

Visualizing Gene Expression in Living Mammals Using a Bioluminescent Reporter

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ABSTRACT

Control of gene expression often involves an interwoven set of regulatory processes. As information regarding regulatory pathways may be lost in *ex vivo* analyses, we used bioluminescence to monitor gene expression in living mammals. Viral promoters fused to firefly luciferase as transgenes in mice allowed external monitoring of gene expression both superficially and in deep tissues. *In vivo* bioluminescence was detectable using either intensified or cooled charge-coupled device cameras, and could be detected following both topical and systemic delivery of substrate. *In vivo* control of the promoter from the human immunodeficiency virus was demonstrated. As a model for DNA-based therapies and vaccines, *in vivo* transfection of a luciferase expression vector (SV-40 promoter and enhancer controlling expression) was detected. We conclude that gene regulation, DNA delivery and expression can now be noninvasively monitored in living mammals using a luciferase reporter. Thus, real-time, noninvasive study of gene expression in living animal models for human development and disease is possible.

INTRODUCTION

We set out to establish a method to access real-time information pertaining to gene expression in living adult mammals. Gene regulation is fundamental to both normal development and pathologic conditions. Consequently, a considerable amount of research effort is devoted to assessing levels of expression from genetic regulatory elements under various conditions. Fusion of regulatory elements to reporter genes can be used to monitor levels of expression, typically via *ex vivo* methods. These methods involve excision of tissue and assessing levels of the reporter molecule in histological sections or in cell lysates using optically distinct sub-

strates for the reporter enzymes (1,2). Such analyses are performed on biopsy or postmortem samples, which eliminates the possibility of understanding regulation in living animals in real-time, and limits these assays to single points in time. Temporal analyses require large numbers of animals, as several animals are used for each time point. Considerably more data could be obtained if the assays were performed *in vivo*, and the cost and effort of animal studies would be reduced.

Monitoring physiological processes using external sources of light has been accepted as a clinical tool (*e.g.* pulse oximetry) and is gaining acceptance as an imaging modality that can reveal both structure and function (3,4). These technologies are based on the ability of photons to pass through mammalian tissue despite its light absorbing and scattering properties (5). Advances in this area of research prompted us to investigate external detection of internal sources of light to determine the location of pathogens (6), and the temporal and spatial expression of mammalian genes. This method could be broadly applied to monitoring expression from transgenes, DNA- or cell-based therapies and host response to infection, as well as the tracking of pathogens and tumor cells in living animal models.

Real-time, *in vivo* monitoring of bioluminescent reporters has been described. However, such assays to date have been limited to organisms that are thin or essentially transparent (7–12) or to stages in development when the organism is translucent (9,13). We previously demonstrated noninvasive monitoring of bacterial pathogens in living adult mice using an internal bioluminescent light source (6), raising the possibility of real-time monitoring of gene expression in adult animals using bioluminescent reporters. Several animal model systems were available for developing this methodology, of which two were selected: a transgenic mouse where the transgene consists of the promoter from the human immunodeficiency virus (HIV)[†] fused to the firefly luciferase gene (*luc*) and an *in vivo* transfection of a luciferase expression vector in rats.

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[†]Abbreviations: CCD, charge-coupled device; DMSO, dimethyl-sulfoxide; HIV, human immunodeficiency virus; ICCD, intensified charge-coupled device; i.p., intraperitoneal; LTR, long terminal repeat; MCP, microchannel plate; p.i., postinfection; TCID, tissue culture infective dose; Tg, transgenic.

In the first luciferase animal model that we describe, the HIV promoter has been placed upstream of the luciferase coding sequence. In the viral genome, the HIV promoter resides in the long terminal repeats (LTR). A transgenic (Tg) mouse line with luciferase expressed from the HIV promoter (LTR-*luc* Tg) has been described (14). Although regulated differently in rodent and human cells, expression from the HIV promoter in rodent cells has some similarities to that in human cells and is inducible with various treatments including illumination with UV light (14,15), skin abrasion (2) and chemical induction (16–18). In the dermis of transgenic mice, the HIV promoter can be induced by topical treatment with dimethyl sulfoxide (DMSO), with maximum induction after 24 h (16).

The development of DNA-based therapies and vaccines would benefit from the ability to externally monitor gene delivery and expression. In our second model, we evaluated *in vivo* transfections using a luciferase expression vector and cationic liposome-mediated delivery as has been suggested for DNA-based therapies and vaccines (19–23). DNA has also been successfully delivered to cells in tissues and expressed in living animals by direct DNA injection, electroporation and particle bombardment. Based on a review of the literature, liposome-mediated gene transfer appeared to be the most efficient and cost effective means of assessing the usefulness of noninvasive luciferase monitoring in the evaluation of *in vivo* transfection. Unlike the transgenic animal model, not all cells in a given tissue are likely to contain the reporter construct, and weak signals may be expected in this type of assay. Therefore, we used a luciferase expression vector in which the luciferase gene was modified for optimal mammalian codon usage and removal of the peroxisome targeting site, which was driven by a strong viral promoter and enhancer (SV-40).

