Retroviral vector infection and transplantation in rats of primary fetal rat hepatocytes

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Summary

Retroviral vectors were used to transduce recombinant DNA encoding firefly luciferase, Escherichia coli β-galactosidase or human factor IX into fetal rat hepatocytes in primary culture. Hepatocytes were transduced optimally during a restricted time interval, 2–4 days post-plating. Although efficient and stable expression of reporter gene products was observed in vitro, it was affected differentially by culture conditions (plating density, media constituents) and chemical modulators of hepatocyte growth and differentiation (gelatin, hydrocortisone, isobutylmethylxanthine). Cultured cells, mock-infected or infected with a luciferase-expressing vector, were harvested non-enzymatically and injected subcutaneously into the dorsal neck fascia of neonatal syngeneic rats. Tissue isolated from injection sites one week later contained hepatocyte foci. In animals transplanted with infected cells, the preliminary results suggest that luciferase activity was present at these sites in proportion to the numbers of infected cells.

These findings and previous observations made with hepatocytes from neonatal and adult primary cultures, indicate that from day 19 in utero through maturity the transient temporal 'period of susceptibility' to infection in vitro is independent of the developmental state of starting tissue. Transplantability of cultured fetal hepatocytes infected with retroviral vectors and stably expressing reporter gene products suggests that such cells might provide promising models for liver gene therapy.

Key words: primary cultures, fetal hepatocytes, retroviral vectors, human factor IX, gene therapy, transplantation.

Introduction

Retroviral vectors are prominent biological tools for models of gene therapy (Friedmann, 1983; McLachlin et al. 1990). Cellular targets of retrovirally transduced genes are frequently of reticuloendothelial and hematopoietic origin, as these tissues are the preferred hosts for infectious and defective retroviruses (Varmus, 1987). Until recently, mature epithelial cells like hepatocytes, often the focal point of genetic disease (McKusick, 1988), were considered unlikely targets for genetic transduction, since hepatic expression of retroviral genes succeeded when viral infections were made in utero during early embryogenesis (Jaenisch et al. 1975; Jaenisch, 1980) but not when infections were made during neonatal or adult stages of development or during adult liver regeneration (Jaenisch and Hoffman, 1979; Stuhlmann et al. 1984). This view changed when we observed that normal adult rat hepatocytes in primary culture, which transiently retro-differentiate during their growth cycle (Leffert et al. 1975a; De Nechaud et al. 1979; Sirica et al. 1979; Gleberman, 1982; Sudhakaran et al. 1986), were competent for efficient infection and long-term expression of recombinant genes transduced by murine leukemia virus (MuLV)-based vectors (Wolff et al. 1987).

A curious observation from primary culture studies was that susceptibility to infection did not depend solely upon proliferative capacity but, instead, was restricted temporally to a period between lag and early logarithmic phases of hepatocyte growth (Wolff et al. 1987; Ledley et al. 1987; Leffert et al. 1988; Wilson et al. 1988a,b; Anderson et al. 1989; Friedmann et al. 1989). Intrigued by this 'time window of susceptibility,' we decided to investigate whether fetal hepatocytes might also manifest this behavior in primary culture, and whether culture conditions or agents that modify proliferation and differentiation of hepatocytes might alter susceptibility to infection or the ability to express recombinant reporter gene products. Furthermore, as no previous reports of studies from laboratories working towards hepatic gene therapy models had described transplantation of fetal hepatocytes infected in vitro, we wanted to determine if such hepatocytes might be suitable, since their proliferative potential in vivo and in vitro exceeds that of more mature cells (Sell et al. 1974; for review, see Koch, 1987).

Materials and methods

Animals

Fischer/344 rats (Charles River, Wilmington, MA), 19–21 days pregnant, were used unless noted. Three- to four-week-old male Fischer/344 rats were used as transplant recipients. Animals
were fed food and water ad libitum and housed at 21°C under alternating 12 h cycles of darkness and light (8 a.m.—8 p.m.).

**Chemical and biological reagents**

Type I collagenase (Cat. no. C0130), type VII bacterial collagenase (EC 1.13.12.7), paraformaldehyde, periodic acid, glutaraldehyde, polyaniline, porcine gelatin (type I, 60 bloom), dimethylformamide and ATP were purchased from Sigma (St. Louis, MO). Fetal bovine serum (Irvine Scientific, Santa Ana, CA) was dialyzed (dPBS) and stored at 20°C (Leffert et al. 1979). Standard reagent-grade organic chemicals and salts were purchased from Mallinkrodt. The substrate for β-galactosidase staining (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was purchased from Boehringer Mannheim.

