Prolonged and Inducible Transgene Expression in the Liver Using Gutless Adenovirus: A Potential Therapy for Liver Cancer

LIN WANG,* RUBÉN HERNÁNDEZ-ALCOCEBA,* VIJAY SHANKAR,† MAIDER ZABALA,* STEFAN KOCHANÉK*,§ BRUNO SANGRO,* M. GABRIELA KRAMER,* JESUS PRIETO,* and CHENG QIAN*

*Division of Hepatology and Gene Therapy, Faculty of Medicine, FIMA, University of Navarra, Pamplona, Spain; †Center for Molecular Medicine, University of Cologne, Cologne, Germany; and §Division of Gene Therapy, University of Ulm, Ulm, Germany

Background & Aims: Gene therapy of liver diseases would benefit from systems allowing prolonged, regulable, and tissue-specific transgene expression. We attempted to produce a vector fulfilling these requirements. **Methods:** We generated gutless adenoviral vectors containing a mifepristone (RU486)-inducible system for controlled and liver-specific expression of human interleukin-12 (hIL-12) (GL-Ad/RUHL-12) and mouse IL-12 (mIL-12) (GL-Ad/RUmIL-12). The properties of these vectors were tested both in vitro and in vivo. **Results:** Infection of cells with GL-Ad/RUHL-12 resulted in high level of hIL-12 expression in the presence of RU486 only in hepatocytic cells. In animals injected with GL-Ad/RUHL-12, the administration of RU486 induced a transient rise of serum hIL-12 that peaked at 10 hours and completely disappeared by 72 hours. The peak value of hIL-12 was dependent on the doses of the vector and the inducer. High and sustained serum levels of hIL-12 could be attained by continuing administration of RU486 every 12 or 24 hours. Repetitive induction of hIL-12 could be obtained over, at least, a period of 48 weeks after a single injection of GL-Ad/RUHL-12. Although the vector was detected in many tissues after systemic injection, transcription of the transgene was only found in the liver. Treatment of liver metastases with 5×10^8 infectious units of GL-Ad/RUmIL-12 plus RU486 resulted in complete tumor regression in all animals. **Conclusion:** Gutless adenoviral vectors allow liver-specific and regulable transgene expression for prolonged periods of time. These vectors are promising tools for gene therapy of liver cancer and could also be useful for other forms of hepatic disease.

Gene therapy is a procedure with great therapeutic potential for a diversity of liver disorders including inherited metabolic conditions (phenylketonuria, tyrosinemia, hemophilia A and B) and acquired diseases (chronic infections, metabolic disorders, primary and metastatic liver tumors).^{1–6} For many of these applications, vectors allowing long-term expression of the transgene would be required. Adeno-associated viruses, lenti-

viral vectors and gutless adenoviral vectors may be used for this purpose.^{7–9}

First generation adenoviral vectors are devoid of E1 and E3 viral genes.^{10–12} Cells transduced by this type of vectors express the transgene but also viral proteins.^{12,13} The highly immunogenic adenoviral antigens stimulate immune rejection of the transduced cells, which results in short duration of transgene expression.^{12,13} In contrast, gutless adenoviruses lack all viral coding regions and consequently exhibit low toxicity and immunogenicity, thus permitting prolonged expression of the transgene.^{11,14–16} In addition, the elimination of all the adenoviral genes leaves room to allocate large expression cassettes, and therefore gutless adenoviruses are also designed as high-capacity adenoviral vectors.^{11,14–16}

For correction of metabolic and/or hereditary diseases, different authors have used gutless vectors with nonregulable promoters showing long-term expression of transgenes such as α 1-antitrypsin,^{17,18} leptin,¹⁹ apolipoprotein E,²⁰ and factor VIII and IX^{21–24} at therapeutic levels without apparent hepatic toxicity.

For gene therapy of primary or metastatic liver cancer, several phase I/II clinical trials have shown very limited efficacy of procedures based on first-generation adenoviruses carrying the tumor-suppressor gene p53 or suicide genes such as thymidine kinase of herpes simplex virus (HSV-tk).^{25,26} Therapy of established tumors with first generation adenoviruses encoding interleukin-12 (IL-12) has been very effective in different animal models of transplanted tumors.^{27–29} However, although in our phase I/II study, the intratumor administration of first-generation adenovirus encoding human IL-12 (hIL-12) has been well tolerated, the antitumor effects were quite

Abbreviations used in this paper: ALT, alanine aminotransferase; HPRT, hypoxanthine phosphoribosyltransferase; PCR, polymerase chain reaction; TTR, transthyretin.

© 2004 by the American Gastroenterological Association

0016-5085/04/\$30.00

doi:10.1053/j.gastro.2003.10.075

weak.³⁰ This lack of efficacy likely depends on short duration of transgene expression and on the fact that the tumor tissue is not easily infected with adenoviral vectors. Thus, to be effective, it would be necessary to infect peritumoral tissue with long-term expression vectors encoding cytokines endowed with potent immunostimulant and antiangiogenic properties such as IL-12.^{31,32} However, these potent biological molecules are potentially toxic.^{33,34} Hence, when using long-term expression vectors for transfer of antitumor cytokines, it would be essential to strictly control their expression to reach therapeutic levels while avoiding unwanted side effects.

The ability to regulate gene expression by using small molecules as ligands has been explored previously.^{35–40} Four major systems have been developed, including regulation by the antibiotic tetracycline, the insect steroid ecdysone or its analogs, the antiprogesterin mifepristone (RU486), and chemical “dimerizers” such as the immunosuppressant rapamycin and its analogs.^{41–43} They all involve the drug-dependent recruitment of a transcriptional activation domain to a basal promoter driving the gene of interest but differ in the mechanism of recruitment.^{41–45} In the mifepristone system, drug-regulated transcription is achieved by fusing a heterologous DNA-binding domain of yeast GAL4 protein and activation domain of VP-16 or NF- κ B p65 proteins to a mutant human progesterone receptor that is unaffected by endogenous hormones but is activated by synthetic antiprogesterins at doses sufficiently low to avoid side-effects in human.^{45,46} The properties of the mifepristone-regulated system have been investigated in transgenic animals and naked DNA plasmid in muscles.^{47–50} Burcin et al.⁴⁵ have incorporated this system into a gutless adenovirus with a human growth hormone target gene. They showed that background transcription was undetectable in vitro and in vivo and that over 50 days’ human growth hormone production could be cycled on and off 3 times or maintained at steady state levels by delivery of the inducer.