In this report, we noninvasively monitored the expression of both stably integrated and transiently transfected bioluminescent reporter gene fusions in both cell culture and living rodents. In our previous pathogenesis model, the bacterial pathogen could be modified to express enzymes for intracellular biosynthesis of the luciferase substrate (6,24). Intracellular biosynthesis of substrate, D-luciferin, is not yet possible in mammalian cells expressing firefly luciferase, thus it must be added exogenously. We demonstrate that D-luciferin can be delivered to living cells in culture and to tissue sites of luciferase expression *in vivo*. Furthermore, we report noninvasive monitoring of gene expression in living mammals from both superficial and deep tissue sites of expression and demonstrate visualization of gene delivery in transient transfections as used in gene therapies and gene vaccines. Finally, we demonstrate efficient control of the HIV promoter in adult transgenic mice and developmental regulation in neonates. These studies indicate that substrate availability and photon passage through tissues are not limiting in the use of bioluminescent reporters in living animals and suggest that promoters, with reasonably high expression, can be noninvasively evaluated in some tissues of living laboratory animal models.

MATERIALS AND METHODS

Cell culture. A human T cell line (Jurkat-derivative; 1G5) stably transfected with an HIV LTR-*luc* construct, originally prepared by

E. Aguilar-Cordova and J. Belmont (25) was obtained from the AIDS Research and Reference Reagent Program of the NIH. This line was propagated in RPMI medium supplemented with penicillin, streptomycin, glutamine (2 mM) and fetal calf serum (10%). Cells were plated in 24 well dishes at 5×10^5 cells per well and infected with 1×10^4 tissue culture infectious doses (TCID) of HIV-1 strain A111 (laboratory isolate of a subtype B North American HIV-1, kindly provided by Drs. R. Shafer and T. Merigan of the Center for AIDS Research at Stanford) in a total volume of 200 μ L. The cells and viral inoculum were maintained in this volume for 4 h after which an additional 800 μ L of medium was added. At 22 h post-infection (p.i.) the substrate luciferin (50 mM; Molecular Probes, Seattle, WA) was added to each well at a final concentration of 0.5 mM. Wells were imaged at 24 h p.i. and at subsequent times as indicated. Cells were maintained for 7 days in luciferin-containing medium.

Animal subjects. Transgenic mice, containing the HIV LTR-luciferase fusion (mouse line 333 generated by Dr. John Morrey and coworkers (14), Utah State University), were initially obtained from Jackson Laboratories (Bar Harbor, ME). Subsequently, a breeding nucleus was obtained directly from Dr. Morrey and then bred and maintained in the Research Animal Facility at Stanford University under strict adherence to institutional guidelines.

To activate LTR-*luc* expression, DMSO (17) (100%, J. T. Baker) was applied twice topically to regions of skin at 24 and 16 h prior to imaging. If necessary, skin was shaved to remove hair just prior to application of DMSO. This method of promoter induction was found to be optimal for expression of luciferase in adult transgenic mice of this line (data not shown). In neonatal LTR-*luc* Tg mice the LTR was not induced chemically or by any other known means. Thus, only basal levels of expression were studied (*i.e.* gene expression in these animals was not modulated by any of the above-described treatments or any other known activators).

For imaging, mice and rats were anesthetized and imaged as previously described (6) using pentobarbital (70 mg/kg body weight). An aqueous solution of the substrate, luciferin (50 mM, 126 mg/kg), was injected into the peritoneal cavity 20 min prior to imaging for systemic delivery or applied topically (50 mM unless otherwise indicated) in 100% DMSO for transdermal delivery. Luciferin was also electroporated into skin of the ear using a caliper electrode (Gentronics/BTX, San Diego, CA) connected to an electroporator (Electro Cell Manipulator, model 600, Gentronics/BTX) under the following conditions: 120 V, 100 μ F, 72 Ω , resulting in a time constant of 6–7 ms.

Imaging. At the time of imaging, animals were placed in a light-tight chamber, and a gray-scale body surface image was collected as a reference in the low room light provided by leaving the chamber door slightly open. Subsequently, the door to the chamber was closed to exclude the room light that obscured the relatively dimmer luciferase bioluminescence. Photons emitted from luciferase within the animal, and then transmitted through the tissue, were collected and integrated for a period of time between 5 and 30 min. A pseudocolor image representing light intensity (blue least intense and red most intense) was generated on an Argus 20 image processor (Hamamatsu, Japan); images were transferred using a plug-in module (Hamamatsu, Japan) to a computer (Macintosh 8100/100, Apple Computer, Cupertino, CA) running an image processing application (Photoshop, Adobe, Mountain View, CA). Gray-scale reference images and pseudocolor images were superimposed using the image processing software, and annotations were added using another graphics software package (Canvas, Deneba, Miami, FL).

An intensified charge-coupled device (CCD) camera (C2400-32, Hamamatsu), fitted with a 50 mm Nikkor lens (Nikon, Japan) and an image processor (Argus 20, Hamamatsu), was used in this study. In the comparison of this system to a cooled CCD (C4880-06, Hamamatsu) equipped with the same lens (subjects at the same distance from the lens), images were collected on a PC and then transferred to a Macintosh computer and processed as above.

