Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice

Ning Zhu, Denny Liggitt, Yong Liu, Robert Debs*

Direct gene transfer into adult animals resulting in generalized or tissue-specific expression would facilitate rapid analysis of transgene effects and allow precise in vivo manipulation of biologic processes at the molecular level. A single intravenous injection of expression plasmid:cationic liposome complexes into adult mice efficiently transfected virtually all tissues. In addition to vascular endothelial cells, most of the extravascular parenchymal cells present in many tissues including the lung, spleen, lymph nodes, and bone marrow expressed the transgene without any apparent treatment-related toxicity. The transgene was still expressed in large numbers of cells in multiple tissues for at least 9 weeks after a single injection. Expression could be targeted to specific tissues and cell types, depending on the promoter element used.

The ability to efficiently transfect large numbers and diverse populations of somatic cells in adult animals would provide researchers a rapid and reproducible source of animals with which to analyze the function of genes transferred and expressed in vivo. This ability could also create new opportunities for directly altering gene expression in living hosts. (i) Loss or gain of function phenotypes could be produced directly in adult animals. (ii) The effects of expressing a transgene at different developmental stages could be assessed. (iii) Transgenes whose expression is lethal during embryogenesis could be expressed after birth. (iv) Multiple transgenes could be expressed in series in the same animal. Cationic liposomes containing *N*[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) have been used to deliver DNA (*1*), mRNA (*2*), or proteins (*3*) into cultured cells. However, their usefulness as an in vivo DNA carrier system has been limited because the expression of transferred genes has been confined to small numbers of cells within a single tissue (*4*, *5*).

By intravenously injecting various mixtures of a cytomegalovirus (CMV)–chloramphenicol acetyltransferase (CAT) expression plasmid and DOTMA:dioleoyl phosphatidylethanolamine (DOPE) liposomes, we determined that a ratio of 1 μg of plasmid DNA to 8 nmol of liposomal lipid produced maximal CAT gene expression in lung, heart, and lymph node tissues (Fig. 1A). The ratio of DNA to cationic liposomes determines the net surface charge on the complex, which can alter the interactions of the complex with potential opsonins in the circulation (6) and alter the ability of the complex to bind and enter cells in vivo. Using the DNA-to-liposome ratio (1:8), we found that doses of more than 50 μg of the CMV-CAT plasmid per 20-g mouse produced generalized CAT gene expression throughout the body (Fig. 1B). With a dose of 100 μg of DNA, high levels of CAT activity (*7*) were present in the lung, spleen, liver, heart, kidney, and lymph nodes (Fig. 1B) as well as in the thymus, uterus, ovary, skeletal muscle, pancreas, bone marrow, stomach, small intestine, and colon (8). When tissues were assayed for CAT activity per milligram of tissue protein, as performed for Fig. 1B, the lung and spleen had the highest levels of CAT activity (9).

In addition to the DNA-to-liposome ratio and DNA dose, conditions which increased levels of transgene expression in vivo were the use of the immediate–early 1 promoter–enhancer element of the human CMV and an intron 5′ to the coding region rather than a 3′ intron (*10*). Similarly, constructs containing the human CMV promoter (*11*) and an intron 5′ to the coding sequence (*12*) significantly increased the level and extent of gene expression in transgenic mice after oocyte injection.

We extracted genomic DNA from mouse tissues and subjected it to both Southern (DNA) blot and polymerase chain reaction (PCR) analyses to probe for the presence of the CAT gene. Significant amounts of plasmid DNA were present in DNA extracted from the lung and liver at day 1 but were undetectable by Southern analysis at day 21 (Fig. 2A). We detected intact plasmid DNA in supercoiled, relaxed, and linearized forms by Southern analysis at day 1 (Fig. 2A), demonstrating that nonintegrated plasmid was present. The CAT gene was detected by PCR analysis in the lung, lymph nodes, heart, and spleen of treated animals at 63 days after injection (Fig. 2B). Transient CAT gene expression in the lung after intravenous (iv) injection has been reported (*4*). Prolonged