In the present study, we generated a gutless adenovirus vector encoding hIL-12 with a liver-specific mifepristone-regulable system and characterized the tissue distribution of the vector and the transgene expression as well as the kinetics of the induction with respect to the dose of the vector, the dose of the inducer, and the periodicity of the induction. We show that this vector enables controlled hIL-12 expression selectively in the liver for more than 48 weeks. In addition, we produced a gutless adenoviral vector encoding murine IL-12 (mIL-12) and we provide evidence that the expression of mIL-12 using this system achieves complete tumor regression in an aggressive model of liver metastases in mice.

Materials and Methods

Cell Lines and Animals

The human cell lines 293, HepG2, PLC/PRF/5, A549, and Hela were obtained from American Type Culture Collection (Rockville, MD). 293, PLC/PRF/5, and Hela cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). HepG2, A549, and MC-38 were grown in RPMI 1640 supplemented with 10% FBS.

C57BL/6J mice, 5 to 8 weeks old, were purchased from Harlan (Barcelona, Spain). The animals were kept under standard pathogen-free conditions and received care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” by the National Academy of Sciences. The experiments were performed in accordance with the local animal commission.

Construction of Liver-Specific Inducible Expression

The pRS21 was constructed as follows: an *Asc I*/*Pac I* fragment containing the TTR promoter and enhancer and the transactivator GLp65 and SV40 polyA from pSTK 119 TA End-TTRB Glp65 SV was cloned into pPAP CMV GLp65 to generate pRS14. The pRS15 plasmid was generated by inserting an oligonucleotide containing *Cla I* and *Swa I* sites to replace the *Pme I*/*Hind III* fragment of the pGene/V5-His (Invitrogen, Carlsbad, CA). pRS16 was generated by the insertion of *Sal I*/*Pvu II* fragment of pRS15 into the *Sal I*/*Sma I* site of plasmid pPNA D *Sal I*/*Kpn I*-CAT. The *Pme I*/*Asc I* fragment containing 17mer GAL4 binding site, unique *Swa I* and *Cla I* cloning sites, and BGH PolyA signal from pRS16 was inserted into the *Pme I*/*Asc I* sites of plasmid pRS14 to form pRS17. The hIL-12 complementary DNA was excised by digesting pBS/hIL-12 with *Xba I* and *Spe I* and was subcloned into the *Swa I* site of pRS17 to generate pRS21. pRS24 was generated by subcloning a *Not I* fragment from pRS21 containing 2 expression cassettes into the *Eclx I* site of the adenovirus vector plasmid pSTK119. The mIL-12 complementary DNA was obtained from pBS/mIL-12 by digestion with *Xba I* and *Spe I* and was subcloned into *Swa I* site of pRS17 to obtain pRS22. pRS25 was generated by subcloning a *Not I* fragment from pRS22 containing 2 expression cassettes into the *Eclx I* site of the adenovirus vector plasmid pSTK119 (GL-Ad/RUmIL-12).

Rescue of Gutless Adenoviral Vectors

After *Pme I* digestion, phenol/chloroform extraction, and ethanol precipitation, 2 μ g of pRS24 DNA (for GL-Ad/RUmIL-12 production) or pRS25 DNA (for GL-Ad/RUmIL-12 production) were transfected into 293 cell-based cre66 cells, which were coinfecting with the loxP helper virus AdLC8cluc. Subsequent amplification steps and large-scale preparations were performed as described previously.¹⁷ All vector preparations were purified twice by CsCl equilibrium density centrifugation. The DNAs from purified vectors were

analyzed by restriction digest showing no rearrangements. Infectious titers and helper virus contamination were evaluated by using the slot-blot method.⁵¹ The ratio of total viral particles to infectious unit (iu) was 20:1. The helper virus particle contamination was about 0.5%–1%.

hIL-12 Expression in Cells Infected With GL-Ad/RUHL-12 In Vitro

HepG2, PLC/PRF/5, A549, and Hela cells were cultured on 24-well plates for 24 hours before infection. The cells were infected with GL-Ad/RUHL-12 at multiplicity of infection (moi) of 100 or with first generation adenoviral vector-expressing hIL-12 (AdCMVhIL-12) at same moi in medium containing 2% FBS for 3 hours at 37°C. The vector-containing medium was replaced with culture media containing 10^{-8} mol/L of RU486. The supernatants were collected after 48 hours. The hIL-12 protein level (p70) was measured by ELISA kit (Pharmingen, San Diego, CA).

Animal Studies by GL-Ad/RUHL-12

GL-Ad/RUHL-12 was administered into C57BL/6J mice by tail vein injection at doses of 3×10^9 or 10^9 iu per animal. The vector was diluted in physiological saline in a total volume of 100 μ L for injection. Single induction was made by intraperitoneal injection of RU486 at 250 μ g/kg, and serum was collected at different time points after induction. RU486 was dissolved in sesame oil (Sigma, St. Louis, MO). As a control, the same volume of sesame oil was injected into animals. The continued induction was carried out by injection of RU486 at a dose of 250 μ g/kg either every 12 hours, 24 hours, or 48 hours. Serum was collected daily. Serum hIL-12 level was measured by using ELISA kit (Pharmingen, San Diego, CA).

Antitumor Effect

Liver metastases were induced by injection of 1×10^6 MC-38 mouse colon cancer cells into the left liver lobe of C57BL/6J syngenic mice, resuspended in 50 μ L saline. Seven days before implantation of tumor cells, mice received GL-Ad/RUHL-12 (5×10^8 iu or 10^8 iu per animal) by intravenous injection or saline as a control. A single tumor nodule (about 8 mm in diameter) was observed in livers 5 days after inoculation of the malignant cells. All groups of animals had a similar tumor size, indicating that, in the absence of induction, the administration of the vector before implantation of tumor cells had no effect on tumor establishment and growth. At this time point, induction of IL-12 expression was initiated by intraperitoneal injection of RU486 at a dose of 250 μ g/kg. Ten daily injections were administered. Blood samples were collected 10 hours after the first RU486 dose for determination of IL-12 levels. Five days after the completion of the induction regime, mice were anesthetized and underwent laparotomy to assess progression of the disease. Tumor size was measured in 2 perpendicular diameters by using a caliper, and tumor volume was calculated by using the formula:

$$V = \text{length} \times \text{width}^2 \times 0.5.$$

Survival was checked daily and animals were killed if moribund.

Determination of Serum Levels of mIL-12 and Alanine Aminotransaminase

Blood samples were collected from the retro-orbital plexus of mice. The mIL-12 protein level (p70) was measured by ELISA kit (Pharmingen). The levels of mIL-12 obtained with this manufacturer's kit are consistently higher than those obtained with another commercially available kit from Endogen (Rockford, IL). Because the same Pharmingen's kit for mIL-12 was used in all our studies with GL-Ad/RUHL-12, this difference does not affect the interpretation of our data.