Biochemical assays. Luciferase activity in tissue homogenates was determined with a commercially available assay kit (Promega, Madison, WI) that included acetyl Co-A to prolong light emission. A standard luminometer (LKB/Wallac, Stockholm, Sweden) was used to measure total light emission in conditions recommended by the manufacturers. Conversely, for substrate assays, luciferase (1

ng/mL) was substituted for luciferin in the same assay system and relative levels of substrate were determined in tissues of BALB/c mice (Stanford University) not expressing luciferase. An aqueous solution of luciferin (50 mM, 126 mg/kg) was injected intraperitoneally (i.p.) into BALB/c mice, and substrate levels in brain, skin and liver were assayed after 20 min.

In vivo DNA delivery. Seven day old Wistar rats were obtained from Simonsen (Gilroy, CA). A cationic liposome solution, Lipofectin (100 μ L, Gibco-BRL, Gaithersburg, MD) was mixed with 100 μ g of plasmid DNA (pGL3control vector, Promega, Madison, WI). The luciferase expression in this commercially available vector is under control of the SV-40 promoter and SV-40 enhancer, and the luciferase gene has been modified for higher levels of expression in some mammalian cells by removal of the peroxisome targeting site and optimization of codon usage for mammalian cells. Following incubation at 22°C for 15 min, DNA and liposome complexes were injected directly into the lungs of 7 day old rats in groups of three animals. Treated animals were then injected with an aqueous solution of luciferin (i.p.), and imaged at 24, 48 and 72 h. Images were obtained with an integration time of 30 min.

RESULTS

Bioluminescence in culture

Luciferase as a transcriptional reporter was used as a means to monitor viral replication in living cultured cells, using LTR activation as a molecular indicator of viral replication. We chose this approach because the LTR of HIV-1 contains elements that respond to both viral encoded factors (Tat protein) and host transcription factors. Thus, these genetic elements can function together as a molecular indicator reporting either viral replication or changes in cellular transcription factor profiles. In the case of infection, viral-encoded Tat can transactivate the LTR in promoter fusions, therefore transactivation can be used as an indicator of infection. Currently, analysis of transactivation of the HIV LTR *via* Tat is performed using lengthy biochemical assays (26). When LTR activity is coupled to luciferase expression, the LTR becomes a molecular switch controlling a biochemical light bulb, permitting noninvasive monitoring. This approach had previously been demonstrated to indicate levels of viral replication as measured in cell lysates (27) and more recently to assess transcription, microscopically, in single living cells (28). However, rapid analysis and detection of transactivation at a macroscopic level had not been previously demonstrated.

Expression of luciferase as a rapid, real-time macroscopic indicator of viral replication in cell culture is shown (Fig. 1). In these studies of infected cell populations, a stably integrated LTR-*luc* construct was used as the reporter (1G5 cell line). Luciferin was added directly to the living cells, and infected cultures were followed over time. Signal above background was apparent at 96 h p.i. and increased to a peak at 7 days. The numbers of syncytia (cytopathic effect due to replication of HIV) corresponded to levels of bioluminescence at each time point (data not shown), suggesting that bioluminescence was a reliable indicator of viral replication. This approach may accelerate viral assays, and reduce handling because detection, photon counting, can be done remotely with minimal human handling.

Substrate availability in cell culture was adequate to detect luciferase activity in this human T cell line. In addition, luciferase enzyme assays could also be performed in living rodent cells (NIH 3T3) and another human cell line (HeLa) transiently expressing luciferase from the pGL3 vector (data

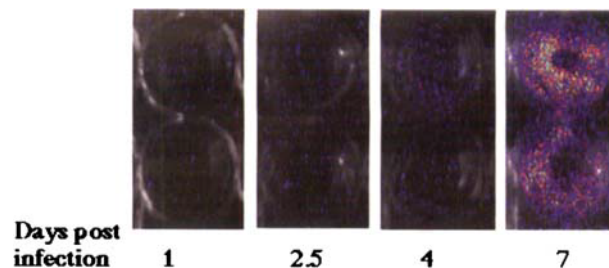


Figure 1. Bioluminescent reporter for promoter activity in cultured cells. Jurkat cells stably transfected with a plasmid containing the HIV LTR (1G5 (25); AIDS Research and Reference Reagent Program) upstream of the coding sequence of firefly luciferase were infected, in duplicate, with a laboratory isolate of HIV-1 (strain A111) using standard laboratory conditions and followed for a period of 7 days for emission of bioluminescent light. Four time points are shown: 1, 2.5, 4 and 7 days. At each time point a gray-scale image of the plate was generated in low room light followed by collection of photons emitted from the cultured cells in complete darkness for a period of 10 min. A color pseudoimage representing the intensity of bioluminescent light was superimposed over the gray-scale image of the plate. At 7 days p.i. a clear signal is present in the duplicate cultures indicating high levels of replication. The images at different time points represent the same two wells over the 7 day period.

not shown). With a single addition of substrate, transiently transfected 3T3 and HeLa cells were uniformly bioluminescent from 48 to 72 h, and the decline after this time likely corresponds to loss of the reporter through cell division. Therefore, the availability of luciferin to intracellular luciferase appeared to be sufficient in these cell lines.

