Exogenous control of mammalian gene expression via modulation of translational termination

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Here, we describe a system for the exogenous control of gene expression in mammalian cells that relies on the control of translational termination. To achieve gene regulation, we modified protein-coding sequences by introduction of a translational termination codon just downstream from the initiator AUG codon. Translation of the resulting mRNA leads to potent reduction in expression of the desired gene product. Expression of the gene product can be controlled by treating cells that express the mRNA with either aminoglycoside antibiotics or several nonantibiotic compounds. We show that the extent of regulation of gene expression can be substantial (60-fold) and that regulation can be achieved in the case of a variety of different genes, in different cultured cell lines and in primary cells *in vivo*. This gene regulation strategy offers significant advantages over existing methods for controlling gene expression and should have both immediate experimental application and possible clinical application.

The ability to exogenously control the expression of genes in mammalian cells has been a powerful tool of biomedical research[1–4]. In particular, gene regulation technology has played a key part in efforts to understand the role of specific gene products in fundamental biological processes and in both normal development and disease states[1]. It is likely that this form of genetic technology will continue to have an impact on a variety of areas of basic research and may even enable new therapeutic paradigms, such as the regulated delivery of protein therapeutics[3]. The technology may also have significant future impact upon the safety of gene therapy strategies.

To date, most of the gene regulation systems commonly used are based on the control of transcription[1]. In spite of their considerable utility, these systems possess some significant limitations because of their reliance on chimeric transcriptional transactivators and specialized promoter elements. Such limitations include the requirement for co-introduction of genes encoding the relevant transcriptional transactivator along with the gene to be regulated, and the inability to provide for the 'on-off regulation' of a gene in the context of its own endogenous transcriptional control elements.

More recently, several RNA-only strategies for the control of gene expression have been developed that may overcome some of the limitations of transcription-based gene regulation systems. Such strategies rely on either RNA interference or siRNA technology[5] or ribozyme-mediated RNA self-cleavage[6]. Here, we provide proof of principle for another RNA-only gene regulation strategy that relies on the control of translational termination. We show that the extent of induction of expression of gene products obtainable using this strategy can be substantial, that the induction of expression is rapid and that regulation of expression can be achieved in both *in vitro* and *in vivo* contexts. This system should extend the experimental utility of existing systems for the regulation of genes in mammalian cells and may have important clinical application in the setting of gene therapy protocols.

RESULTS

Design features of translation-based gene regulation system

The strategy for controlling gene expression via the modulation of translational termination is shown in **Figure 1a**. First, a translational termination (nonsense) codon is introduced into transgene coding sequences, close to the AUG codon that serves to initiate translation of the complete protein. Upon introduction of vectors encoding transgene sequences into cells, translation of the resulting mRNA should result in production of only a short nonfunctional peptide. Addition of small molecules capable of suppressing translational termination should result in production of the desired full-length protein. Based on previous studies that have shown that aminoglycoside antibiotics are capable of suppressing nonsense mutations in mammalian cell lines and in animal models[7–11], and may act similarly in humans[12], we expected that aminoglycoside antibiotics could serve as inducers of gene expression in such a system. We chose G418 (or Geneticin)[13] for our initial studies, based on a number of published reports that it was the most effective aminoglycoside for suppressing nonsense mutations in mammalian cells.[7,10,11]

