doi:10.1006/mthe.2001.0276, available online at http://www.idealibrary.com on IDEAL

ARTICLE

Transduction of Liver Cells by Lentiviral Vectors: Analysis in Living Animals by Fluorescence Imaging

Alexander Pfeifer,* Torsten Kessler,[†,1] Meng Yang,[‡,1] Eugene Baranov,[‡] Neeltje Kootstra,* David A. Cheresh,[†] Robert M. Hoffman,[‡] and Inder M. Verma*[,2]

*The Salk Institute, La Jolla, California 92037
[†]The Scripps Research Institute, La Jolla, California 92037
[‡]AntiCancer, Inc., San Diego, California 92111

Received for publication December 1, 2000; accepted in revised form February 2, 2001.

Viral vectors based on lentiviruses, such as the human immunodeficiency virus, are able to transduce a broad spectrum of nondividing cells *in vivo.* This ability of lentiviral vectors makes them an attractive vehicle for gene transfer into the liver. In order to determine the requirements for efficient lentiviral gene transfer, we used a fluorescence imaging system, which allows the detection of cells and tissues that express fluorescent reporter genes (e.g., green fluorescence protein) in the living animal. We show that the latest generation of lentiviral vectors efficiently transduces the murine liver. Further analysis demonstrated that neither cell-cycle activation nor division of liver cells is a prerequisite for lentiviral gene transfer *in vivo.*

Key Words: **lentiviral vectors; gene transfer; fluorescence imaging; GFP; liver; hepatocytes.**

INTRODUCTION

Lentiviral vectors are a promising tool for gene therapy. They have been shown to transduce a broad spectrum of nondividing cells *in vivo*, such as neurons, retina, liver, muscle, and hematopoietic stem cells (1–4). However, it was recently reported (5) that hepatocytes are refractory to lentiviral transduction unless they progress into the cell cycle or divide. The ability to infect nondividing cells is a highly desirable feature of gene therapy vectors especially in the context of gene delivery to the liver, since most of the liver cells normally do not divide in the healthy adult. Here we describe that lentiviral vectors can efficiently transduce hepatocytes *in vivo.*

MATERIALS AND METHODS

Animals. Twelve-week-old female nude (Hsd:Athymic Nude-*nu*) mice (Harlan Sprague–Dawley, Indianapolis, IN) were used.

Virus production. A third-generation, Tat-free packaging system (6) was used to produce recombinant lentivirus. The GFP-LV (7) plasmid together with the two packaging plasmids [encoding human immunodeficiency virus (HIV) gag, pol, and rev] and the plasmid coding for VSV-G envelope were transfected into 293T(HEK) cells using the calcium phosphate method, as described (1, 8). In brief, we transfected 24 15-cm dishes and harvested the virus by collecting the cell culture medium 24, 48, and 72 h. After filtering the collected medium through 0.45-μm filters, we concentrated the virus by spinning at 68,400*g* for 2 h followed by a second spin (59,000*g*, 2.5 h at room temperature). The resulting pellet was resuspended in 200 μl Hanks' buffer. The titer of lentiviral vectors was determined by measuring the amount of HIV p24 Gag antigen by ELISA (Alliance; NEN Life Science Products, Boston, MA).