Bioluminescence in whole animals

Application of the method described above for cells in culture to living mammals required demonstration that luciferin could be delivered to cells in target organs. As the intracellular biosynthesis of the substrate, D-luciferin, is not yet possible in mammalian cells, it must be delivered exogenously. We evaluated the delivery of luciferin to different cells in the body after i.p. injections. In this experiment, D-luciferin was injected i.p. into BALB/c mice, tissues were removed after 20 min, and luciferin assays, in which luciferase replaced luciferin in the buffer system, were performed on tissue homogenates from brain, skin and liver. Detectable levels of the substrate were found in all tissues analyzed (data not shown) indicating that substrate availability may not limit the ability to assess gene expression in various tissues of living animals.

Use of luciferase as a rapid, real-time *in vivo* indicator of genetic transfection in mammals is shown (Fig. 2). In these experiments, a luciferase expression vector was used to transfect cells of tissues in living rodents. Young Wistar rats were given a single unilateral injection of DNA-liposome complexes intrapleurally. The pGL3-control vector encoding a modified firefly luciferase was used in these assays. After 48 h, bioluminescence was detected unilaterally (a representative animal is shown in Fig. 2). Signals above background were not detected at other times. The contralateral lungs in these rats were used as internal negative controls as only the liposome preparation without DNA was injected at this site. These data suggest that peak levels of expression



Figure 2. Monitoring transient gene expression in chimeric animals. The luciferase expression vector, pGL3control, was delivered to cells in the lung of neonatal rats via cationic liposome delivery, and substrate was injected i.p. at 24 and 48 h post-DNA delivery. A representative image at the 48 h time point is shown. This image indicates that luciferase expression (color) from transient *in vivo* transfections of the lung can be monitored externally.

in the transfected tissues occurred after 2 days and indicate that luciferase expression, as measured by photon emission, can be used to noninvasively monitor *in vivo* transfections of cells in deep tissues of laboratory rodents. Moreover, the i.p. injection of luciferin appeared to adequately supply the intrapleural luciferase reaction with substrate. Such monitoring of reporter gene expression in deep tissues may be limited by absorbing and scattering tissues such as liver, and weak signals may not be apparent with external monitoring. Subsequent empirical determinations of the limits of detection will enhance our understanding of these anticipated limitations.

To further investigate use of *in vivo* luciferase monitoring for studying gene regulation in living animals, we used a transgenic mouse line where the promoter fusion (LTR-*luc*) is present in all cells. In this transgenic mouse, the promoter can be regulated and luciferase expressed in a variety of tissues. This transgene was initially selected for evaluation due to known activity of the HIV LTR in the skin; given the uncertainty of tissue penetration by photons from a weakly bioluminescent internal signal, a superficial site of expression was preferred for preliminary studies monitoring luciferase expression *in vivo*.

Use of luciferase as a rapid, real-time *in vivo* indicator of gene activation in a mammal is shown (Figs. 3 and 4). In the skin of LTR-*luc* Tg mice, expression of the HIV LTR has been reported to possibly occur in Langerhans cells (14,15); in skin cells the promoter can be activated with DMSO. Topical application of substrate appeared to be the most direct means of delivery. Of the various methods for transdermal delivery of small molecules, use of DMSO appeared to be suitable given the size and charge of the luciferin molecule. The extent of our ability to control and monitor gene expression in the skin of transgenic mice was tested by activating the gene in cells of approximately half of the shaved back and one ear of each LTR-*luc* Tg mouse. Twenty-four hours after initial activation with DMSO, the entire surface and both ears of each mouse were treated with 50 mM luciferin in 100% DMSO and a representative image is shown (Fig. 3). The effects of DMSO, which was used to deliver substrate to the expressing cells, on LTR expression was negligible in the time from substrate addition to imaging (less than 1 h).

A uniform bioluminescent signal from the region of the

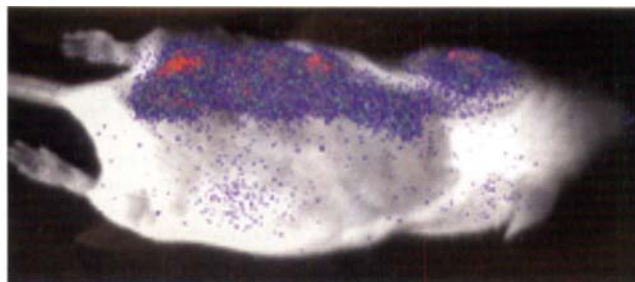


Figure 3. Unilateral induction of expression. The left half of the shaved dorsal surface of animals and the left ear were treated twice daily for 2 days with DMSO to activate expression of the HIV-1 LTR. Luciferin was applied topically over the entire surface of the back and both ears, and animals were imaged immediately after addition of substrate. Unilateral emission of bioluminescence (color) corresponding to the induced region was observed in groups of mice and a representative image is shown.