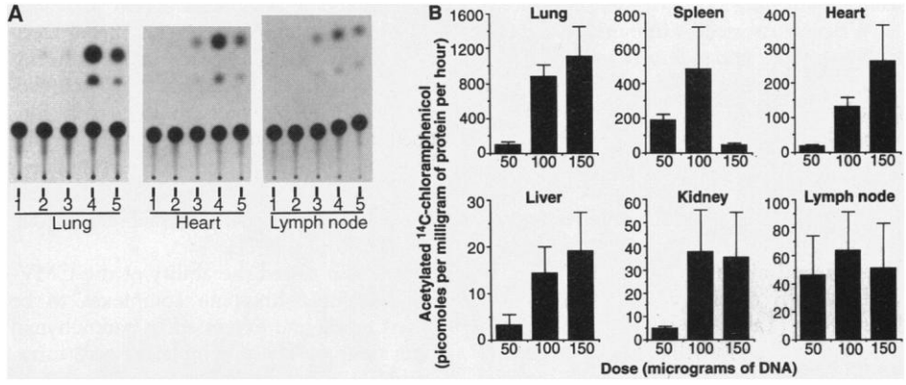

Fig. 1. The effect of the DNA-to-liposome ratio on CAT gene expression in vivo. (**A**) Female ICR mice (20 g) (Simonson) were injected in their tail veins with 100 μg of a human CMV-CAT plasmid (pCIS-CAT, obtained from C. Gorman, Genentech) complexed to 200 (lane 2), 600 (lane 3), 800 (lane 4), or 1200 nmol (lane 5) of DOTMA:DOPE liposomes in 200 μl of 5% dextrose in water. (Lane 1, untreated control.) We prepared liposomes from DOTMA and DOPE in a 1:1 molar ratio as described (*3*). Mice were killed 48 hours later, and tissues were harvested and homogenized. We prepared extracts and assayed for protein by means of a Coomasie blue–based assay (Bio-Rad). Each tissue was normalized for protein and assayed for CAT activity as described (*21*), with the following modification: 0.3 μCi of ^{14}C-labeled chloramphenicol (55 mCi/mmol) was added to 200 nmol of acetyl coenzyme A for a final volume of 122 μl. (**B**) The effects of the injected dose of DNA:liposome complexes on CAT gene expression in vivo. We gave ICR mice (in groups of three) a tail vein injection of 50, 100, or 150 μg of plasmid DNA complexed to 400, 800, or 1200 nmol of DOTMA:DOPE liposomes, respectively, and killed the mice 48 hours later. Paraoxon (1 mM) was added to liver and kidney extracts. To quantitate CAT activity in tissue extracts, we cut areas corresponding to [^{14}C]chloramphenicol and its acetylated derivatives from the thin-layer chromatography plate and counted them in a scintillation counter. The enzymatic activity of CAT in tissue extracts was expressed as picomoles of [^{14}C]chloramphenicol acetylated per milligram of protein per hour under assay conditions. Values represent mean ± SD.

N. Zhu, Y. Liu, R. Debs, Cancer Research Institute, University of California, San Francisco, CA 94143–0128.
D. Liggitt, Department of Comparative Medicine, University of Washington School of Medicine, Seattle, WA 98195.
*To whom correspondence should be addressed.