Alanine aminotransferase (ALT) levels were determined by using a Boehringer Mannheim Hitachi 911 Automatic Analyzer (Mannheim, Germany) at day 1 and 7 of RU486 induction. In a group of animals, ALT values were also analyzed at day 45 after induction.

Histological Studies

Additional groups of tumor-bearing mice receiving GL-Ad/RUHL-12 with and without induction with RU486 and control animals receiving RU486 alone ($n = 4$ per group) were killed at day 4 after initiation of induction to collect liver samples. In addition, we obtained liver specimens from mice treated with vector plus RU486 induction 35 days after the end of the complete 10-day induction period. These animals had rejected metastases and were free of tumor. Livers were fixed in formalin and embedded in paraffin. Three-micrometer sections were stained with H&E and examined for histopathological changes.

Isolation of DNA and RNA

The mice receiving GL-Ad/RUHL-12 were killed at the indicated time together with naive C57BL/6 mice. Liver, lung, spleen, and kidney were obtained. Total DNA and RNA from these tissues were isolated with TRI Reagent (Sigma, St. Louis, MO) according to the instruction of manufacturer.

Polymerase Chain Reaction Analysis

For the detection of vector in tissues, we used polymerase chain reaction (PCR) with primers corresponding to the region of GLp65 transactivator. They included GLp65s: 5'-AGCCAGATCTGAAGCTAC-3'; GLp65as: 5'-TGCTTGATATCTCGTCA-3'. DNA, 0.5 μ g, from tissues was used for amplification in 25- μ L reaction volume including 6 pmol of each primer and 1 unit of Red Taq polymerase (Sigma). PCR amplification was carried out for 1 cycle of 5 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 48°C, and 1 minute at 72°C with a final extension of 5 minutes at 72°C. Fifteen microliters of amplification products were separated by electrophoresis on 2% agarose gel and visualized with ethidium bromide. The predicted size for GLp65 was 1103 bp.

Reverse-Transcription PCR Analysis

Sixteen micrograms of total RNA from each tissue was treated with 15 units of RNase-free DNase I (Roche) and 20 units of RNase inhibitor (Invitrogen) for 15 minutes at room temperature. After extraction with phenol/chloroform and precipitation with ethanol, RNA was redissolved in 20 μ L diethylpyrocarbonate (DEPC)-treated H₂O. One microgram of RNA was subjected to reverse transcription (RT) using 100 units of M-MLV Reverse Transcriptase (GIBCO-BRL, Barcelona, Spain) in 12 μ L volume for 60 minutes at 37°C and followed by 5 minutes at 95°C. After RT, 6 μ L of this mixture was used for PCR amplification in 50 μ L total volume containing 10 pmol of each primer as described before and 2 units of Red Taq DNA polymerase (Sigma). PCR conditions were the same as before. Fifteen microliters of PCR products were analyzed by electrophoresis on 2% of agarose gel and visualized with ethidium bromide. RT-PCR for mouse β -actin was used as control. The primers selected for β -actin were Act-s: 5'-ACTGCGCTTCTTGCCGC-3' and Act-as: 5'-CATGACGC-CCTGGTGTC-3'. PCR amplification was carried out in total 50 μ L volume with 6 μ L of RT mixture, 10 pmol of each primer, and 2 units of Red Taq DNA polymerase. The predicted size was 182 bp.

Statistical Analysis

When indicated, the statistical differences were calculated by using a nonparametric test (Mann-Whitney *U* test, 2-tailed) for unpaired samples. *P* values smaller than 0.05 were considered significant.

Results

Characterization of GL-Ad/RUHL-12 In Vitro

A gutless adenoviral vector carrying hIL-12 genes (GL-Ad/RUHL-12) was generated (Figure 1A). First, the vector was used to examine transgene induction in different cell lines after infection at moi of 100. We found no expression of hIL-12 in the absence of RU486 in any of the cell lines tested, whereas in hepatocytic cell lines HepG2 and PLC/PRF/5 the levels of hIL-12 in the presence of the inducer were similar to those obtained by using first-generation adenovirus with the strong constitutive cytomegalovirus (CMV) promoter (Figure 1B). Moreover, hIL-12 expression in liver-derived cells was dependent on RU486 in a dose-dependent manner from 10^{-10} mol/L to 10^{-7} mol/L (data not shown). In contrast, in nonhepatocytic cells A549 and Hela, transgene expression in the presence of mifepristone was absent or very poor (Figure 1B).

Kinetics and Dose Dependency of the Induction of hIL-12 In Vivo

To characterize the kinetics and dose dependence of transgene induction in vivo, mice received 3×10^9 or

Figure 1. (A) Construction of GL-Ad/RUHL-12 vector. pRS21 contained the inducible transactivator GLp65 inserted under control of the liver-specific TTR promoter and enhancer and human IL-12 (including p35 and p40 subunits linked by IRES) cloned into downstream of GAL4 DNA-binding site and E1b TATA box. The whole expression cassettes from pRS21 were cloned into *EclXI* site of AdSTK119 to generate GL-Ad/RUHL-12. (B) Characterization of GL-Ad/RUHL-12 in vitro. HepG2, PLC/PRF/5, A549, and Hela cells were infected with GL-Ad/RUHL-12 at moi of 100 or AdCMVhIL-12 at same moi for 3 hours. The media were removed, and the cells were incubated in the absence or presence of RU486 (10^{-8} mol/L) for 48 hours. The supernatant was collected for determination of hIL-12 by ELISA. The results are represented as mean $\pm$ standard error of the mean of 4 independent experiments.

1×10^9 iu of GL-Ad/RUHL-12 per animal. Two weeks after vector administration, animals were given a single injection of RU486 at 250 μ g/kg. As shown in Figure 2A and B, hIL-12 was detectable in serum at 4 hours after RU486 administration, peaked at 10 hours, and declined sharply to low values by 24 hours. After 72 hours, hIL-12 could not be detectable in serum. Transgene expression was not observed at baseline or in mice that received sesame oil without RU486. Peak values of serum hIL-12 were 12-fold higher in mice that were treated with 3×10^9 iu of GL-Ad/RUHL-12 than in those injected with 1×10^9 iu (Figure 2A and B). To determine whether the expression level of hIL-12 was related to the dose of inducer, 3 doses of RU486 (125, 250, and 500 μ g/kg) were administered to 2 groups of mice treated with 3×10^9 iu or 10^9 iu of GL-Ad/

Figure 2. (A, B) Kinetics of hIL-12 induction in vivo. C57BL/6J mice were treated by intravenous injection of GL-Ad/RUhIL-12 at 10^9 (A) or 3×10^9 iu (B) per animals. Two weeks later, induction was done by single intraperitoneal injection of RU486 at 250 $\mu\text{g}/\text{kg}$ or sesame oil as a control. The blood samples were taken before and at different time points after induction for determination of hIL-12 level. The results are represented as mean $\pm$ standard error of the mean of $n = 5$ for RU486 induction and $n = 3$ for sesame oil control. (C, D) Dose-dependent expression of hIL-12 in vivo. Mice receiving GL-Ad/RUhIL-12 vector at 10^9 (C) or 3×10^9 iu (D) per animal were subjected to induction with RU486 at different doses (125, 250, and 500 $\mu\text{g}/\text{kg}$). Ten hours after induction, serum was collected for measurement of hIL-12. The results are represented as mean $\pm$ SEM of 3 animals per group.