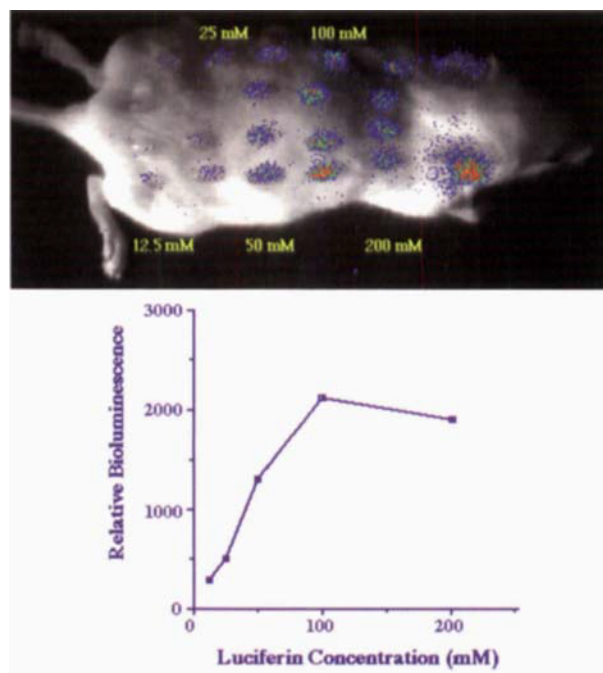


Figure 4. Transdermal delivery of substrate using DMSO. The HIV-1 LTR was induced in the skin of mice with twice daily treatments of DMSO over the entire surface of the back and the right ear for 2 consecutive days. Substrate was applied to the skin in solutions prepared in DMSO at a concentration of 200, 100, 50, 25 and 12.5 mM. A volume of 5 μ L of each concentration was spotted in quadruplicate on skin using a multichannel pipette, with the highest concentrations near the head. Five microliters of the 50 mM luciferin solution was applied to each ear. (A) Two minutes after application, the animal was imaged as described in Fig. 1, and the bioluminescent response is shown (color). (B) The bioluminescent response appeared to increase linearly over the concentrations from 12.5 to 100 mM. Bioluminescence from 200 mM luciferin was roughly equivalent to, or slightly less than, that from 100 mM. DMSO solutions of luciferin containing water (25% H₂O in DMSO to 100% H₂O) resulted in no detectable bioluminescence (data not shown).

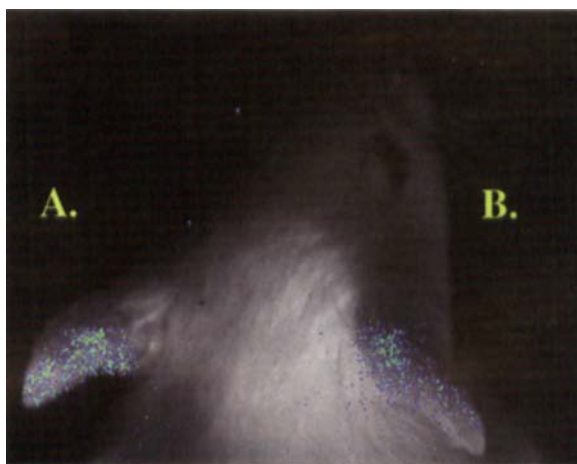


Figure 5. Transdermal delivery of substrate electrically and chemically. The LTR was activated in each ear of three mice, and then 24 h later substrate was delivered to the cells in DMSO or *via* electroporation and the animals were imaged. Bioluminescent signals were detected whether the substrate is delivered (A) electrically or (B) *via* electroporation.

backs in which the gene was activated was observed (Fig. 3), whereas signal from the nonactivated skin remained at background levels despite the presence of substrate in DMSO at the time of imaging. The ears served as internal negative and positive controls. Within the limits of this system, Fig. 3 demonstrates fairly good control of the HIV promoter in living animals and the ability to assay luciferase in large numbers of cells over large surface areas.

In experiments using various ratios of H₂O and DMSO for transdermal delivery of luciferin, the fraction of DMSO in these mixes ranged from 12.5 to 100%, bioluminescence could not be detected with luciferin made in solutions containing even 12.5% water (data not shown). A preparation of D-luciferin in pure DMSO, however, mediated transdermal delivery, as bioluminescence was easily detected from treated areas (Fig. 3) (24). To determine optimal substrate concentration for transdermal delivery, solutions of 12.5–200 mM luciferin in DMSO were spotted in quadruplicate directly on the skin 24 h after LTR activation (Fig. 4). In these experiments, the backs of mice were shaved, and the HIV-1 LTR was activated with twice daily treatments of DMSO 24 h and 16 h prior to analysis. Signals were quantified on images and data were plotted (Fig. 4). Bioluminescence increased linearly from 12.5 to 100 mM but remained relatively constant from 100 to 200 mM luciferin. These data suggest that 100 mM luciferin in 100% DMSO is the optimal concentration for transdermal delivery of this small molecule.

Gene activation due to the DMSO used to carry substrate into the expressing cells would not be apparent in the time from substrate addition to imaging. However, the use of DMSO for transdermal substrate delivery may be limiting in some studies of the HIV LTR as the promoter is simultaneously activated by DMSO. Therefore, electrodelivery of substrate to the skin was investigated as an alternative method. A representative comparison of chemical transdermal delivery (DMSO) and electrodelivery is demonstrated in Fig. 5. In three of three animals, electrodelivery of luciferin was