**Primary culture**

Fetal rat hepatocytes (≥30 fetuses/4 rats) were isolated by sequential collagenase digestion, and plated into 3.5 or 9 cm Falcon plastic tissue culture dishes (Leffert and Paul, 1972; Leffert, 1974a,b; Koch and Leffert; Koch, 1987). Specific plating conditions are described in the figure and table legends. Arginine-free growth media formulations and general culture conditions employed in these studies have been published (Leffert et al. 1979).

The numbers of cells per culture were quantified electronically (Coulter, Hialeah, FL); attached cells were dispersed vigorously and suspended in tryptic solutions (Leffert et al. 1979). Under these conditions, >99% of cells in aggregates were reduced to single cells. Protein measurements were made (Lowry et al. 1951) with bovine serum albumin (BSA) as the standard (1×10⁶ cells=1 mg soluble proteins).

Photomicrographs were made with a Nikon Diaphot phase-contrast microscope equipped with a Nikon Microflex HFX exposure control system and Polaroid or 35 mm Nikon camera.

**Retroviral vectors**

The vector encoding *Escherichia coli* β-galactosidase (pLZRLN) was constructed (Xu et al. 1989) from pBAB, obtained originally from C. Cepko (Cepko et al. 1984). The construction and growth in appropriate packaging cell lines of pLNRLL and pLZRLN (obtained from T. Friedmann and J. Wolff) and other vectors have been described in detail (Wolff et al. 1987; Friedmann et al. 1989; Xu et al. 1989). A cell line, pFFIXmne04, secreting an ecorotopic defective factor IX (human) retrovirus was a gift from I. Verma (St. Louis, MO; Verma et al. 1988). This expression factor IX from a factor IX provirus, pAFFIXSVNeo, integrated into the chromosome of the packaging cell line, pW2. The titers of each retroviral vector preparation, determined by infection of rat 208F cells or some of the packaging cell line, W2. The titers of each retroviral vector preparation, determined by infection of rat 208F cells or some of the packaging cell line, W2. The titers of each retroviral vector preparation, determined by infection of rat 208F cells or some of the packaging cell line, W2. The titers of each retroviral vector preparation, determined by infection of rat 208F cells or some of the packaging cell line, W2. The titers of each retroviral vector preparation, determined by infection of rat 208F cells or some of the packaging cell line, W2. The titers of each retroviral vector preparation, determined by infection of rat 208F cells or some of the packaging cell line, W2. The titers of each retroviral vector preparation, determined by infection of rat 208F cells or some of the packaging cell line, W2. The titers of each retroviral vector preparation, determined by infection of rat 208F cells or some of the packaging cell line, W2. The titers of each retroviral vector pLZRLN (retroviral vector encoding firefly luciferase); pLZRLN (retroviral vector encoding *E. coli* β-galactosidase); pAFFIXSVNeo (retroviral vector encoding human plasma factor IX).

**Infection conditions and reporter gene assays**

Infections of hepatocyte cultures with pLNRLL and pLZRLN vectors were performed as indicated in the respective figure and table legends. Usually, 0.5 ml of infectious fluid (diluted with serum-free arginine-free plating media as needed) was added together with 4 μg polybromo monol. This procedure was altered when infections were performed with defective factor IX retrovirus, since this contained significant levels of secreted human factor IX (St. Louis and Verma, 1988), which would interfere with the subsequent infection assay. In these experiments, the cultures were infected for a period of 24 h. The culture fluids were then aspirated (the dishes were washed with 2 ml fresh plating medium) and replaced with 2 ml of conditioned medium from unperturbed sister-cultures.

Luciferase assays: the dishes were washed five times with 2 ml of TisNaCl buffer, pH 7.4 (Leffert et al. 1979). Exhaustive washing was necessary to remove adsorbed serum proteins, which might otherwise interfere with measurements of cellular proteins. The attached cells were extracted in a lysis buffer (0.5%, v/v, Triton X-100, 100 mM potassium phosphate, pH 7.8, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.25% Triton X-100) for 10 min at 0°C. Cell extracts were centrifuged at 15,000 g for 10 min at 21°C and the recovered supernatants stored at −70°C. Freshly thawed samples were used for protein determinations (as above; or with Coomassie Protein Assay Reagent (Pierce Chemical)) and luciferase measurements (Rippe et al. 1990). Enzyme activity was quantified for 10 s at 25°C using a luminometer (Monolight 2001, Analytical Luminescence, San Diego, CA). Under these conditions, 1000 light units =0.26 pg luciferase (S. Subramani, personal communication). Similar procedures were followed with transplanted tissue from neonatal rats (see below).

Visualization of pLZRLN-infected cultured cells expressing *E. coli* β-galactosidase was performed by cytochemical staining (Rippe et al. 1990). Blue reaction products were allowed to develop overnight in a humidified chamber at 37°C. ELISA (enzyme-linked immunosorbant assay) measurements (Anson et al. 1986) were used to quantify human factor IX levels in culture fluids of factor IX-retrovirus-infected or mock-infected cells using MAb A5 (Smith, 1985). This assay detects 1-2 ng factor IX antigen ml⁻¹. A5 did not cross-react with rat factor IX. This property was essential, because dPBS contains factor IX and because primary cultures of fetal rat hepatocytes contain demonstrable levels of rat factor IX mRNA and, therefore, presumably synthesize and secrete the clotting factors (P. M. Crosseley, G. G. Brownlee and H. L. Leffert, unpublished results).