RUhIL-12 and serum hIL-12 was measured at 10 hours after induction. Figure 2C and D show that increasing the dose of RU486 caused higher induction of hIL-12 both in mice receiving the high and the low dose of the vector. Thus, the intensity of transgene expression can be modulated by changing the dose of the vector and/or the dose of the inducer.

Repetitive Induction of hIL-12 In Vivo

To characterize the effect of repeated inductions on the pattern of hIL-12 serum levels, mice injected with 3×10^9 iu of GL-Ad/RUhIL-12 were treated with 250 $\mu\text{g}/\text{kg}$ of RU486 every 12, 24, or 48 hours over 6 days (Figure 3A–C). We found that induction every 48 hours resulted in a saw-like pattern of hIL-12 expression with peak levels (about 6000 pg/mL) on the day of induction alternating with 6- to 8-fold lower values on the following day. In contrast, sustained levels of hIL-12 could be achieved by administering mifepristone every 12 or 24 hours (Figure 3B and C). In these 2 protocols, a moderate reduction in the induction of hIL-12 was observed after the second day of the study; this decrease was less noticeable when the induction was repeated every 24 hours. In a different group of mice, induction every 24 hours was maintained during 21 days. In this group of mice, the serum level of hIL-12 was kept constant (around

3000 pg/mL) until the ninth day of sustained induction and decreased moderately after this time point but still was above 1500 pg/mL on day 21 (Figure 3D).

Mice were also stimulated with RU486 at weeks 19, 28, 33, and 48 to determine the duration of the activity of GL-Ad/RUhIL-12. In all cases, hIL-12 was undetectable in serum before the injection of RU486 and increased to values around 2000 pg/mL after induction, indicating that the system remains operative for at least near 1 year. Figure 4 summarizes the hIL-12 values 10 hours after induction at different time points of the same group of animals during a period of 48 weeks. It can be seen that the level of transgene induction at week 28 is similar to that observed at week 2 but declined to about 43% of these values at week 48.

Distribution of GL-Ad/RUhIL-12 and Tissue-Specific Expression of Transactivator Glp65 In Vivo

Animals receiving GL-Ad/RUhIL-12 at 3×10^9 iu were killed at weeks 2, 28, and 48 after vector administration. Liver, lung, spleen, and kidney were obtained from these animals and 2 naive controls. In naive mice, no PCR signals of transactivator were found in any of the organs analyzed, whereas in animals injected with GL-Ad/RUhIL-12, the sequence of GLp65

Figure 3. Sustained induction of hIL-12 in vivo. (A) Animals receiving GL-Ad/RUHL-12 vector at 3×10^9 iu per animal were given RU486 at 250 $\mu\text{g}/\text{kg}$ for every 12 hours over 6 days at week 9 after vector administration. (B) The same animals were induced with RU486 at 250 $\mu\text{g}/\text{kg}$ for every 48 hours over 6 days at week 11 after vector administration. (C) The same animals were induced with RU486 at 250 $\mu\text{g}/\text{kg}$ every 24 hours over 6 days at week 14 after vector administration. Serum was collected daily for determination of hIL-12 level. Data are presented as mean and standard error of the mean of $n = 5$ for RU486 induction and $n = 3$ for sesame oil control. (D) Long-term and sustained induction of hIL-12 in vivo. Mice were infected with GL-Ad/RUHL-12 vector at 3×10^9 iu and were given RU486 at 250 $\mu\text{g}/\text{kg}$ every 24 hours over 21 days. Serum was collected daily for the first 6 days and once for every 3 days for the rest of time for determination of hIL-12 level. Data are presented as mean and standard error of the mean of $n = 4$ for RU486 induction and $n = 3$ for sesame oil control.

was found in all organs sampled being the signal more intense in the liver (Figure 5), indicating that the gutless vector can persist in all these tissues for a long period of time. By using RT-PCR to investigate the expression of GLp65, we observed that GLp65 messenger RNA could not be found in lung, spleen, or kidney but was detected specifically in the liver where it was present at all time points until week 48 after vector administration. These data indicate that the TTR promoter and enhancer that control GLp65 transcription allow transgene expression specifically in liver tissue.

Antitumor Efficacy of GL-Ad/RUmIL-12

Because hIL-12 has no biological effect on mouse immunocytes, we generated a gutless adenovirus expressing mIL-12 to evaluate its antitumor activity and potential toxicity in murine models. Similar to GL-Ad/RU-

Figure 4. Long-term and repetitive induction of hIL-12 in vivo. Mice receiving GL-Ad/RUHL-12 vector at 3×10^9 iu per animal were scheduled for repetitive inductions with RU486 at the indicated times. The serum hIL-12 level was measured 10 hours after each induction. The data are presented as mean and standard error of the mean of $n = 5$ for RU486 induction. The line joins each individual measurement.

Figure 5. Distribution of vector and tissue specific expression of transactivator GLp65 in vivo. Animals receiving GL-Ad/RU-hIL-12 at 3×10^9 iu were killed at weeks 2 ($n = 2$) and 28 ($n = 2$) after vector administration and one animal was killed at week 48. Liver, lung, spleen, and kidney were obtained from these 5 animals and 2 naive mice as controls. (A) Total DNA was extracted from those organs to determine distribution of viral vector by PCR. (B) Total RNA was extracted for determination of transcription of transactivator GLp65 by RT-PCR. (C) RT-PCR for amplification of housekeeping gene β -actin was used for internal control of RNA input.