To prevent the constitutive generation of truncated transgene products that might engender immune responses *in vivo*, we positioned nonsense codon sequences very close to the initiator AUG, such that translation termination would result in the production of a short two-to-three amino-acid peptide, a peptide size insufficient for classic antigen presentation[14]. Such a configuration of sequences was modeled after a naturally occurring nonsense mutation in the human gene encoding apolipoprotein CII ($APOC2_{Paris2}$), which results in the near-complete loss of the corresponding gene product in an
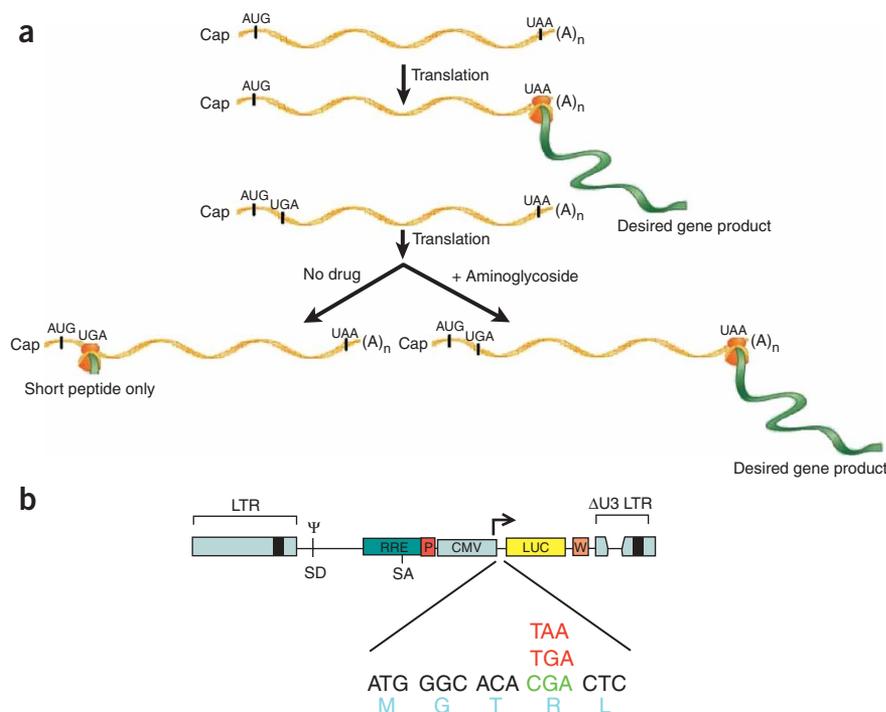
Figure 1 Design features of translation-based system for gene regulation. (**a**) Schematic diagram of gene regulation strategy. Relative positions of cap and polyadenylation sequences and initiation and termination codons within mRNA are shown, as well as products of translation in presence and absence of aminoglycoside. Brown structure and green 'ribbon' denote ribosome translating mRNA and nascent polypeptides produced, respectively. (**b**) Sequence configuration of initiation and termination codons used to generate initial luciferase fusion proteins are shown, as well as structure of lentiviral vector used for studies.

individual carrying the mutation[15]. The relevant nucleotide sequences of wild-type and mutant forms of *APOC2* are shown in **Figure 1b**.

Gene regulation *in vitro* by suppression of translation termination

To facilitate quantitative measurement of both spontaneous read-through of specific termination codon configurations and the ability of specific small molecules to suppress translational termination, we first generated a lentiviral vector encoding a luciferase reporter in which the first five codons of either wild-type or mutant (TGA-containing) *APOC2* sequences were fused to luciferase-coding sequences (**Fig. 1b**) and used the resulting virus to transduce human FG293 cells. We then exposed transduced cells to G418 at various drug concentrations for 48 h and subsequently assayed extracts of the cells for luciferase expression. Cells transduced by vectors encoding wild-type *APOC2*-luciferase sequences expressed high levels of luciferase that were not dependent upon the presence of antibiotic (**Fig. 2a**). In contrast, cells transduced by vectors encoding the mutant *APOC2*-luciferase expressed only low levels of luciferase in the absence of G418 (approximately 1.2% the activity of the wild type) but could be induced to strongly express luciferase by the addition of the antibiotic (**Fig. 2b**). Maximum induction of expression was observed in the presence of 300 μg/ml G418, representing an approximately 60-fold increase in reporter expression relative to the uninduced state. The efficiency of suppression of translational termination was extremely high (more than 70% of the level of wild-type reporter expression observed in the absence of the nonsense codon). Concentrations of G418 greater than 300 μg/ml did not lead

to greater levels of induction (**Fig. 2b**). Northern analysis of RNA isolated from cells transduced with viruses encoding either wild-type or mutant Apo CII–luciferase fusion proteins showed that comparable amounts of mRNA were produced, whether or not G418 was present (data not shown).

To establish the kinetics of induction of gene expression after administration of aminoglycoside antibiotic, we exposed the same transduced FG293 cells described above to G418 (200 μg/ml) and measured the level of reporter activity as a function of time (**Fig. 2c**). As soon as 1 h after induction, the first time period tested, we detected levels of luciferase substantially higher than the levels observed before addition of drug (1.4% at $t = 1$ h versus 0.8% wild-type activity at $t = 0$). At 6, 12 and 24 h after induction, 6.1%, 25.3% and 38.1%, respectively, of wild-type activity was achieved, with maximal induction noted at 48 h after exposure of the cells to G418 (60-fold induction; 51.3% of wild-type activity). Cells cultured for more than 1 month in G418 (200 μg/ml) did not show any overt signs of toxicity or alteration in growth rate (data not shown).