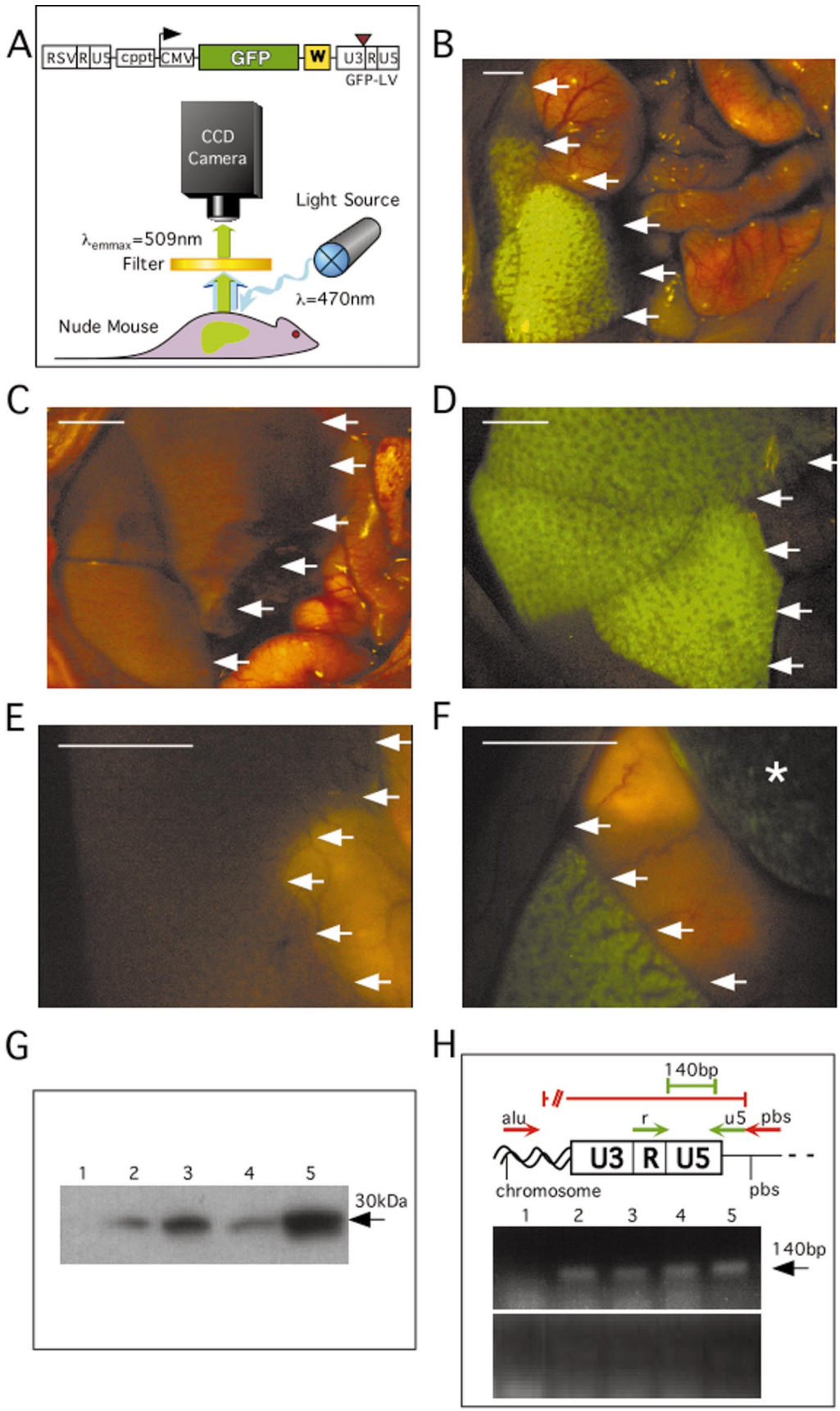
In vivo fluorescence optical imaging. The fluorescence imaging setup has been described in detail (9, 10). Briefly, a Leica stereomicroscope (Model LZ12) equipped with a mercury 50-W lamp was used with a Hamamatsu C5810 three-chip cooled color charge-coupled-device camera (Hamamatsu Photonics Systems, Hamamatsu City, Japan) attached. Excitation of green fluorescence protein (GFP) was achieved at 470 nm (using a D425/60 band-pass filter and 470 DCXR dichroic mirror). The emitted fluorescence was collected through a long-pass filter (GG475; Chroma Technology, Brattleboro, VT). Images were analyzed using Image Pro Plus 3.1 software (Media Cybernetics, Silver Springs, MD). Images were captured directly on an IBM PC or recorded on a high-resolution Sony VCR, Model SLV-R1000 (Sony, Tokyo).

Immunhistochemistry. Three weeks after injection of GFP-LV the mice were sacrificed and the livers dissected. The tissue samples were fixed in 4% paraformaldehyde in PBS overnight at 4°C, followed by an incubation for 48 h in 20% sucrose. Thereafter, the tissue was embedded in OCT (Tissue-Tek), frozen, and sectioned on a cryostat. GFP expression was detected using a confocal laser scanning MRC1024 microscope (Bio-Rad, Hercules, CA). Analysis of 5′-bromo-2′-deoxyuridine (BrdU) incorporation into tissue sections was done using an anti-BrdU antibody (Accurate), after incubating the slides for 30 min with 2 N HCl.

[1] These authors contributed equally to the work.

[2] To whom correspondence and reprint requests should be addressed at the Laboratory of Genetics, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037. Fax: (858) 558-7454. E-mail: verma@salk.edu.