As a crude measure of the extent of γ-carboxylation (a secondary modification of glutamyl residues necessary for biological activity) of factor IX, the proportion adsorbing to BaSO₄ was also estimated by ELISA using MAb A5 (Anson et al. 1985).

**Preparation of gelatin solutions**

Gelatin stock solutions were prepared by dissolving 100 mg proteins ml⁻¹ serum-free growth medium at 60°C. The solutions were brought to pH 7.4 (with 12 mM NaOH) at 37°C, autoclaved (15 lbf in⁻¹, 1 lbf in⁻²≈6.9 Pa; 120°C, 30 min), dialyzed and stored at 4°C (as a gel). By preincubating these preparations at 37°C, the gel was converted to a liquid; these solutions were then used for additions to the cultures (10% gelatin solutions begin to gel at 21°C within 6–10 min) or as substrates for collagenase treatment. The latter was performed by mixing equal volumes of 10% gelatin stock solution with hepatocyte digestion buffer (Leffert et al. 1979) containing purified bacterial collagenase (480 IU ml⁻¹, final concentration). The resulting 5% gelatin solution was then incubated at 37°C for 4–6 h. Control incubations consisted of 5% gelatin solutions (minus collagenase) or collagenase solutions (mixed with serum-free growth medium) minus gelatin. All solutions were autoclaved, dialyzed and stored at 4°C. Under these conditions, enzyme-treated gelatin no longer gelled; whereas, mock-treated 5% gelatin solutions, gelled at 4°C, could be liquified at 37°C and remained so at 21°C.

**Hepatocyte transplantation**

Freshly isolated hepatocytes were plated into 9 cm Falcon 3003 dishes (4×10⁶ cells/10 ml of arginine-free medium supplemented with 5% dPBS) and infected with pLNRLL (see Table 3, below). Parallel cultures were made for eventual luciferase measurements to validate infection in vitro. At varying times, the cultures were washed five times with 10 ml sterile PBS, pH 7.4, and shifted into Ca²⁺⁻free PBS containing 0.1% EDTA. After 10–15 min at 21°C, the cells were dislodged gently using a plastic cell scraper (Scientific Products) and hand-pipetted vigorously (using a 2 ml glass pipette) to generate a suspension containing small clumps of cells. This suspension was centrifuged for 2 min at 300 g at 21°C. The cell-free supernatant was saved and the cell

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Results

Effect of culture conditions on time of susceptibility to infection

To determine the effect of growth-regulating culture conditions on susceptibility to infection with retroviral vectors, hepatocytes were plated at different concentrations of dFBS (5 or 15%), and at different cell inoculum concentrations (2 or 5 x 10^5 cells/culture) in arginine-free media without or containing L-ornithine. Because of the sensitivity of luciferase assays, initial infections were performed with retrovirus pLNRLL (Fig. 1). Cultures were infected daily with a constant amount of infectious particles per dish through day 9 post-plating, and luciferase activities were measured on day 12, as shown in Fig. 2A and B. Despite varying conditions and observed differences among growth curves (see Table 1), luciferase expression peaked when cultures were infected 2-3 days post-plating*. Luciferase levels in mock-infected controls did not exceed background light levels (50-100 light units per 10^5 protein).

Effect of culture conditions on quantitative levels of expression of reporter gene products

When hepatocytes were plated without ornithine (conditions generating limited proliferation but more stable differentiation; Leffert and Paul, 1972, 1973), susceptibility to infection by pLNRLL was diminished when cultures were established at higher inoculum (5 x 10^5/dish) or higher serum concentration (15%), as shown in Fig. 2A. These trends were maintained when hepatocytes were plated into media containing ornithine (Fig. 2B), a nutrient that reduces the growth requirement of serum (Leffert and Paul, 1973; Koch and Leffert, 1974; Koch, 1987) and increases production of growth-promoting 'conditioning factors' (Koch and Leffert, 1974; Koch, 1987).

Complex effects of serum were evident in two additional ways. First, the inhibitory effects of serum on luciferase expression were partially attenuated in ornithine-supplemented cultures, particularly at high inoculum (Fig. 2B). Second, when cultures (5 x 10^5 cells plated, minus ornithine) were infected and assayed on days 3 and 9 post-plating, respectively, luciferase activity behaved biphasically as a function of initial serum concentration.