hIL-12 vector, mIL-12 could be produced only in hepatocytic cells in the presence of RU486 after infection with GL-Ad/RUmIL-12 vector (data not shown). For the determination of antitumor activity, we established a liver metastatic tumor model by implantation of the mouse colon cancer cell line (MC-38) into liver of syngenic C57BL/6J mice. When liver tumor became about 8 mm in diameter (5 days after implantation of tumor cells), the tumor-bearing animals that had been injected intravenously with 10^8 ($n = 4$) or 5×10^8 ($n = 7$) iu of GL-Ad/RUmIL-12 were treated with RU486 at 250 μ g/kg daily for 10 consecutive days. Five days after the end of induction, tumor size was checked at laparotomy. As represented in Figure 6A, control animals receiving saline ($n = 8$) showed progressive tumor growth. The animals treated with high dose of vector but no RU486 ($n = 3$) also showed tumor progression. In contrast, all animals receiving high dose of vector and induction with RU486 experienced complete tumor regression. The animals treated with low vector dose and RU486 showed a clear reduction of the tumor mass as compared with controls, but the disease eventually progressed and they finally died as a consequence of massive liver metastases (Figure 6B). Figure 7 shows representative photographs of metastatic liver tumors before and after therapy in 2 animals that were injected with 5×10^8 iu of GL-Ad/RUmIL-12 vector. One of these animals (Figure 7A and C) was not induced with RU486, whereas the other (Figure 7B and D) received the inducer for 10 days. Before induction, the tumor mass was evident in both animals (in fact the one that received RU486 had at baseline a tumor of bigger size than the other mouse). As

shown in Figure 7, the mouse treated with RU486 was free of tumor when killed 35 days after the end of the induction period (histological examination confirmed the absence of tumor in the area that was previously inoculated with colon cancer cells). In contrast, the mouse that did not receive RU486 (Figure 7C) presented tumor progression and died 20 days after the end of the induction period with a tumor mass exceeding 20 mm in diameter, peritoneal dissemination, and lung metastases.

The strong antitumor effect of continuous induction of IL-12 within the liver using GL-Ad/RUmIL-12 was confirmed by the fact that this therapy resulted in disease-free, long-term survival in 90% of treated mice (Figure 6B). Tumor eradication was associated with increased serum mIL-12 levels. As shown in Figure 6C, no mIL-12 could be detected in animals injected with saline or in animals receiving high dose of vector without induction. In contrast high levels of mIL-12 up to 8000 ng/mL were found in sera from animals receiving the high vector dose (5×10^8) and induction with RU486. The lowest mIL-12 value obtained with this viral dose, that still showed antitumor efficacy, was 170 ng/mL. However, mice treated with the low vector dose (10^8) produced mIL-12 around 1–2.5 ng/mL after induction. These data indicate that the efficacy of treatment is related to the level of mIL-12 induced by RU486.

Toxicity of GL-Ad/RUmIL-12 Plus Induction With RU486

To evaluate the toxicity of the therapy with GL-Ad/RUmIL-12 plus RU486 in animals with liver tumors, we analyzed the level of ALT in treated and control

mice at day 1 and 7 of the induction with RU486. As shown in Figure 8A, mice treated with GL-Ad/RUmIL-12 but no RU486 showed ALT values similar to control animals receiving saline. However, animals treated with GL-Ad/RUmIL-12 and RU486 exhibited significantly increased serum ALT values at day 1 and even higher values at day 7. In these animals, which were able to eliminate the liver metastasis, ALT levels were normal at day 35 after completion of induction. Liver samples obtained at day 4 of RU486 induction from mice treated with high vector dose showed an inflam-

matory infiltration mainly localized around the central vein (Figure 8D). In contrast, normal liver histology was observed at the same day of the study in animals that received only RU486 (Figure 8B) or GL-Ad/RUmIL-12 without RU486 induction (Figure 8C). In mice treated by GL-Ad/RUmIL-12 plus RU486, the liver was free of tumor and showed no histological changes when examined at day 35 after the end of RU486 administration (Figure 8E). These data indicate that gutless adenoviral vector alone in the absence of RU486 induction does not cause liver toxicity and that vector plus RU486 administration causes an inflammatory liver reaction that is of moderate intensity at the doses of vector and inducer used in this work and that subsides on cessation of IL-12 induction.

Discussion

Gene therapy offers considerable promise to treat a great diversity of conditions involving the liver, including metabolic, infectious, and neoplastic diseases. In many cases, sustained expression of the transgene for long periods of time is required. Long-term expression can be achieved by using vectors with the ability to integrate into the host genome such as retroviruses or adeno-associated viruses. Problems with these vectors include low transduction efficiency and the risk of insertional mutagenesis. Adenoviruses have the advantage of possessing marked hepatotropism, high transduction efficiency, and persistence in an episomal form.¹⁰⁻¹² However, expression of adenoviral proteins induce a strong humoral and cellular immune response that, on the one hand, limits the duration of transgene expression and, on the other hand, prevents successful readministration of the vector.^{12,13}

Figure 6. Antitumor effect of GL-Ad/RUmIL-12. Liver metastases of colon carcinoma were established in syngenic C57BL/6J mice by intrahepatic injection of 10⁶ MC-38 cells. One week before cell implantation, the GL-Ad/RUmIL-12 vector or saline was injected intravenously at 10⁸ or 5 × 10⁸ iu per animal. Five days after cell implantation some of the mice injected with the vector received daily intraperitoneal injections of 250 µg/kg of the inducer mifepristone (RU486) for a total of 10 days, and others were left untreated. Five days after the last dose of RU486, mice underwent laparotomy to evaluate tumor progression. (A) Tumor size of control animals that did not receive GL-Ad/RUmIL-12 (black dots, n = 8), mice injected with 5 × 10⁸ iu of the vector without RU486 (empty circles, n = 3), mice injected with 10⁸ iu of the vector plus RU486 (black squares, n = 4), and mice treated with the vector at 5 × 10⁸ iu plus RU486 (black triangles, n = 7). (B) Survival of these groups of mice. The percentage of surviving mice over time is represented. The day 0 corresponds to the inoculation of the tumor cells. The survival of the control group is represented with a discontinuous line. (C) IL-12 levels in the serum of the same animals, measured 10 hours after the first administration of RU486. The symbols of panels B and C are the same as panel A.

Gutless adenoviruses have been developed to circumvent these problems. They are devoid of all adenoviral genes (2 inverted terminal repeats and packaging signals are the only conserved sequences), and therefore transduced cells do not express any adenoviral product and do not elicit cellular immune response against the vector, thus permitting prolonged transgene expression.^{14,15} Moreover, the persistence in the cells for a long period of time in an episomal form adds to the safety of this type of vector.^{14,15} Additionally, the removal of all adenoviral genes leaves a large space to allocate in the vector genes or combination of genes with complex regulatory sequences.^{14,15}

For many gene therapy applications, it is necessary to use long-term expression vectors encoding molecules capable of inducing powerful biological effects but also having the risk of significant potential toxicity. The clinical use of such therapeutic genes makes mandatory using regulatory systems, allowing a strict control of transgene expression. IL-12 is a potent cytokine with robust antitumor effects.^{27–29,32} The systemic administration of the recombinant protein to treat cancer has found the limitation of toxicity due to the ability of IL-12 to strongly induce interferon gamma production.^{33,34} Adenovirus-mediated gene transfer of IL-12 to the tumor or peritumoral tissue causes a gradient of IL-12 concentration with higher values at the site of the neoplasm and lower systemic levels, resulting in increasing antitumor efficacy and wider therapeutic window.^{28–30} For treatment of primary and metastatic liver cancer, sustained expression of IL-12 within the liver is a very attractive option because of the potent immunostimulant and antiangiogenic properties of this cytokine.^{28–32} However, the clinical implementation of this promising therapy implies full characterization of the kinetics of the regulatory system selected to control IL-12 expression.