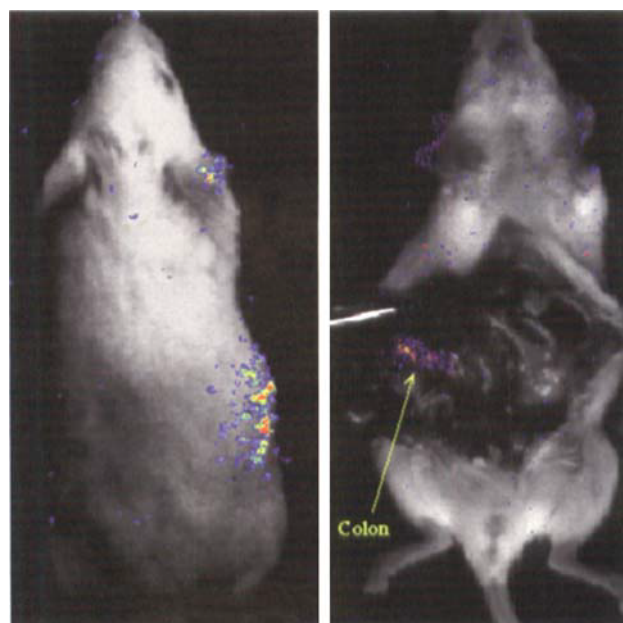


Figure 6. Detectable bioluminescence from internal tissues. (A) Bioluminescent signals were detectable from the abdomens of animals treated with DMSO on one ear only. This signal is assumed to be due to ingestion of DMSO during grooming. (B) Animals demonstrating signal from the abdomen were laparotomized and imaged. Bioluminescent signal localized to the colon in each of four animals studied, and in the animals shown in this figure, bioluminescence was tightly localized to a region of the colon (arrow), about 1 cm in length. In some other animals, the entire colon appeared to emit light.

comparable to chemical delivery. Cells in the ear may be more accessible than other tissues and with similar conditions electrodelivery of substrate was not achieved on the shaved backs of mice (data not shown). Electrodelivery is unidirectional relative to electron flow. Therefore, optimal bioluminescence would likely be obtained from the ears if the substrate was delivered to both sides by pulsing the tissue, reversing the polarity and giving an additional pulse.

During the initial evaluation of this imaging method, the active, inducible nature of the HIV LTR in cells of the skin was useful. However, following successful visualization of transient *in vivo* transfection with the mammalian expression vector (pGL3) and the initial studies demonstrating detection of LTR expression in superficial tissues, it appeared that photonic detection of luciferase expression from a variety of tissue sites was possible. After a single dose of luciferin (126 mg/kg) was injected into the peritoneal cavity, photon emission from the skin of the ears peaked at 20 min (29). With systemic rather than topical application of substrate, additional sites of expression from the LTR, other than the skin, could be identified (Fig. 6A). Photons originating from cells in the colon (Fig. 6B) were apparent externally, indicating that LTR-*luc* expression from deep tissues was also possible. This was also observed with expression from pGL3control vector in the lungs (Fig. 2). In earlier studies, externally viewed bioluminescent signals from the colon were not as intense as those obtained after removal of the skin and peritoneal wall exposing the viscera (6). This reduction in signal may affect studies using either weak bio-

luminescent signals, larger animal models or signals originating from more absorbing and scattering tissues such as liver.

Comparison of CCD detectors

Two different types of imaging systems were compared, as the optimal system for use in low-light imaging of biological processes in living animals was not previously determined. We focused on systems based upon the CCD, as it is a readily available, low-cost detector with many features supporting use for luciferase imaging. A CCD operates by converting photons that strike a CCD pixel into electrons and accumulating a charge that represents the number of photons striking each pixel. Maximum conversion of photons to electrons occurs at wavelengths between 400 and 1000 nm, and thus a CCD can detect visible through near infrared light. However, there are several important limitations in using a standard video rate CCD for *in vivo* luciferase imaging. First, in order to quantitate the electrons in each pixel, the stored charge must be read out. The signal produced when the photons first strike the CCD is too small to be sensed by the readout circuitry. Thus, to be recognized as a signal over noise, there is a minimum number of photons that must strike each pixel before the stored charge is converted to signal. This read noise is about 150 electrons for a typical video rate CCD design. Second, not all photons striking the detector are converted to electrons. The response of a CCD is wavelength dependent, and the standard efficiency of video rate CCD is about 35% (*e.g.* the probability that a given photon will strike the CCD and produce a change in stored charge is about 1 in 3). Last, there are background count sources, such as thermal energy or gamma rays, that can trigger false charges not originating from the luciferase construct. A good detector is shielded from background radiation and uses materials and components that are low in emitter-containing substances.

These CCD limitations can be overcome in several ways. First, intensified CCD (ICCD) amplify the signal relative to the noise by using an image intensifier that has both a photocathode and a microchannel plate (MCP) attached to a high-voltage element, such that many electrons are released when struck by a single photon. The camera noise is then discriminated by photon counting imaging. One advantage of these devices is that a single photon now results in a detectable signal, and single photon detection becomes possible. With an intensifier, some loss of image resolution occurs as the electron spray produced by the intensifier results in a blurring of the image, particularly if more than one intensifier stage is used in series. An advantage of the ICCD approach is that the intensifier can be designed to serve as a high-pass filter that amplifies only those photons with an energy at or above a desired level. In the ICCD system used in this study, amplification occurred only for photons with a wavelength of 600 nm or shorter, thus the background noise is minimal. A second alternative to the ICCD is a system where the CCD detector is cooled to reduce the background thermal noise, the signal is read slowly to reduce the read noise (less than 10 electrons), and the CCD chip used has higher photon to electron conversion rate (higher than 60%). In this cooled CCD the improvement scales linearly with