* Similar trends in the curves shown in Fig. 2 were obtained when the data were plotted as luciferase activity per culture. Therefore, the y-axis values shown in Fig. 2 were not corrected for possible artificial increases in cell numbers (which, if occurring, would have preferentially affected cell growth in media lacking ornithine) that could have resulted from the artificial introduction of trace arginine by addition of infectious fluids.

Dependence of infection on the concentration of infectious particles

In cultures infected with pLNRLL, luciferase expression was proportional to retrovirus titers (0.01-1.0 m.o.i.; multiplicity of infection, defined as the ratio of vector titer to number of cells per culture) during a period of 28 days in vitro (Fig. 3A).

The dependence of luciferase expression on exogenous arginine — introduced into cultures by retroviral fluids, and otherwise limiting in cultures plated without ornithine — seemed unlikely for two reasons. First, luciferase expression was almost always unaffected by ornithine deficiency (see Fig. 2). Second, when parallel experiments were performed (infection on day 2 and assay on day 9), high extracellular arginine levels (purposely set ~2-fold higher than levels expected at infection: 0.8-80 µM over the m.o.i. range of 0.01-1.0) failed to change increases in luciferase expression that depended on vector m.o.i. (data not shown). Whereas 80-160 µM arginine levels stimulated additional growth, determined indirectly from cellular protein measurements (Fig. 3B), no significant growth differences occurred at arginine levels <1.6 µM, although the relation between luciferase expression and m.o.i. was still evident (Fig. 3A).

Visualization of hepatocytes infected with retroviral vector pLZRNL

Primary hepatocyte cultures contain macrophages and endothelial cells, although hepatocytes outnumber them by 4:1 (Koch and Leffert, 1978). It was necessary, therefore, to quantify directly infected cells. The results of these experiments, employing pLZRNL, revealed that hepatocytes were the principle infectious targets (Fig. 4A).

Whether or not the clustering of β-galactosidase-stained cells arose from colonial growth and/or aggregation is unknown. High proportions of hepatocytes (70-90%) could be infected under various conditions (Fig. 4B and C). Endothelial cells or macrophages expressing β-galactosidase were observed rarely (<10% of total stained cells).

Expression of recombinant genes in hepatocytes

Fig. 1. Structure of integrated vectors. Abbreviations are: LTR, long terminal repeat (C); Neo, Tn5 neomycin-resistance gene (D); RSV, Rous sarcoma virus promoter (E); Luc, luciferase gene (F); LacZ, β-galactosidase gene (G); human factor IX gene (H); and SV40, simian virus 40 enhancer and early promoter (I). The direction of transcription (5'-3') at each promoter is shown by arrows. Both 5' and 3' LTRs were derived from cloned murine sarcoma and leukemia viruses, respectively.

Expression of recombinant genes in hepatocytes

Fig. 2. Dependence of luciferase expression on arginine concentration. Data are expressed as fold increase in luciferase activity determined 9 days post-infection. Data were obtained from 10 hepatocyte cultures infected with retrovirus pLNRLL, with the exception of the lo µM arginine value (data not shown). Bars represent standard errors.

Expression of recombinant genes in hepatocytes

Fig. 3. Visualization of β-galactosidase-stained hepatocytes infected with retroviral vector pLZRNL. Primary hepatocyte cultures were incubated with 1 µM of X-gal for 24 hours, then fixed with 4% paraformaldehyde and stained with 0.1% Toluidine Blue. Scale (kb)

Expression of recombinant genes in hepatocytes

Fig. 4. Comparison of the expression of recombinant genes in hepatocytes infected with retroviral vectors pLNRLL and pLZRNL. Primary hepatocyte cultures were incubated with 1 µM of X-gal for 24 hours, then fixed with 4% paraformaldehyde and stained with 0.1% Toluidine Blue. Scale (kb)
Fig. 2. Susceptibility of cultured hepatocytes to infection with pLNRL. Fetal rat hepatocytes were prepared as described in Materials and methods and plated into 3.5 cm dishes (at low, 2×10^5 cells/dish (broken lines and open symbols) or high, 5×10^6 cells/dish (continuous lines and filled symbols) inoculum concentrations) containing 2 ml arginine-free medium, without (A) or with (B) 0.4 mM ornithine, and further supplemented with 5% (O, •) or 15% (C, □) dFBS. Retroviral vectors (1×10^6 infectious units per culture) were added at the indicated times (abscissae) and luciferase activities were determined 12 days post-plating as described in Materials and methods. The values shown on the ordinates represent luciferase activity per culture divided by cell number per culture (cell numbers were obtained from sister cultures in the identical plating; see Table 1). Errors of measurement on single points ranged from 5 to 10%.