1525-0016/01 $35.00



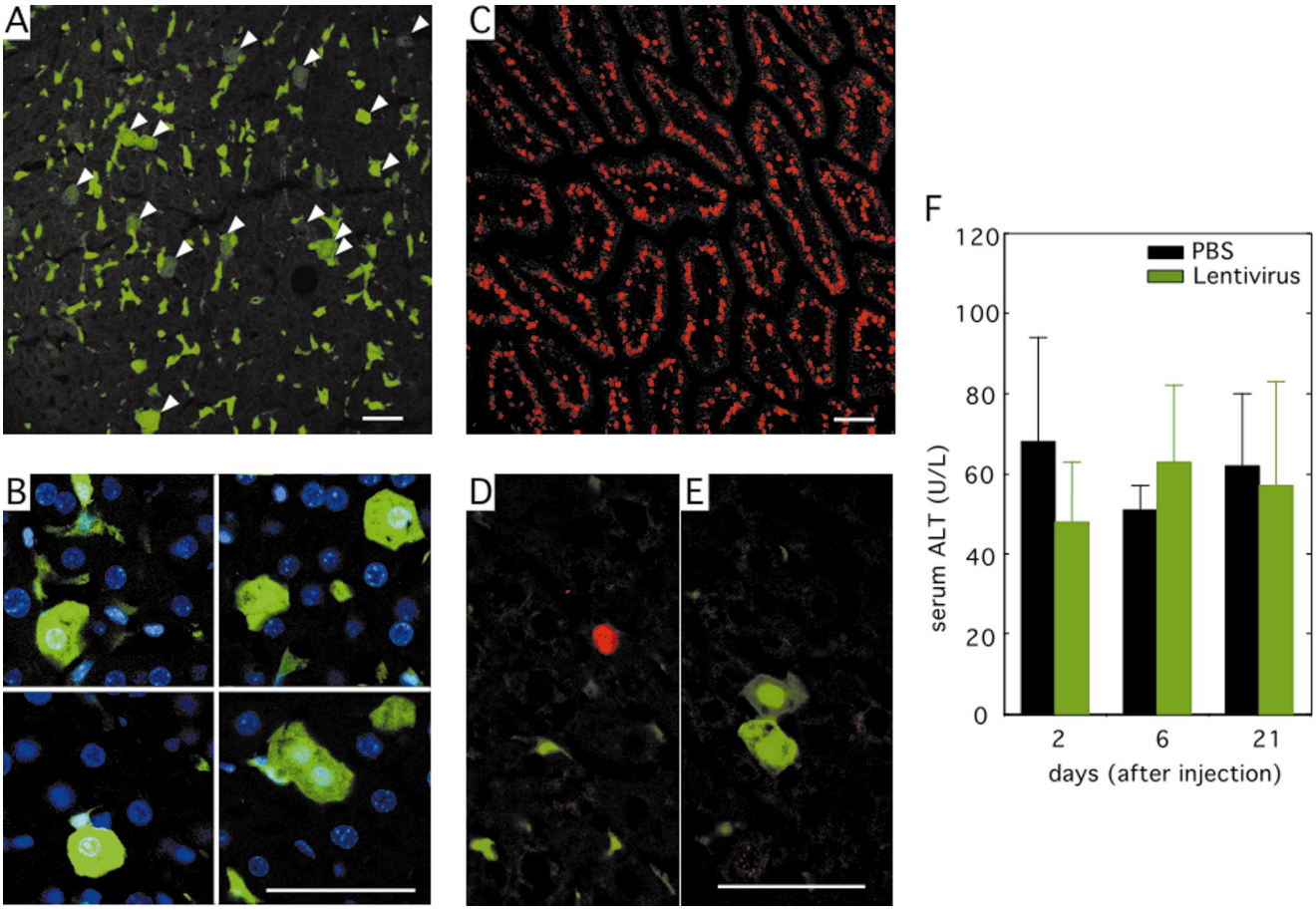


FIG. 2. Histochemical analysis of transduction of liver cells *in vivo.* (A) Confocal microscopy analysis of green fluorescence 3 weeks after portal vein injection of GFP-LV (1×10^9 IU). Hepatocytes are indicated with the arrowheads. (B) Typical examples of transduced hepatocytes. The nucleus was stained with TO-PRO (Molecular Probes) (blue). (C–E) Labeling of cells in S phase by BrdU incorporation. BrdU labeling (red) and GFP expression (green) in a GFP-LV-injected animal: duodenum (C) and liver (D, E) 3 weeks after injection. Bar, 50 μm. (F) Analysis of serum ALT as a marker for liver toxicity. Mice were injected with PBS (black bars) or lentiviral vectors (1×10^9 IU) in the portal vein. Mean values are presented with SEM (n = 3–4).