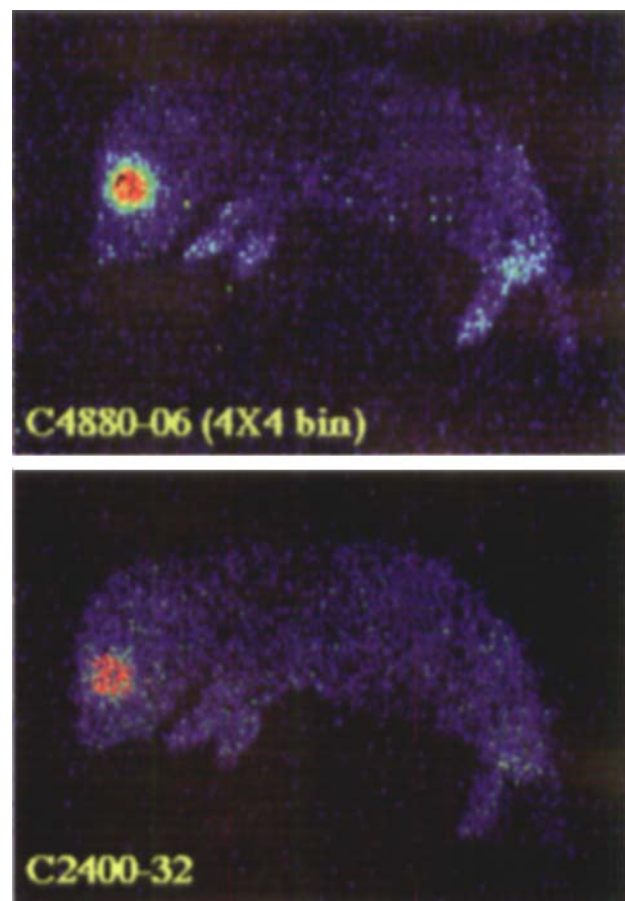


Figure 7. The LTR expression in neonatal mice and comparison of intensified and cooled CCD cameras. Four day old LTR-*luc* transgenic mice were imaged after luciferin injection with (A) a cooled CCD detector (C4880-06) and (B) an intensified CCD detector (C2400-32). Pixels were binned (4×4) with the cooled device, and 10 min integration times were used with each system. The images of bioluminescent light emission are nearly equivalent with each device. Gray-scale reference images are not shown.

integration time. Thus, short exposures benefit minimally from this approach. A combination of technologies can also be employed in which the intensifier of an ICCD is cooled. Cooled ICCD or alternatives to CCD-based systems were not explored in this study.

A comparison of images from cooled and intensified CCD cameras using the neonatal LTR-*luc* Tg mouse model is shown (Fig. 7). Two images that were taken within minutes of each other of the same neonatal mouse (4 days of age) are presented (Fig. 7). These images were obtained with an ICCD (C2400-32) and a cooled CCD (C4880-06 cooled to -30°C , 16 pixels were binned; 4×4). The two images appear approximately equivalent. Thus, in the current model, no significant difference was seen between the two types of systems. Practically, however, an ICCD with the photon counting imaging method offers the advantage of showing real-time images as the signal accumulates, allowing for continuous monitoring of the process.

Developmental expression of the LTR

In Fig. 7 expression from the LTR in the 4 day old mouse was not induced by chemical treatment or by any other

known exogenous method. That is, the observed expression was under endogenous control of the regulatory element (HIV LTR). In these neonatal LTR-*luc* Tg mice, luciferase expression appeared to be elevated in the eye and limbs, and an overall basal level of activity was apparent over the entire animal. These patterns of expression were consistent with newborn LTR-*luc* Tg mice up to 4 days of age, at which time the elevated levels of expression in the eye decreased to background. Elevated levels of expression in the limbs appeared to be more variable. This suggests that the HIV LTR may serve as a probe for developmental gene regulation that marks steps in development of this animal model.

DISCUSSION

This study demonstrates our ability to visualize genetic responses of living cells in culture and in intact mammals by using bioluminescent light to report gene activity under varied conditions, such as viral replication and gene activation. Further, we demonstrate that the substrate for the luciferase reaction can be provided to mammalian cells in the intact animal. Delivery was optimized for systemic and transdermal delivery. Transdermal delivery was achieved by chemical or electrical means. All of the events described here can be monitored using commercially available ICCD detectors.

Mammalian development, host response to infection and house-keeping operations of organisms are ultimately controlled by a complex genetic program allowing the organism to respond to and interact with numerous environmental factors. This genetic program includes regulatory elements that act as switches for spatiotemporal control of gene expression in response to specific signals. Organisms have the ability to modulate gene expression over a wide range of dose-response relationships, where no expression and full-blown expression represent the ends of a continuous spectrum. An ability to study these genetic events noninvasively will increase our understanding of these processes in living mammals.