Fig. 3. Dependence of luciferase expression in pLNRL-infected cultures on multiplicity of infection. Fetal rat hepatocytes were prepared as described in Materials and methods and plated into 3.5 cm dishes (5×10^5 cells) containing 2 ml arginine-free medium. Two days later (7.5×10^4 cells/dish), the cultures were infected with pLNRL (m.o.i.=0.01 (A); 0.1 (B) or 1.0 (□)). Fresh media changes were made on day 13 post-plating (arginine-free medium supplemented with 2%, v/v, dFBS). Luciferase activity (A) and cell proteins per dish (B) were measured as described in Materials and methods.

Stained hepatocytes were not observed in mock-infected cultures under similar conditions.

Optimal infections were generally obtained 3 days post-plating (Fig. 4B and C). This trend was maintained in cultures plated at 5% dFBS at low or high inoculum concentrations, and with (Fig. 4C) or without (Fig. 4B) ornithine, but not when hepatocytes were plated at 15% dFBS and high inoculum. The latter cultures displayed, instead, continuously rising curves of β-galactosidase-positive hepatocytes through day 4. In addition, when cultures were established at low inoculum, in contrast to similar cultures infected with pLNRL (Fig. 2), the levels of reporter gene expression by cells infected 3 days post-plating were two- to threefold higher if plating media contained ornithine (Fig. 4C).

Production of human factor IX by fetal hepatocytes

When cultures were infected with pAFFIXSVNeo, reporter gene expression was approximately linear with time (Fig. 5). Results of control experiments (not shown) indicated (1) no artifactual carry-over of factor IX from infectious fluids into infected cultures, and (2) no instability of human factor IX antigen in media during the time interval examined in vitro.

The accumulation of human factor IX in media of infected cultures was a stable property of such cells. If fluid in cultures was changed on day 18, then the amount of factor IX that accumulated until day 36 was approximately the same as was found between 1.5 and 18 days (data not shown).

Accurate measurements of the amount of factor IX produced/cell could not be made, since the proportion of infected cells was unknown. However, assuming that 100% of hepatocytes were infected productively, the 'optimal' daily rate of human factor IX production was 7 ng/ml⁻¹ per 10^6 cells, a rate about half that measured for pAFFIXSVNeo-infected NIH3T3 fibroblasts, but uncorrected for differences in m.o.i. and non-parenchymal contaminants. At least 80% (average of 3 experiments) of human factor IX molecules secreted by infected hepatocytes adsorbed and could be recovered from BaSO₄. This indicated, as expected from the known enzymatic proper-
Table 1. Effect of plating conditions on proliferation of fetal rat hepatocytes in primary culture

<table>
<thead>
<tr>
<th>Time after plating (day)</th>
<th>Minus ornithine</th>
<th>Plus ornithine</th>
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<tr>
<td></td>
<td>Low density</td>
<td>High density</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>15%</td>
</tr>
<tr>
<td>1</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
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<tr>
<td>7</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>40</td>
</tr>
</tbody>
</table>

Isolated hepatocytes were prepared as described in Materials and methods and plated into 35 cm dishes (at low, 2 x 10^5 cells/dish, or high, 5 x 10^5 cells/dish, inoculum concentrations) containing 2 ml arginine-free medium or identical medium supplemented with 0.4 mM ornithine (and 5% or 15% dFBS). Supplement conditions are given in the Table column headings. Cell numbers were determined at the indicated times as described in Materials and methods (N=3; measurement errors, ±10%).

Ties of hepatocytes, the occurrence of γ-carboxylation on most of the glutamic acid residues located in the N-terminal domain of factor IX (Brownlee, 1988).

Effects of growth modulators on expression of reporter gene products

Since proliferation and differentiation of fetal rat hepatocytes are hormonally controlled (Leffert et al. 1978), we examined the effects of three compounds that enhance differentiation (gelatin, HS and IBMX) on luciferase expression in pLNRL-infected cultures. Additions were made either at infection (zero hours) or 24 h post-infection; luciferase and cellular proteins were measured 10–12 days later.

Table 2 shows that each compound depressed luciferase expression 86–98% if added at infection. Interactions among the three compounds were suggested, since their inhibitory effects were attenuated slightly when the complete mixture was tested at or 24 h post-infection (70–73% inhibition). Cytotoxicity could not account for inhibitory effects, since no significant differences from controls were observed among cell numbers in the different treatment groups, except for cell protein decreases of 20–25% in HS-treated cultures, as expected from previous growth curve measurements in this system (Fig. 4).

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with cultures subjected to high concentrations of this hormone (Leffert et al. 1978; Leffert and Koch, 1978). However, addition of either HS or IBMX at 24 h post-infection abrogated the depression in luciferase expression, which was hence inhibited by only 31 and 13 %, respectively; in contrast, gelatin was essentially totally inhibitory whether added at infection or 24 h thereafter. With gelatin preparations pretreated with collagenase, inhibitory activity was retained (71 % inhibition) following addition at infection, or lost when added 24 h post-infection (Table 2).