Western blotting and detection of proviral DNA. Western blot analysis was performed using polyclonal antibodies to GFP (Clontech). For the detection of proviral DNA a nested PCR approach was used (11).

RESULTS AND DISCUSSION

We used the latest generation of HIV-1-based lentiviral vectors that contain the GFP coding sequence (GFP-LV, Fig. 1A). To enhance transgene expression and transduction of the target cells, the woodchuck hepatitis virus posttranscriptional element (WPRE) and a central polypurine tract (cppt) were included in the vectors (7, 12, 13) (Fig. 1A). Transduction of the liver by GFP-LV was monitored in the living animal using *in vivo* fluorescence optical imaging (9, 10) (Fig. 1A).

Six days after injection of GFP-LV (1×10^9 IU) into the portal vein of nude mice (Hsd:Athymic Nude-*nu*), green fluorescence was detectable in the liver of the injected animals using *in vivo* imaging (data not shown). At day 21 all liver lobes of mice injected with GFP-LV exhibited a homogeneous green fluorescence (Figs. 1B and 1D), but

FIG. 1. Detection of lentiviral gene delivery using *in vivo* fluorescence optical imaging. (A) Schematic representation of GFP-LV lentiviral vector (top) and the imaging system (bottom). The lentiviral vector contains a self-inactivating mutation of the U3 region (brown triangle), a posttranscriptional element (W), and an internal CMV promoter. cppt is the central polypurine tract derived from HIV-1 *pol.* (B–D) Analysis of green fluorescence after opening the abdominal cavity by a median-line incision. Representative mice are shown 21 days after portal vein injection with GFP-LV (1×10^9 IU) (B, D) or PBS (C). (E and F) *In vivo* fluorescence imaging 21 days after ip injection of PBS (E) or GFP-LV (1×10^9 IU) (F). The arrows and the asterisk indicate the liver and the spleen, respectively. White bar, 0.25 cm. (G and H) Western blot analysis of GFP expression (G) and PCR analysis of vector integration (H) 21 days after injection of lentiviral vectors. Lane 1, PBS-injected control; lane 2, vector without cppt ($cppt^-$) (1×10^9 IU); lane 3, vector containing cppt ($cppt^+$) (1×10^9 IU); lane 4, $cppt^+$ (0.5×10^9 IU); lane 5, $cppt^-$ (2.5×10^9 IU). A "nested" PCR protocol was used to detect proviral DNA using a primer pair that binds in the *Alu* sequences (alu) of the mouse genome and the primer binding site (pbs) of HIV-1 (shown in red), followed by a PCR amplifying the R/U5 region (green) that results in a 140-bp fragment. To confirm that only integrated proviral DNA was detected using this PCR method, an additional PCR (using r and u5) was performed with samples 1–5 without the primers of the first reaction (alu and pbs).

not in the PBS-injected control (Fig. 1C). Intraperitoneal (ip) injection of GFP-LV also resulted in a high level of transduction of the liver and the spleen (Fig. 1F), while in the PBS-injected controls no fluorescence was detectable (Fig. 1E). GFP expression was detectable even 6 months after injection (not shown). Western blot analysis demonstrated a dose-dependent effect of the lentiviral vector on liver GFP expression (Fig. 1G). In addition, inclusion of a cppt element further increased (~two- to threefold) GFP expression (Fig. 1G). PCR analysis of proviral DNA showed vector integration in the liver 3 weeks after injection of the lentiviral vectors (Fig. 1H).