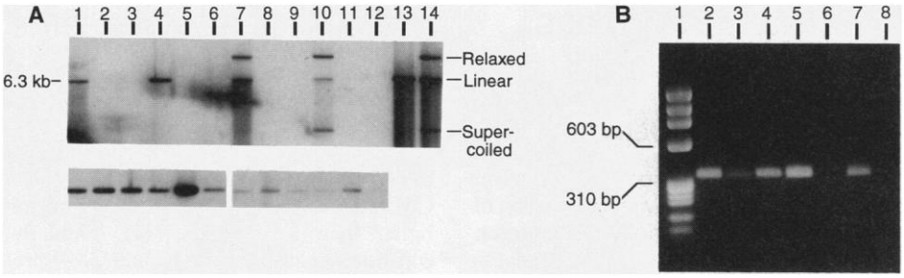

Fig. 2. Southern and PCR analyses to detect the CAT gene in mouse tissues. (**A**) We gave mice iv injections of DNA:liposome complexes or left the mice untreated, then 1 or 21 days later we harvested tissues and isolated nuclear DNA (*22*). For Southern analysis, DNA was digested with Eco RV, which does not cut within the plasmid, and Bgl II, which cuts at a single site within the plasmid. The DNA fragments were separated by electrophoresis on a 1% agarose gel and transferred to Hybond-N+ nylon membranes (Amersham). The blot was hybridized with a ^{32}P-labeled Bam H1–Pst 1 fragment of the mouse β-globin gene, washed, and rehybridized with the CMV-CAT plasmid. Lanes 1 through 6 were cut with Bgl II: (lane 1) day-1 lung; (lane 2) day-21 lung; (lane 3) control lung; (lane 4) day-1 liver; (lane 5) day-21 liver; and (lane 6) control liver. Lanes 7 through 12 were cut with Eco RV: (lane 7) day-1 lung; (lane 8) day-21 lung; (lane 9) control lung; (lane 10) day-1 liver; (lane 11) day-21 liver; and (lane 12) control liver. The remaining two lanes contain (lane 13) CAT plasmid–Bgl II and (lane 14) CAT plasmid–Eco RV. (**B**) Mouse DNA extracted from the lung, lymph nodes, heart, and spleen of an animal injected 63 days before, as well as DNA extracted from the spleen of a control mouse, was subjected to PCR analysis using two primers specific for the CAT gene, ACGTTTCAGTTTGCTCATGG and AGCTAAGGAAGCTAAAATGG, which yield 320-bp fragments. We performed the PCR analysis in a final volume of 100 μl of 2.5 mM $MgCl_2$ with 100 pmol of each primer. The cycles were 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min. After 27 cycles we analyzed the products on 1.6% agarose gels. Lane 1, øX174 ladder (BRL); lane 2, day-63 lung; lane 3, day-63 lymph node; lane 4, day-63 heart; lane 5, day-63 spleen; lane 6, control spleen; lane 7, CAT plasmid; and lane 8, water control.

Table 1. Immunohistochemical localization of CAT antigen in tissues harvested 24 to 48 hours after iv injection with CMV-CAT:cationic liposome complexes. Initial evaluations were always conducted in a blinded fashion.

Organ	Tissues staining positive for CAT antigen	Relative expression of CAT antigen in tissues*
Lung	Alveolar walls†	4
	Bronchioles	3
Heart	Endothelium	3
	Myocytes	1
Bone marrow	Mononuclear cells	2
Liver	Endothelium	1
	Hepatocytes	1
Spleen	Red pulp	4
	White pulp	3
Lymph node	Paracortex	4
	Germinal center	1
Kidney	Endothelium	2
Lung‡	Metastatic tumor cells	2–3

*A semiquantitative estimation of the frequency of positively stained cells in a representative series of immunohistochemical preparations (range: 1, staining of <25% of cells; to 4, staining of >75% of cells). These results were compiled from a minimum of three different experiments consisting of a total of at least 10 animals, and all the results were consistent. †Includes endothelium and alveolar lining cells (indistinguishable). ‡From mice bearing B16 melanoma lung metastases.

expression of episomal transgenes in nondividing cell types has been reported after intramuscular injection of naked DNA into mice (*13*) or after cationic liposome–mediated transfection of freshly isolated cells from rodent lung (*14*).

We next assessed by immunohistochemistry the types and numbers of cells transfected in vivo after iv injection. Although circulating DNA:liposome complexes would be expected to be confined to the vascular compartment, we found that iv injection of CMV-CAT:liposome complexes transfected the majority of all cells present in the lung, including most of the alveolar and airway parenchymal cells, as well as cells of the vascular endothelial lining cells (Table 1). The staining of both alveolar and endothelial cells was diffuse (Fig. 3A). Staining intensity varied throughout the lung, but negatively staining areas were rare.

Because CAT activity was present in large numbers of extrapulmonary tissues after iv injection of CMV-CAT:liposome complexes, we assessed the cellular pattern of CAT gene expression by immunohistochemistry in several other tissues as well. Two patterns of transgene expression were apparent: (i) generalized expression throughout the tissue (as seen in the lung) or (ii) expression largely confined to the vascular endothelial compartment. Specifically, CAT gene expression was present in large numbers of extravascular parenchymal cells in the spleen, liver, lymph nodes, and bone marrow after injection into normal animals (Table 1 and Fig. 3C). [The lung and spleen had the largest amounts of CAT protein as revealed by immunostaining (Fig. 3 and Table 1) and by enzymatic activity measurements (Fig. 1B).] Thus, circulating DNA:liposome complexes appear to readily extravasate across vascular endothelial barriers in these tissues. In contrast with this diffuse pattern of parenchymal cell transfection, CAT gene expression was largely confined to the vascular compartment in the heart and kidney. In these tissues, most endothelial cells lining small blood vessels, but few extravascular parenchymal cells, were transfected (Table 1).