In this study, we incorporated the mifepristone (RU486) regulatory system into a gutless adenoviral vector to mediate long-lasting, regulable and liver-specific expression of IL-12. The system is based on two expression cassettes: one encoding a mifepristone-inducible transactivator under the control of a liver-specific promoter (transthyretin) and the other encoding IL-12 under the control of a minimal promoter, which is operative only in the presence of the active transactivator. Our *in vitro* data show that the expression of hIL-12 occurred only in hepatocytic cells in a dose-dependent manner. In the absence of RU486, leakage was minimal or absent and the expression level mediated by GL-Ad/RUhIL-12 in the presence of mifepristone was similar to that induced by first generation adenovirus carrying

hIL-12 driven by CMV promoter. Our *in vivo* studies in mice that received GL-Ad/RUhIL-12 at 10^9 or 3×10^9 iu by intravenous injection showed that although the vector preferentially infected the liver, it was also found in other tissues including lungs, kidneys, and spleen. Importantly, however, the expression of the transgene took place selectively in the liver, a feature of the system that may focus the therapeutic effect of hIL-12 in the diseased liver and that may contribute to the safety of the therapy. Our *in vivo* data also showed no leakage of hIL-12 in the absence of mifepristone, but a potent induction of the cytokine occurred after administration of this compound with a wave of serum hIL-12 that lasted 24 hours. The absolute levels achieved depend on both the dose of the virus and the dose of the inducer. Because in the clinical setting stable and prolonged expression of hIL-12 at a desirable level should be required, we investigated different induction protocols to determine which would be suitable to achieve constant serum concentration of the cytokine. We found that inducing every 12 hours or every 24 hours resulted in maintained serum levels of hIL-12. In both cases, the concentration of the cytokine was higher the first 2 days and reached a plateau at a value 40%–60% of the initial level after the third day (Figure 3). Based on these data, we selected induction every 24 hours to analyze whether prolonged administration of the inducer could maintain its efficacy at stimulating hIL-12 expression. In experiments of daily RU486 administration for 21 days, we found that after the initial peak there was a plateau until day 9 and then serum levels decreased slowly to values 50% of those of the plateau by day 21. Interruption of the induction for 2 days permitted a restoration of the expression levels to those of the plateau upon reintroduction of RU486 (data not shown). These findings provide insight into the functioning of the mifepristone-based regulatory system and are relevant for appropriate regulation of transgene expression in clinical trials.

An important point is to know for how long this therapy could be applied after a single administration of the adenovirus. This information is of interest because of the fact that antiadenovirus antibodies generated after the first dose of the vector may block cell transduction after a second dose. In the present study, we found that in the normal liver GL-Ad/RUhIL-12 persists for more than 48 weeks and that the regulatory system remains functional during all this time. Interestingly, the induction levels attained are similar at 2 weeks and at week 28, and after this time point there is a slow decrease in the efficacy of the induction consistent with the half-life of transduced hepatocytes. Thus, as shown in Figure 4, about a year after the injection of GL-Ad/

Figure 7. Photographs of representative tumors in mice treated with GL-Ad/RUmlL-12. Liver metastases of colon carcinoma were established in syngenic C57BL/6J mice by intrahepatic injection of 10^6 MC-38 cells and one week before cell implantation GL-Ad/RUmlL-12 (5×10^8 iu per animal) were injected intravenously. *A* and *B* corresponds to livers of 2 mice 5 days after cell implantation. The margins of the tumor mass are indicated with white arrows. From this moment, the mouse shown in *panel A* was left untreated, and the one in *panel B* received daily intraperitoneal injections of 250 μ g/kg of the inducer mifepristone (RU486) for a total of 10 days. *C* shows tumor progression in the untreated animal that died 20 days after end of induction period showing at necropsy massive tumor (indicated as *T*) that occupied most of the liver (indicated as *L*), peritoneal dissemination (white asterisk), and lung metastases (black asterisk). *D* shows tumor eradication in the mouse treated with the vector plus RU486 and killed 35 days after the end of therapy; the liver appeared normal and no evidence of tumor was found in any other organ.

A

Figure 8. Toxicity of GL-Ad/RUmlL-12. C57BL/6J mice were injected intravenously with 5×10^8 iu of GL-Ad/RUmlL-12. One week later, liver metastases were induced by inoculation 10^6 MC-38 cells. Five days later, animals received daily intraperitoneal injections of 250 μ g/kg RU486 for 10 days, whereas others were not treated with the inducer. (A) ALT levels in serum at day 1 and day 7 of induction with RU486 (5 mice) and in mice ($n = 3$) treated with the vector without RU486 induction. The empty bars show the values of control animals that did not receive the GL-Ad/RUmlL-12 vector ($n = 5$). The right black column corresponds to 2 of the animals treated with vector plus RU486 35 days after the end of the therapy. * $P < 0.05$ versus vector without induction; ** $P < 0.05$ versus controls and vector without induction. (B) Microphotographs of liver sections stained with H&E corresponding to a mouse treated with RU486 alone, (C) liver section from a mouse treated with high vector dose without RU486 induction, and (D) liver section from mouse that received treatment with high vector dose vector plus RU486 at day 4 of induction. The inflammatory infiltrate surrounding the central vein is indicated with black point arrows. (E) Liver histology of a mouse that was treated with vector plus 10 daily injections of RU486 and was killed 35 days after the end of therapy. Original magnification of *B* to *E* is $\times 200$.

RUhIL-12 the serum concentration of IL-12 obtained is about 45% of that found in the second week. Pastore et al.⁵² showed that the use of a liver-specific promoter in adenoviral vectors prevented the generation of antibodies against the transgene, an eventuality that may occur when a ubiquitous promoter is used. Thus, the liver-specific promoter in our vector might have been helpful not only in focusing the therapeutic properties of hIL-12 to the liver but also in avoiding host immune responses to transgenic proteins and in prolonging transgene expression *in vivo*.