Transplantation of hepatocytes infected in vitro into syngeneic neonatal rats

To determine if cultured fetal hepatocytes expressing reporter genes could be successfully transplanted into animals, 2- to 3-day-old primary cultures were infected with pLNRLL (m.o.i. =1.0). The bulk of the cultures were used as a source of transplanted tissue. The remaining dishes were assayed for luciferase activity to validate the productivity of infection. Infected cells were harvested between 9 and 14 days post-plating and transplanted subcutaneously into the dorsal neck fascia of neonatal rats; a total of eight rats were used without fatalities. One week later, tissues at the injection sites were recovered for assays of luciferase activity and histological staining. The results of three independent experiments are shown in Table 3 and Fig. 6.

Microscopic examination of transplanted tissue (experiments II and III) revealed small hepatocytic foci (Fig. 6). Non-parenchymal liver cells were not observed. Significant levels of luciferase activity were detected in transplanted tissue when >4 x 10⁵ cells, regardless of ornithine-supplementation were injected (experiments II and III). When the numbers of injected cells, originating from infected, un-supplemented cultures, were reduced 10-fold, only threshold levels of luciferase activity were detectable (experiment I). No luciferase activity was detected at injection sites when either mock-infected cells, supernatants from infected cell pellets (hurrying recombinant infectious or helper virus, if present), sterile cell-free buffer vehicles, or an amount of purified luciferase enzyme (20 x 10⁶ light units) equivalent to the amount of luciferase activity retained by transplanted injected cells, were injected into recipient rats under identical conditions.

A crude estimate of transplantation efficiency was made (using data from Table 3) by elucidating the ratio of the average luciferase activity (per mg protein) in transplant

![Fig. 5. Human factor IX expression in cultured hepatocytes infected with pAFFIXSVNeo. Liver tissues were obtained from 19- to 21-day pregnant PVG rats (MRC, Immunology Laboratory, Sir William Dunn School of Pathology, Oxford). Fetal rat hepatocytes were prepared as described in Materials and methods and plated into 3.5 cm dishes (5 x 10⁶ cells) containing arginine-free medium supplemented with 5 % dFBS and 0.4 mM ornithine. The cultures were infected (m.o.i. =1.0) for a period of 24 h (1.5-2.5 days post-plating) and the factor IX levels in the media were measured as described in Materials and methods.](#)

![Fig. 6. Morphology of transplant sites in rats injected with hepatocytes infected in primary culture with pLNRLL. Fetal rat hepatocytes were plated, infected, harvested and injected as described in Materials and methods. The photomicrograph shows two nests of transplanted foci of Toluidine Blue-stained hepatocytes, one week following subcutaneous injection of luciferase-expressing cells into dorsal neck fascia (bar, 50 μm).](#)

<table>
<thead>
<tr>
<th>Additions to media</th>
<th>Hours post-infection</th>
<th>Luciferase activity % of control</th>
<th>Cell proteins % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Gelatin</td>
<td>24</td>
<td>6</td>
<td>110</td>
</tr>
<tr>
<td>Collagenase-treated gelatin</td>
<td>24</td>
<td>29</td>
<td>107</td>
</tr>
<tr>
<td>Hydrocortisone-succinate (HS)</td>
<td>24</td>
<td>84</td>
<td>91</td>
</tr>
<tr>
<td>Isobutyl-methyl xanthine (IBMX)</td>
<td>24</td>
<td>87</td>
<td>89</td>
</tr>
<tr>
<td>Gelatin, HS, IBMX</td>
<td>24</td>
<td>30</td>
<td>110</td>
</tr>
</tbody>
</table>

* Isolated hepatocytes were prepared as described in Materials and methods and plated into 3.5 cm dishes containing 2 ml arginine-free medium supplemented with 5 % dFBS. Additives were (final concentrations): gelatin (5 %), v/v); collagenase-treated gelatin (10 %, v/v); HS (10 μg ml⁻¹), and IBMX (0.1 mM).

† Cultures incubated with collagenase-treated gelatin were infected on day 3 post-plating; all other cultures were infected on day 2 post-plating.

‡ Absolute levels of luciferase activity and cell proteins in the no addition control group (day 2 infection; day 14 assay) were 626 000 light units mg⁻¹ proteins and 262 μg proteins/dish, respectively. For cultures infected on day 3 (day 12 assay), the levels were 77 900 light units mg⁻¹ and 211 μg proteins/dish, respectively (100 % controls).

