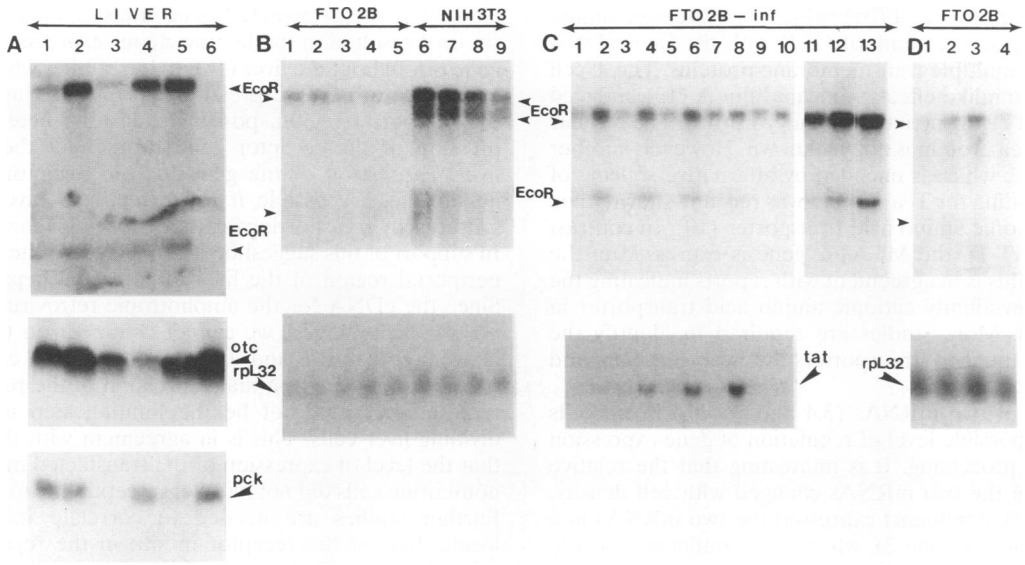

FIG. 4. Effects of hormones on the expression of the gene for the Ecor in rats. Northern blot analysis of RNA isolated from the livers of adult rats (A) or from cells (B, C, and D). (A) The rats were injected with dexamethasone (lane 1), arginine (lane 2), and insulin (lane 3), or not treated (control) (lane 6). Lane 4 contains RNA from the regenerating liver of a rat that was injected with dexamethasone for 1 h 24 h after partial hepatectomy. Lane 5 contains RNA from the liver of a rat that was fed a low-protein diet (81.5% sucrose, 12.2% casein, 0.3% DL-methionine, 4% cottonseed oil, 2% brewer's yeast and 1% mineral mix plus vitamins) for 3 days. Three hybridization probes were used sequentially, Ecor, *otc*, and *pck* (see Materials and Methods). All the experiments were performed at 9 a.m., and the treatment lasted for 2 h. (B) FTO2B and NIH 3T3 cells were treated for 4 h with serum-free medium containing increasing amounts of arginine (1× to 20×). Cells were cultured in serum-containing medium supplemented with 0.84 mM arginine (1×). The medium was changed to serum-free medium containing arginine at 1× (lanes 5 and 9), 2× (lane 4), 5× (lanes 3 and 8), 10× (lanes 2 and 7), or 20× (lanes 1 and 6). (The hybridization probes used in the Northern blot analysis were the cDNAs for the mouse Ecor [top] and the cDNA for the rat L32 ribosomal protein [bottom].) (C) Time course (40, 60, 120, 180, and 240 min) of expression of the rat Ecor gene (top) or *tat* gene (bottom) in FTO2B cells stably selected with G418 after infection with an ecotropic retrovirus (FTO2B-inf) is shown. Cells were changed to serum-free medium (lanes 1, 3, 5, 7, and 9) or medium containing dexamethasone (lanes 2, 4, 6, 8, and 10) at the time points stipulated above. Lanes 11 to 13 contain RNA from an independent experiment, in which the same cells were changed to serum-free medium (lane 11) or treated for 4 h with dexamethasone alone (lane 12) or with insulin (lane 13). (D) Expression of the rat Ecor in insulin-treated FTO2B cells is shown. Confluent FTO2B cells were changed to serum-free medium for 6 h before treatment with insulin. Fresh serum-free medium with 50 nM insulin (lanes 1 to 3) or without insulin (lane 4) was added to the cells for 30 min (lane 1), 1 h (lane 2), or 2 h (lanes 3 and 4). The hybridization probe was the mouse Ecor (top) or *rpL32* (bottom) cDNA.