We also tested the ability of the CMV-CAT:cationic liposome complexes to be taken up by and expressed in parenchymal lung tumors. These complexes were intravenously injected into mice bearing established B16 melanoma lung metastases. We found that both metastatic lung tumors and tumor emboli within the pulmonary vascular compartment were efficiently transfected (Table 1).

Next we assessed the duration of CAT gene expression in tissues by immunohistochemistry. Expression of the CAT gene, as detected by positive staining, was still present in approximately 40% of all lung cells, as well as in approximately 30% of lymph node and spleen cells, 9 weeks after a single iv injection of CMV-CAT:liposome complexes (*8*). The half-life of CAT activity in freshly isolated rodent hepatocytes transfected with the CAT gene ex vivo is approximately 20 hours (*15*). Thus, the persistence of CAT activity does not appear to result from stability of the CAT protein within cells. Furthermore, iv injections of DNA:liposome complexes repeated at 2-week intervals produced peak levels of transgene expression comparable to those produced by the first dose (*8*).

We have also tested the expression of the human gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) driven by the human CMV promoter. Using immunohistochemistry, we found that the human CFTR transgene is still expressed in large numbers of lung cells for at least 150 days after the last iv injection of CMV-CFTR plasmid:cationic liposome complexes into mice (*16*). These results indicate that transgene expression can persist in tissues for prolonged periods of time after cationic liposome–based in vivo gene delivery.

Sections from a wide variety of tissues from treated animals were also analyzed for potential toxicity. We observed no treatment-related toxicity in any treated animal. Specifically, hematoxylin- and eosin-stained histologic sections from treated animals were indistinguishable from comparable sections from untreated animals, and organ weights were not significantly different between control and treated animals. Furthermore, treated mice appeared normal