§ Mock-digestion additions (autoclaved medium minus gelatin, containing collagenase) gave values of luciferase activity and cell proteins per dish (relative to 100 % controls defined in footnote †) as follows: when added at 0 h post-infection, 72 % and 113 %, respectively; when added at 24 h post-infection, 86 % and 99 %, respectively.
tissue extracts (uncorrected for host-tissue proteins derived from the transplant site) to the average luciferase activity assumed to be present in hepatocyte suspensions destined for transplantation (assayed at or after in vitro harvest). Assuming an in vitro infection efficiency of 100%, the value obtained was roughly 0.81% ([6735/\(33.177\times10^4\))×100] at ‘inputs’ of 4×10⁷ to 4.5×10⁷ cells [=40–45 mg proteins/site]. If relevant, this value would imply that for every 10,000 productively infected cells, the average luciferase activity would be 0.81%. Thus far, neither the structure of the infectious agents nor culture conditions employing different matrix coatings on plastic dishes (Wilson et al. 1988a; Anderson et al. 1989) have influenced this response pattern. Except for one experimental observation, the transient-window of susceptibility has been observed regardless of the retroviral vector sub-type (ecotropic or amphotropic), vector promoter structure (5'-LTR (long terminal repeat), internal simian virus 40 (SV40) or Rous sarcoma virus (RSV)), encoded gene product (HPRT (human hypoxanthine phosphoribosyl transferase), neo- mycin-resistance, luciferase, or β-galactosidase), or species of recipient hepatocyte (rat, mouse, rabbit) employed for primary culture.

Only pLZRNL-infected cells plated initially at high cell density (0.4 mM or more) were transduced. By contrast, no luciferase activity was observed in pLNRLL-infected cells planted initially at high cell density (0.4 mM or more).

### Table 3. Expression of luciferase activity in transplanted tissue derived from fetal rat hepatocytes infected in vitro with retroviral vector pLNRLLL

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Injection (day)</th>
<th>Harvest (day)</th>
<th>Supplement</th>
<th>Injected material</th>
<th>Cells per site</th>
<th>Cultured cells (days in vitro)</th>
<th>Transplanted tissue</th>
<th>Luciferase activity (light units mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3</td>
<td>9</td>
<td>None</td>
<td>pLNRLLL-infected cells</td>
<td>4.5×10⁶</td>
<td>47 700</td>
<td>Left</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>0</td>
<td>NM</td>
<td>Right</td>
<td>38</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>9</td>
<td>None</td>
<td>pLNRLLL-infected cells</td>
<td>4.5×10⁷</td>
<td>903 000</td>
<td>Left</td>
<td>1736</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Buffer</td>
<td></td>
<td>0</td>
<td>NM</td>
<td>Right</td>
<td>13 845</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>14</td>
<td>0.4 mM L-ornithine</td>
<td>pLNRLLL-infected cells</td>
<td>4.0×10⁷</td>
<td>553 020</td>
<td>Left</td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td>Buffer</td>
<td></td>
<td>0</td>
<td>NM</td>
<td>Right</td>
<td>0</td>
</tr>
</tbody>
</table>

* Injected cells at this site merged with contralateral site.

### Discussion

Experimental observations made with primary cultures of fetal rat hepatocytes, expressing gene products encoded by retroviral vectors, suggest three conclusions about transduction phenomena in normal hepatocytes. First, the time interval of susceptibility to retroviral vector infection, optimal 2–4 days post-plating in primary culture, is independent of hepatic developmental state and species of recipient hepatocyte (rat, mouse, rabbit) employed for primary culture.

Only pLZRNL-infected cells plated initially at high cell density in vitro and in serum concentrations exhibited continuously rising curves of susceptibility to infection through day 4 (Fig. 4B and C). This observation could be spurious, since later time points were not measured. Or, it might point to the nature of measurement protocols, as single time points for measurements of reporter gene expression products were taken in all such experiments following variable time points of infection. Time course studies of reporter gene expression coupled with Southern blots to define the integrated status of the provirus (for example, see Wilson et al. 1988a), following various times of infection, might help to interpret this anomaly – particularly in light of recent findings that reporter genes tend to be expressed more stably in cells infected with vectors using upstream as opposed to internal regulatory promoters (Xu et al. 1989). Although replicative DNA synthesis is necessary for retroviral vector gene expression (Varmus and Swanson, 1986), the varying degrees of proliferative competence among different primary hepatocyte systems tested could not explain the susceptibility window, since DNA synthesis rates are high during both inclining and declining portions of the susceptibility curve in all such systems. In addition, reinitiation of DNA synthesis in quiescent adult hepatocytes...
cyte cultures fails to restore susceptibility to infection (Wolff et al. 1987) in agreement with the liver regeneration studies of Jaenisch and Hoffmann (1979).

Alternatively, the susceptibility window might reflect transient accessibility of retroviral cDNA to preferred chromosomal integration sites (Weider et al. 1987; Shih et al. 1988) or re-expression of pre-19-day gestational functions, which confer a permissive state (Wolff et al. 1987). Such functions might regulate viral receptor or post-receptor mechanisms or be attuned specifically to transcriptional requirements of strong tumor virus promoter elements (Rippe et al. 1990). Such functions might also restrict the communal behavior of hepatocytes engaged in forming maturation-related electrically coupled intercellular aggregates (Gleberman et al. 1989).