It may be possible that in the normal human liver and in the normal hepatic tissue surrounding metastatic tumor nodules the duration of transgene expression may be similar to that found in our study in mice. It seems also possible that in livers with chronic inflammation the duration of transgene expression may be shortened because of a shorter half-life of hepatocytes. It should be noted, however, that the hIL-12 serum concentration obtained on induction after 48 weeks is much higher than the values that should be sought in clinical trials, indicating that the procedure is robust enough to ensure appropriate levels of transgene expression during a considerable period of time.

To evaluate the effectiveness of this therapy on liver tumors, we have treated established metastatic liver tumors in an experimental animal model. Our data indicate that *in vivo* gene therapy with GL-Ad/RUmIL-12 at 5×10^8 iu per animal with RU486 induction resulted in complete tumor regression in all treated animals with long-term tumor-free survival. Toxicological studies showed that the gutless vector without RU486 induction does not cause any apparent toxicity. However, induction of IL-12 production within the liver at the level attained in this work is associated with an inflammatory reaction of moderate intensity characterized by pericentral mononuclear cell infiltration and ALT elevation, which is reversible and subsides on cessation of IL-12 induction. It is possible that higher doses of the vector and/or inducer might cause severe inflammation. Because most of the toxicity of IL-12 is caused by interferon- γ production, the use of this therapeutic strategy in patients with liver cancer should be performed with close monitoring of IFN- γ levels to allow reduction of the RU486 dose when this cytokine reaches potentially toxic values. The possibility of controlling toxicity by modifying or interrupting the dose of the inducer is one of the attractive features of this system.

In conclusion, our data indicate that the vector and regulatory system used in the present work are capable of mediating high-level, long-term, tissue-specific, and regulable transgene expression in the liver, offering a promising tool to treat liver conditions necessitating effective therapies.

References

- Shetty K, Wu GY, Wu CH. Gene therapy of hepatic diseases: prospects for the new millennium. *Gut* 2000;46:136–139.
- Ghosh SS, Takahashi M, Thummala NR, Parashar B, Chowdhury NR, Chowdhury JR. Liver-directed gene therapy: promises, problems and prospects at the turn of the century. *J Hepatol* 2000;32(1 Suppl):238–252.
- Qian C, Drozdik M, Caselmann WH, Prieto J. The potential of gene therapy in the treatment of hepatocellular carcinoma. *J Hepatol* 2000;32:344–351.
- Mohr L, Geissler M, Blum HE. Gene therapy for malignant liver disease. *Expert Opin Biol Ther* 2002;2:163–175.
- Schmitz V, Qian C, Ruiz J, Sangro B, Melero I, Mazzolini G, Narvaiza I, Prieto J. Gene therapy for liver diseases: recent strategies for treatment of viral hepatitis. *Gut* 2002;50:130–135.
- Sangro B, Qian C, Schmitz V, Prieto J. Gene therapy of hepatocellular carcinoma and gastrointestinal tumors. *Ann N Y Acad Sci* 2002;963:6–12.
- Ferry N, Heard JM. Liver-directed gene transfer vectors. *Hum Gene Ther* 1998;9:1975–1981.
- Galimi F, Verma IM. Opportunities for the use of lentiviral vectors in human gene therapy. *Curr Top Microbiol Immunol* 2002;261:245–254.
- Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* 2001;7:33–40.
- Graham FL. Adenovirus vectors for high-efficiency gene transfer into mammalian cells. *Immunol Today* 2000;21:426–428.
- Benihoud K, Yeh P, Perricaudet M. Adenovirus vectors for gene delivery. *Curr Opin Biotechnol* 1999;10:440–447.
- Wilson JM. Adenovirus-mediated gene transfer to liver. *Adv Drug Deliv Rev* 2001;46:205–209.
- Yang Y, Jooss KU, Su Q, Ertl HC, Wilson JM. Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes *in vivo*. *Gene Ther* 1996;3:137–144.
- Kochanek S. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum Gene Ther* 1999;10:2451–2459.
- Morsy MA, Caskey CT. Expanded-capacity adenoviral vectors—the helper-dependent vectors. *Mol Med Today* 1999;5:18–24.
- Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 1996;93:13565–13570.
- Morral N, Parks RJ, Zhou H, Langston C, Schiedner G, Quinones J, Graham FL, Kochanek S, Beaudet AL. High doses of a helper-dependent adenoviral vector yield supraphysiological levels of alpha1-antitrypsin with negligible toxicity. *Hum Gene Ther* 1998;9:2709–2716.
- Morral N, O'Neal W, Rice K, Leland M, Kaplan J, Piedra PA, Zhou H, Parks RJ, Velji R, Aguilar-Cordova E, Wadsworth S, Graham FL, Kochanek S, Carey KD, Beaudet AL. Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc Natl Acad Sci U S A* 1999;96:12816–12821.
- Morsy MA, Gu M, Motzel S, Zhao J, Lin J, Su Q, Allen H, Franklin L, Parks RJ, Graham FL, Kochanek S, Bett AJ, Caskey CT. An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. *Proc Natl Acad Sci U S A* 1998;95:7866–7871.
- Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci U S A* 2001;98:13282–13287.
- Chuah MK, Schiedner G, Thorrez L, Brown B, Johnston M, Gillijns V, Hertel S, Van Rooijen N, Lillcrap D, Collen D, Vanden-