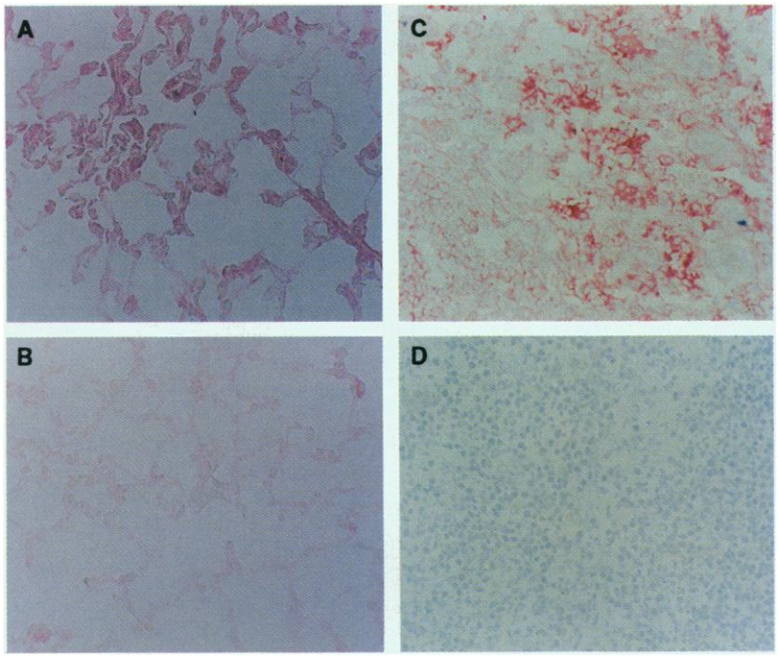

Fig. 3. Immunohistochemical analysis to detect CAT enzyme in mouse tissues. We injected ICR mice intravenously with 100 μg of CMV-CAT complexed to cationic liposomes, as described in Fig. 1B, or left the mice untreated and then killed them 48 hours later. For tumor localization studies, female C57B mice were injected intravenously with 5×10^4 B16 melanoma cells, received 14 days later 100 μg of the DNA:liposome complex or no treatment, and then killed 24 hours later. We then prepared the appropriate tissues for analysis (*23*). Red staining indicates the location of CAT antigen. (**A**) and (**B**) are adjacent lung sections from a mouse treated with CMV-CAT:liposome complexes. (**C**) and (**D**) are spleen sections from a CMV-CAT:liposome–treated mouse and a control mouse, respectively. To verify the specificity of CAT immunostaining, we first adsorbed the primary rabbit polyclonal anti-CAT against purified CAT (Sigma). For the liquid phase absorption we diluted the rabbit primary antibody 1:300 with a solution of purified CAT (CAT diluted 1:50 with a solution of PBS containing 0.5% casein and 2% normal goat serum) (B). Nonabsorbed antibody was similarly prepared but without the addition of purified CAT (A). The solutions were incubated for 1 hour at 37°C, placed on ice for an additional hour, and then subjected to immunostaining. The positive CAT immunostaining signal (A) is specifically blocked by absorbing the anti-CAT with purified CAT enzyme (B).

from the time we injected them with DNA:liposome complexes until they were killed. In the absence of inflammation or injury, the ability of circulating DNA:liposome complexes to extravasate into many of these tissues cannot be explained by altered vascular permeability. Consistent with our findings, other investigators have concluded that the systemic injection of DNA:cationic liposome complexes into animals appears nontoxic (*17*).

This somatic cell transgenic model provides simple, rapid, and reproducible transfer and expression of heterologous genes directly in adult animals. It could facilitate the identification of cis-acting sequences that regulate gene expression in vivo. Because the expression plasmid appears to be present primarily in episomal form, the use of these somatic cell transgenic animals may avoid unpredictable regulatory effects resulting from integration of transgenes into random sites within chromosomal DNA after the plasmid is injected into oocytes (*18*). Furthermore, specific tissues and cell types can be targeted in vivo by several, potentially complementary approaches. These include (i) using promoter-enhancer elements that are tissue- and cell type–specific, (ii) administering the plasmid regionally into selected tissue compartments (*19*), and (iii) coupling targeting ligands to the liposome surface (*20*). High level, generalized or tissue-specific expression of heterologous genes directly in adult animals could facilitate both the study and the control of molecular events in vivo and the range of diseases that can be treated by gene therapy.

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7. CAT activity produced per gram of tissue extract was approximately 1 to 2% of CAT activity produced per gram of cell extract. The cell extracts were prepared from several highly transfectable cell lines that we transfected in culture with CMV-CAT:DOTMA:DOPE liposome complexes. (Significant amounts of extracellular, and therefore nontransfectable, protein are present in tissue extracts, but not in cultured cells.) Furthermore, the amount of CAT activity in lung tissue from intravenously treated animals was within a factor of 3 of that produced by cells transfected in culture [single-cell suspensions prepared from whole lungs of untreated mice (*8*)]. This suggests that the process of in vivo transfection is relatively efficient.
8. Data not shown.
9. We injected 100 μg of plasmid DNA complexed to 800 nmol of cationic liposomes per mouse for subsequent experiments. The 100-μg dose of CMV-CAT DNA contained $\sim 1.8 \times 10^{13}$ DNA molecules. These were associated with $\sim 4 \times 10^{12}$ cationic liposomes, with a mean liposome diameter of ~100 nm (*3*). Estimating that a 20-g mouse contains $\sim 2 \times 10^{10}$ cells [I. F. Tannock, in *Cancer: Principles and Practice of Oncology*, V. DeVita, S. Hellman, S. Rosenberg, Eds. (Lippincott, Philadelphia, PA, ed. 3, 1989), p. 4], we injected each mouse with $\sim 1 \times 10^3$ CMV-CAT DNA molecules per mouse cell.
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23. Tissues were quick frozen, embedded in O.C.T. (Miles), sectioned at 6 μm, collected onto sialinized glass slides, fixed for 10 min in 4°C acetone, and washed in a solution of 0.1% Tween-20 in phosphate-buffered saline (PBS). Tissue sections were blocked with 10% goat serum, blotted, and incubated for 24 hours with a polyclonal antibody to CAT (anti-CAT) or a nonspecific control antibody at the appropriate dilution. The slides were washed, incubated for 60 min with a biotinylated antibody directed against the primary antibody, washed, then incubated with a streptavidin–alkaline phosphatase or peroxidase complex for 60 min, and washed again. Lastly, we applied a substrate-chromagen appropriate for the enzyme label (Zymed). Endogenous alkaline phosphatase activity was inhibited with levamisole. Spurious binding of streptavidin was blocked by application of free avidin and biotin (Zymed).

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