can speculate that a possible role of the receptor is to import ornithine to be used in polyamine synthesis during liver regeneration (16). In support of this hypothesis is the fact that the activity of the key enzyme in polyamine synthesis, ornithine decarboxylase, peaks during the G_1 phase (16) and that ornithine levels rise progressively in the livers of partially hepatectomized rats (17). Furthermore, the Ecor mRNA, similar to the ornithine decarboxylase mRNA is induced by the synergistic action of dexamethasone and insulin (Fig. 4C, lane 13). During the G_1 phase, the activity of the urea cycle enzymes, including arginase, is minimal (5), while expression of Ecor is induced and falls to undetectable levels in the quiescent liver. This observation supports the hypothesis suggested by Closs and coworkers (11) that the quiescent liver may have a role in guarding the plasma arginine pool against the high levels of arginase.

In general, changes in the concentrations of the urea cycle enzymes, including arginase, reflect changes in the rate of transcription of the corresponding genes (38). Diet and hormones regulate expression of these genes (38). Low-protein diet and insulin are negative regulators of expression, while cyclic AMP, dexamethasone, and high-protein diet stimulate expression (39). We demonstrated that the expression of the silent gene coding for the rat receptor in the liver was induced by insulin, dexamethasone, and arginine, while cyclic AMP had no effect (data not shown). Induction by dexamethasone or arginine was independent of insulin, which was also observed in tissue culture rat hepatoma FTO2B cells. This suggests that dexamethasone might be involved in the induction of the receptor gene in the regenerating liver, since the portal concentration of glucocorticoids peaks immediately after partial hepatectomy (7). The induction of expression of the genes for the receptor and the urea cycle enzymes by dexamethasone may question our hypothesis of their inverse regulation. However, consistent with our position is the suggestion that under conditions of induction the gene for the receptor is expressed in a subset of hepatocytes which express marginal levels of the urea cycle enzymes. Reports in the literature indicate that while the expression of the urea cycle enzyme carbamoyl phosphate synthetase in the adult liver is localized in periportal hepatocytes, after induction by dexamethasone the expression of the gene is localized in pericentral hepatocytes (38). Therefore, it is conceivable that the pattern of distribution of the receptor is not homogeneous in the liver acinus. Further experimentation involving in situ localization of the receptor in the liver is required to test our hypothesis. However, while the expression of the gene is rapidly induced, it quickly returns to undetectable levels, indicating that it is also subject to negative regulation. The factors involved in this regulation of expression are not yet known. Although confluent cultures of rat cells express Ecor in much lower levels than those of rapidly growing cells (30 to 60% density), it is not clear whether the cell contact of the quiescent liver cells inhibits expression of the gene (11).

Another gene subject to regulation of expression is the

T-cell early activation gene (*Tea*), which has extensive amino acid and DNA sequence similarity (33) with the Ecor cDNA (2); both encode multiple transmembrane proteins. The T-cell mitogen with insulinlike effects, concanavalin A (13), induced expression of the *Tea* gene in T lymphocytes (33). The cellular function of the Tea protein is not yet known. However, another protein, MCAT-2, which is encoded by alternative splicing of the same gene coding for Tea protein was recently shown to be a low-affinity cationic amino acid transporter (10). In contrast with Ecor (MCAT-1), the MCAT-2 gene is expressed in the quiescent liver. This is in agreement with reports indicating the presence of a low-affinity cationic amino acid transporter in hepatocytes (51). More studies are required to identify the involvement of the two transporters in liver function and development.