- Driessche T, Kochanek S. Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. *Blood* 2003;101:1734–1743.
22. Balague C, Zhou J, Dai Y, Alemany R, Josephs SF, Andreason G, Hariharan M, Sethi E, Prokopenko E, Jan HY, Lou YC, Hubert-Leslie D, Ruiz L, Zhang WW. Sustained high-level expression of full-length human factor VIII and restoration of clotting activity in hemophilic mice using a minimal adenovirus vector. *Blood* 2000;95:820–828.
 23. Reddy PS, Sakhuja K, Ganesh S, Yang L, Kayda D, Brann T, Pattison S, Golightly D, Idamakanti N, Pinkstaff A, Kaloss M, Barjot C, Chamberlain JS, Kaleko M, Connelly S. Sustained human factor VIII expression in hemophilia A mice following systemic delivery of a gutless adenoviral vector. *Mol Ther* 2002;5:63–73.
 24. Ehrhardt A, Kay MA. A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. *Blood* 2002;99:3923–3930.
 25. Ruiz J, Mazzolini G, Sangro B, Qian C, Prieto J. Gene therapy of hepatocellular carcinoma. *Dig Dis* 2001;19:324–332.
 26. Sung MW, Yeh HC, Thung SN, Schwartz ME, Mandeli JP, Chen SH, Woo SL. Intratumoral adenovirus-mediated suicide gene transfer for hepatic metastases from colorectal adenocarcinoma: results of a phase I clinical trial. *Mol Ther* 2001;4:182–191.
 27. Caruso M, Pham-Nguyen K, Kwong YL, Xu B, Kosai KI, Finegold M, Woo SL, Chen SH. Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc Natl Acad Sci U S A* 1996;93:11302–11306.
 28. Mazzolini G, Qian C, Xie X, Sun Y, Lasarte J, Drozdik M, Prieto J. Tumor regression and long-term protection for colon cancer after intratumoral injection of adenovirus expressing IL-12. *Cancer Gene Ther* 1999;5:514–522.
 29. Barajas M, Mazzolini G, Genové G, Bilbao R, Narvaiza I, Schmitz V, Sangro B, Melero I, Qian C, Prieto J. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. *Hepatology* 2001;33:52–61.
 30. Sangro B, Mazzolini G, Ruiz J, Herraiz M, Quiroga J, Herrero I, Benito A, Larrache J, Pueyo J, Subtil J, Olage C, Sola J, Sadaba B, Lacasa C, Melero I, Qian C, Prieto J. A phase I clinical trial of intratumoral injection of an adenovirus encoding interleukin 12 for advanced digestive tumors. *J Clin Oncol* 2004; (In press).
 31. Hendrzak JA, Brunda MJ. Interleukin 12: biological activity, therapeutic utility, and role in disease. *Lab Invest* 1995;72:619–637.
 32. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol* 1998;70:83–243.
 33. Orange JS, Salazar-Mather TP, Opal SM, Spencer RL, Miller AH, McEwen BS, Biron CA. Mechanisms of interleukin 12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids. *J Exp Med* 1995;181:901–914.
 34. Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, Sosman JA, Dutcher JP, Vogelzang NJ, Ryan JL. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 1997;90:2541–2548.
 35. Harvey DM, Caskey CT. Inducible control of gene expression: prospects for gene therapy. *Curr Opin Chem Biol* 1998;2:512–518.
 36. Rossi FM, Blau HM. Recent advances in inducible gene expression systems. *Curr Opin Biotechnol* 1998;9:451–456.
 37. Miller N, Whelan J. Progress in transcriptionally targeted and regulatable vectors for genetic therapy. *Hum Gene Ther* 1997;8:803–815.
 38. Clackson T. Regulated gene expression systems. *Gene Ther* 2000;7:120–125.
 39. Agha-Mohammadi S, Lotze MT. Regulatable systems: applications in gene therapy and replicating viruses. *J Clin Invest* 2000;105:1177–1183.
 40. Ye X, Schillinger K, Burcin MM, Tsai SY, O'Malley BW. Ligand-inducible transgene regulation for gene therapy. *Methods Enzymol* 2002;346:551–561.
 41. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995;268:1766–1769.
 42. Rossi FM, Guicherit OM, Spicher A, Kringstein AM, Fatyol K, Blakely BT, Blau HM. Tetracycline-regulatable factors with distinct dimerization domains allow reversible growth inhibition by p16. *Nat Genet* 1998;20:389–393.
 43. Hoppe UC, Marban E, Johns DC. Adenovirus-mediated inducible gene expression in vivo by a hybrid ecdysone receptor. *Mol Ther* 2000;1:159–164.
 44. Ye X, Rivera VM, Zoltick P, Cerasoli F Jr, Schnell MA, Gao G, Hughes JV, Gilman M, Wilson JM. Regulated delivery of therapeutic proteins after in vivo somatic cell gene transfer. *Science* 1999;283:88–91.
 45. Wang Y, O'Malley BW Jr, Tsai SY, O'Malley BW. A regulatory system for use in gene transfer. *Proc Natl Acad Sci U S A* 1994;91:8180–8184.
 46. Burcin MM, Schiedner G, Kochanek S, Tsai SY, O'Malley BW. Adenovirus-mediated regulatable target gene expression in vivo. *Proc Natl Acad Sci U S A* 1999;96:355–360.
 47. Wang Y, DeMayo FJ, Tsai SY, O'Malley BW. Ligand-inducible and liver-specific target gene expression in transgenic mice. *Nat Biotechnol* 1997;15:239–243.
 48. Wang XJ, Liefer KM, Tsai S, O'Malley BW, Roop DR. Development of gene-switch transgenic mice that inducibly express transforming growth factor beta1 in the epidermis. *Proc Natl Acad Sci U S A* 1999;96:8483–8488.
 49. Abruzzese RV, Godin D, Burcin M, Mehta V, French M, Li Y, O'Malley BW, Nordstrom JL. Ligand-dependent regulation of plasmid-based transgene expression in vivo. *Hum Gene Ther* 1999;10:1499–1507.
 50. Abruzzese RV, Godin D, Mehta V, Perrard JL, French M, Nelson W, Howell G, Coleman M, O'Malley BW, Nordstrom JL. Ligand-dependent regulation of vascular endothelial growth factor and erythropoietin expression by a plasmid-based autoinducible Gene-Switch system. *Mol Ther* 2000;2:276–287.
 51. Kreppel F, Biermann V, Kochanek S, Schiedner G. A DNA-based method to assay total and infectious particle contents and helper virus contamination in high-capacity adenoviral vector preparations. *Hum Gene Ther* 2002;13:1151–1156.
 52. Pastore L, Morral N, Zhou H, Garcia R, Parks RJ, Kochanek S, Graham FL, Lee B, Beaudet AL. Use of a liver-specific promoter reduces immune response to the transgene in adenoviral vectors. *Hum Gene Ther* 1999;10:1773–1781.

Received May 16, 2002. Accepted October 9, 2003.

Address requests for reprints to: Cheng Qian, M.D., or Jesus Prieto, M.D., Division of Hepatology and Gene Therapy, Department of Medicine, Medical School, University of Navarra, 31080 Pamplona, Spain. e-mail: cqian@unav.es; j.prieto@unav.es; fax: (34) 948-425649.

Supported in part by grants QLK3-1999-00364 from EU and SAF 2002-0327 of the Ministry of Science and Technology (Spain), grant Instituto Carlos III C03/02 (Spain), and by grant FKZ 01KS9502 from German Federal Ministry of Education and Research and the Center of Molecular Medicine Cologne (ZMMK) and Sander foundation.

The authors thank Dr. Matilde Bustos for histological examination, Helena Villanueva, Celia Gomar, Pilar Alzuguren, Paula Garces, and Africa Vales for their expert assistance, and Frank Graham for providing AdLC8luc.

L.W. and R.H. contributed equally to this work.

Dr. Wang's present address is Department of Pathology, School of Medicine, Ji Lin University, China.