The latter hypothesis raises the possibility that the disruption of cellular contacts, a consequence of liver tissue digestion required for primary culture, might expose or fortuitously generate cryptic receptors for infection. According to this view, low susceptibility to infection would be lost as disaggregated cells regroup and restore communal differentiated structure.

Unlike susceptibility to infection, the levels of reporter gene expression/cell and the proportion of productively infected hepatocytes seemed to be influenced by both retroviral vector structure and the conditions of primary culture. Conditions that stimulated the rate and magnitude of proliferation (optimal serum concentrations (low>high; see Leffert, 1974a; Koch, 1987), optimal plating densities (low>high; see Paul et al. 1972), and ornithine-supplemented media (reviewed by Koch, 1987)) were associated with increased reporter gene expression per cell in pLNRLL-infected cultures and with greater proportions of cells expressing β-galactosidase in some of the cultures exposed to pLZRNL. In contrast, conditions that favored differentiation of hepatocytes were associated with reduced levels of reporter gene expression. These generalizations are complicated, however, by discrepant observations with pLZRNL-infected cultures, particularly those plated at low inoculum with ornithine. These cultures showed higher proportions of infected cells compared to cultures plated without ornithine. Here, too, protocol design, the position of the internal RSV-promoter (Fig. 1; Xu et al. 1989) and the status of proviral integration will require further study before we understand the discrepancies.

A relationship between differentiation and attenuated reporter gene expression was further suggested by cellular responses to chemical agents that stimulate fetal hepatocyte differentiation in vitro. Gelatin, which increases alpha-fetoprotein production (Belanger et al. 1978), hydrocortisone, which depresses proliferation at high concentration and stimulates albumin production (Leffert et al. 1978b; Chou, 1988), and IBMX, which increases infectivity per cell and for a AMP analog with differentiated function (Leffert et al. 1978b; Chou, 1988), blocked luciferase expression when added to cultures 24 h post-infection (94, 31 and 13%, respectively).

Some insight into mechanism(s) of action of these agents can be inferred from findings that gelatin's acute inhibitory effects resisted enzymatic hydrolysis, whereas its post-infection inhibitory effects were abolished by pretreatment with collagenase*. The latter observation supports the above interpretation, that by facilitating differentiation after infection, type I collagen solutions attenuate reporter gene expression. On the other hand, a regulatory role around the time of infection is suggested for a collagenase-resistant substance, possibly bound to collagen or derived from proteoglycans, as well as for HS and IBMX, since these agents all interfered acutely with the infectious process. One possible focus of such interactions is retroviral integration, a process that requires 24 h in culture infected with pAFFIXSVNeo 2–3 days post-plating, as determined by Southern blotting (G. G. Brownlee, unpublished observations).

Transplantation of cultured fetal liver cells expressing reporter gene products into syngeneic neonatal rats raises the possibility that this system could provide a model for studies of gene therapy for liver disease. While several complex methods for transplanting suspensions of freshly isolated adult rat hepatocytes into various tissue sites in adult rats have been reported (Darby et al. 1986; Anderson et al. 1987; Antersing et al. 1989), our results suggest that cultured fetal hepatocytes can tolerate damaging harvesting procedures when introduced into the flank fascia of neonatal animals. The conclusion that such cells survive the procedure is based upon the morphological appearance of intact hepatocytes in multicellular foci detected in histological sections of tissue injection sites, as well as the accompanying luciferase activity at these sites, 7 days following injection of the infected cells. However, whereas foci of epithelial cell aggregrates with hepatocyte-like morphology were observed at transplantation sites, direct evidence that such hepatocytes were luciferase-positive and functionally differentiated has yet to be obtained. We favor this interpretation, since Toluidine Blue-stained non-parenchymal liver cells were not observed in histological sections of fascial tissue taken from transplant sites. And, the proportion of productively infected cells from culture is enriched fourfold with hepatocytes, over non-hepatocytes.

While it is not yet clear if the fetal nature of these donor hepatocytes and the neonatal status of the recipients are beneficial to transplantation, the rationale for these studies involved suppositions that hepatocytes with enhanced proliferative potential might generally fare better, and that animals with constitutively proliferating livers and, presumably, appropriate circulating humoral hepatocellular growth factors (Koch et al. 1990), would provide the most permissive environment. In addition, the non-enzymatic method of harvesting could have been important in alleviation of massive inflammatory reactions previously found at subcutaneous injection sites of enzymatically harvested cultured cells (H. Leffert and S. Sell, unpublished results). Further experimentation is clearly necessary for precise quantification of transplantation efficiency and of long-term survivability of the gene, and for an assessment of the many variables upon which these two parameters, and a successful gene therapy regimen, certainly depend.

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