GFP expression in the liver was further analyzed by confocal microscopy of frozen sections 21 days after GFP-LV injection. Sections of transduced livers exhibited a strong green fluorescence throughout the whole organ (Fig. 2A). Statistical analysis of the frequency of GFP-positive cells revealed that 26.4 ± 1.2% of all the liver cells present in the sections were transduced by the lentiviral vectors. The majority of GFP-positive cells (78.7%) were nonhepatocytes, such as perisinusoidal cells, Kupffer cells, and endotheliocytes (Figs. 2A and 2B). Morphological analysis revealed that 5.3 ± 0.5% of the hepatocytes present in the sections were GFP positive. To analyze cell-cycle status of the transduced liver cells, we administered 50 mg/kg of BrdU by daily ip injections for 3 weeks (14), which resulted in a strong labeling of dividing cells in the duodenum (Fig. 2C). In contrast, only a small percentage (less than 1%) of all liver cells present in the sections were labeled with BrdU. No significant difference in the number of BrdU-labeled cells was observed between the PBS- and the lentivirus-injected liver. In most of the sections analyzed, the GFP-positive hepatocytes were not labeled with BrdU. Only in one (of eight) sections was colabeling with GFP and BrdU of hepatocytes observed; the BrdU-labeled cells account for less than 1% of all transduced hepatocytes (Figs. 2D and 2E). We therefore conclude that cell cycle progression is not required for transduction of hepatocytes *in vivo*. To study the possible liver toxicity of lentiviral vectors, we analyzed serum alanine aminotransferase (ALT) levels in transduced animals. No significant changes in serum ALT concentrations were observed after injection of lentiviral vectors (1×10^9 IU) into the portal vein (Fig. 2F).

Taken together our data indicate that lentiviral vectors transduce nondividing liver cells *in vivo* without inducing any detectable liver damage. Using first-generation lentiviral vectors we observed efficient transduction of liver parenchymal cells (2). The discrepancy between the results reported here and those published by Park *et al.* (5) is not clear but could be due to improved vector production. Additionally, the use of minimal lentivectors (third-generation self-inactivating vectors, lacking all accessory proteins) combined with WPRE and cppt elements further facilitates transgene expression in liver cells and other nondividing cells. Finally, we show that *in vivo* fluorescence imaging is a powerful technique to analyze transduction efficacy of viral vectors in the living animal.

ACKNOWLEDGMENTS

A.P. receives a Heisenberg Scholarship from the Deutsche Forschungsgemeinschaft, T.K. is supported by the Mildred Scheel Stipendium from the Deutsche Krebs Hilfe. D.A.C. is supported by CA45726, CA50286, and CA 78045. I.M.V. is an American Cancer Society Professor of Molecular Biology. He is supported by grants from the NIH, the March of Dimes, the Wayne and Gladys Valley Foundation, and the H. N. and Frances C. Berger Foundation.

REFERENCES

[1] Naldini, L., *et al.* (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272:** 263–267.

[2] Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997). Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* **17:** 314–317.

[3] Takahashi, M., Miyoshi, H., Verma, I. M., and Gage, F. H. (1999). Rescue from photoreceptor degeneration in the rd mouse by human immunodeficiency virus vector-mediated gene transfer. *J. Virol.* **73:** 7812–7816.

[4] Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M., and Torbett, B. E. (1999). Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* **283:** 682–686.

[5] Park, F., Ohashi, K., Chiu, W., Naldini, L., and Kay, M. A. (2000). Efficient lentiviral transduction of liver requires cell cycling in vivo. *Nat. Genet.* **24:** 49–52.

[6] Dull, T., *et al.* (1998). A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72:** 8463–8471.

[7] Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M., and Naldini, L. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat. Genet.* **25:** 217–222.

[8] Pfeifer, A., Kessler, T., Silletti, S., Cheresh, D. A., and Verma, I. M. (2000). Suppression of angiogenesis by lentiviral delivery of PEX, a noncatalytic fragment of matrix metalloproteinase 2. *Proc. Natl. Acad. Sci. USA* **97:** 12227–12232.

[9] Yang, M., *et al.* (2000). Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. *Proc. Natl. Acad. Sci. USA* **97:** 1206–1211.

[10] Yang, M., Baranov, E., Moossa, A. R., Penman, S., and Hoffman, R. M. (2000). Visualizing gene expression by whole-body fluorescence imaging. *Proc. Natl. Acad. Sci. USA* **97:** 12278–12282.

[11] Kootstra, N. A., and Schuitemaker, H. (1999). Phenotype of HIV-1 lacking a functional nuclear localization signal in matrix protein of gag and vpr is comparable to wild-type HIV-1 in primary macrophages. *Virology* **253:** 170–180.

[12] Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., and Verma, I. M. (1998). Development of a self-inactivating lentivirus vector. *J. Virol.* **72:** 8150–8157.

[13] Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73:** 2886–2892.

[14] Kempermann, G., Kuhn, H. G., and Gage, F. H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature* **386:** 493–495.