The presence of two mRNAs (3.4 and 7.9 kb) in rat cells suggest another possible level of regulation of gene expression through mRNA processing. It is interesting that the relative concentrations of the two mRNAs changed with cell density. FTO2B cells (70% confluent) expressed the two mRNAs in a ratio of 1.4/1 (Fig. 1A, lane 2), whereas in confluent cells the ratio was 4.6/1 (Fig. 1B, lane 2). The ratio of intensities of the two mRNA bands was determined with a phosphoimager. When FTO2B cells (70% confluent) were maintained in serum-free medium for 24 h, the 7.9-kb mRNA was undetectable (Fig. 1A, lane 1). The 2.85-kb cDNA which we reported here is closer to the size of the 3.4-kb transcript. Since we did not find any cDNAs resulting from alternative splicing within the coding region, we suggest that the 7.9-kb transcript is generated by an alternative poly(A) site 3′ to the 3.4-kb transcript. The rat cDNA encodes a protein that is 97% identical to the mouse receptor protein (2) and has 88% homology to the human transporter protein (53). Thus, the function of the mouse receptor as a cationic amino acid transporter is highly conserved in different species. The human protein does not have a viral receptor function (1). As with the mouse and human proteins, the domain with the greatest diversity in the rat is the putative 3d extracellular domain, a region found to be critical in determining the ability of the protein to function as a receptor for the ecotropic retrovirus. By using amino acid substitutions between the human and mouse proteins, it has been demonstrated that the residues in the NVKYGE amino acid sequence at position 235 of the mouse protein are critical for viral receptor function (1). This sequence in the rat cDNA is identical to that in the mouse cDNA, thus explaining the susceptibility of the rat cells to infection by ecotropic retroviruses. The divergence of the sequences of rats and mice in the region amino terminal to the NVKYGE sequence supports the previous finding that this region does not play a significant role in the function of the protein as a receptor.

In this study we have also demonstrated that infection of the regenerating rat liver with ecotropic retroviruses parallels expression of the gene for the receptor. However, cell cycle factors may be involved in cell infectibility. It has been suggested that amphotropic retroviruses infect the liver more efficiently 24 h after partial hepatectomy (18), which is the peak of DNA synthesis. Also, the efficiency of infection of T lymphocytes by amphotropic retroviruses is cell cycle dependent (45). Cell cycle-dependent infectibility by ecotropic retroviruses has been reported (27). In our study, liver cells were infected when expression of the receptor was induced in the regenerating liver 24 h after hepatectomy. However, the expression of the proviral β-Gal gene was not homogeneously distributed throughout the liver. This is different than the observation by Ferry et al. (18), who reported that amphotropic retrovirus-mediated gene transfer in the regenerating rat liver resulted in the homogeneous expression of the β-Gal gene throughout the liver (18). In our studies where we used an ecotropic retrovirus, expression appeared in clusters of cells in the periportal region, possibly indicating heterogeneous expression of the receptor gene throughout the regenerating liver. Expression of the gene for the receptor in periportal hepatocytes is possible if the receptor is involved in DNA synthesis by transporting ornithine for polyamine biosynthesis. In support of this suggestion is that DNA synthesis starts in the periportal region of the liver after partial hepatectomy (49). Since the cDNA for the amphotropic retrovirus receptor has not yet been cloned, we cannot comment on the differences between our data and the data of Ferry et al. (18). In conclusion, we suggest that expression of the receptor gene is required but may not be the limiting step in infection of dividing liver cells. This is in agreement with the observation that the level of expression of the transfected mouse cDNA in nonmurine cells did not parallel susceptibility to infection (41). Further studies are needed to correlate infectibility with localization of the receptor in situ in the regenerating liver cells. The use of the stimulators described in this study to induce expression of the gene during the process of liver regeneration will allow future study of the effects of cell cycle factors on susceptibility to infection by ecotropic retroviruses.

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