Since luciferin is taken up by mammalian cells in culture without overt signs of cytotoxicity and provides reasonable bioavailability of the substrate in living mammals, it appears that luciferase can function as a reporter for DNA delivery directly to living tissues. Monitoring expression of a modified luciferase gene fused to the SV-40 promoter transfected by cationic liposome delivery was used as a rapid assay for testing delivery of substrate to tissue sites. The ability to observe a bioluminescent signal from cells expressing luciferase in this model for DNA-based therapies and vaccines suggests that the substrate reaches useful concentrations in cells of the lung and perhaps other tissues following i.p. injections. Codelivery of the reporter gene or co-expression from a single promoter may allow rapid evaluation of different methods of delivery and duration of expression in the development of emerging DNA- and cell-based therapies. The luciferase expression detected optically in various tissues, and our data on substrate levels in different tissues indicate that substrate availability is not limiting and that a variety of promoters expressed in different tissues can be studied using this method.

Reproducible, qualitative signals can be obtained from both superficial and deep tissue photon emissions, indicating

a qualitative correlation between detected light levels and two-dimensional detection of the desired event. It is likely that, at least within the same tissue, levels of light can be qualitatively compared, because graded responses were obtained from skin, ears and eyes. At present, quantitation of a genetic event coupled to luciferase by measurement of the bioluminescent light traversing live, intact tissue has not been achieved. This is due in part to the unknown kinetics of the reaction (*e.g.* uncertain local physiologic conditions, substrate and enzyme concentrations) as well as uncertainties regarding the tissue optics (*e.g.* variable transmission of the generated light based upon depth, and the dependence of transmission upon variable and unknown tissue-specific absorbance and scattering characteristics of the tissues interspersed between light generation and light detection).

The *in vivo* study of LTR-*luc* Tg mice demonstrated that in this mammalian model: (1) a number of variables can be tested simultaneously in a single animal noninvasively, (2) luciferase can be used as a reporter for expression in deep tissues, (3) developmental control of expression from the HIV promoter can be assessed in living animals and (4) transdermal delivery of small molecules (luciferin) can be evaluated in living mice. Therefore, *in vivo* monitoring of luciferase expression can be used to titrate, in real-time, the effective doses of individual compounds or their combinations for transfection of living tissue, which is useful for optimizing the DNA delivery and expression of DNA-based therapies. In addition, a number of inhibitory molecules as potential therapeutic agents could be tested rapidly and in real-time, accelerating their development for use in human therapies. A significant amount of data from each rapid assay can be collected, allowing evaluation of several different conditions simultaneously, at different time points and without *ex vivo* sample processing (*e.g.* removing the tissues, lysis of the cells or other sample preparations). Performing the assays directly on tissues in the living animal can be an alternative to *in vitro* biochemical assays or a complementary *in vivo* approach to test predictions made from *in vitro* data.

The ability to use such probes in living animals is a powerful tool for revealing steps in the complex regulatory pathways involved in development and response to infection. Toward this end we have evaluated the HIV promoter in culture and in living transgenic mice. Viral promoters utilize host and viral transcription factors to achieve levels of expression that are commensurate with requirements of viral replication. Some viral proteins are expressed to relatively high levels during infection, and promoters for genes encoding these proteins have been exploited to achieve elevated expression of experimental and therapeutic genes (*e.g.* SV-40 promoter in pGL3control vector). In neonatal LTR-*luc* Tg mice, a basal level of bioluminescence was detected in the absence of chemical or physical induction, and elevated signals have been observed in the developing eye and limbs with a rapid postnatal decline in all tissues over the first few weeks of life (Contag *et al.*, unpublished results). The reliance of viral promoters on host factors may thus make them useful as molecular probes for understanding host cell gene regulation.

The HIV LTR is regulated differently in mouse cells compared to cells of human origin; however, the ability to con-

trol LTR expression in rodent cells with DMSO and UV light has significance in understanding the role of host transcription factors in viral replication. The regulatory region (-167 to +80) in the HIV LTR contains numerous canonical binding sites for cellular transcription factors (30,31). Ultraviolet light-mediated activation of HIV-1 replication has been proposed in infections of humans, and the Tg mice serve as a model for this environmental factor (32). Moreover, Langerhans cells in the skin of infected humans appear to support HIV-1 replication (33), and increased tropism for Langerhans cells has been linked to heterosexual transmission (34). Because LTR-*luc* appears also to be expressed in these cells in Tg mice, analysis of these animals may reveal host factors that mediate expression from the LTR in human Langerhans cells thus providing clues to the role of different cell tropisms in increased transmission and the involvement of UV light in human disease.

Even though the relationship of LTR regulation in transgenic mice to the pathogenesis of HIV in humans remains speculative, the use of the LTR-*luc* fusion in mouse cells serves as an excellent model for demonstrating the potential of *in vivo* monitoring and may help to identify and characterize human cellular factors that interact with viral-encoded Tat (35). The Tat transactivation of the HIV LTR differs in cells derived from humans and rodents (36); thus, studies comparing host factors from these two species may lead to an understanding of the transactivation mechanism and ultimately to the generation of rodent models for studying HIV pathogenesis. Small animal models for HIV pathogenesis would be invaluable in the discovery of vaccines and new antiviral therapies for HIV. Despite the obvious caveats related to using HIV LTR-*luc* Tg mice as a model for HIV pathogenesis at this time, initial data suggest that the differential expression of this promoter during murine development may also hold tremendous potential for understanding gene regulation involved in mammalian ontogeny. Moreover, development of therapies for a wide variety of human diseases could be accelerated with access to real-time gene expression data from living mammalian animal models.

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