We next performed analogous induction experiments in other commonly used cell lines, including A549, Vero, Cos 7, B16 and C2C12, to establish the general utility of the gene regulation system. In the case of each cell line, we observed a reasonable range of induction of gene expression in response to addition of G418 (300 μg/ml; **Fig. 2d**).

To confirm that the ability to regulate gene expression was not specifically related to the choice of reporter, method of gene delivery or both, we generated both lentiviral and standard plasmid expression vectors (pHDM[16]) encoding a regulatable form of a destabilized ZS-Green reporter (designed similarly to the luciferase reporter described above) and used them to transfect FG293 cells. Cells transfected with the ZS-Green reporter virus displayed little or no fluorescence in the absence of inducer, yet readily expressed the reporter 72 h after administration of G418 (300 μg/ml; **Fig. 2e**). We obtained similar results after transfection of either the lentiviral plasmid DNA construct used to generate virus or the pHDM-derived expression vector (data not shown).

Finally, to further demonstrate the general utility of the regulation strategy, and to explore the specific application of the approach to the regulation of secreted gene products, we modified cDNA sequences encoding human growth hormone (hGH) by introducing a UGA termination codon seven nucleotides downstream of the initiator ATG. We then introduced the resulting coding sequences into a lentiviral vector. After transduction of FG293 cells with the hGH-encoding virus, we cultured the transfected cells for an additional 48 h in the presence of increasing concentrations of G418 (or no drug), and then we assayed supernatant from the transfected cells for hGH. In the absence of G418, only low levels of hGH could be detected in the culture media of the transfected cells (**Fig. 2f**). Addition of G418, however, led to the efficient induction of hGH expression (approximately 40-fold induction).
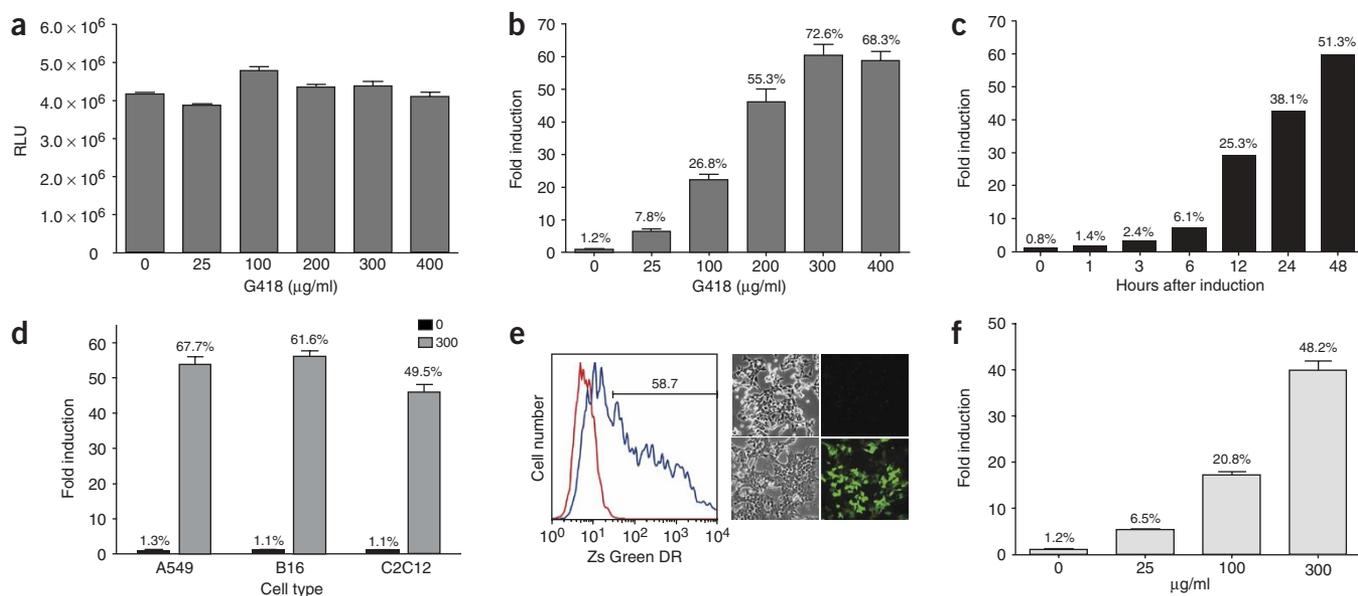
Figure 2 Aminoglycoside-induced suppression of translational termination can be used to regulate gene expression in cultured cells. (**a,b**) Expression of luciferase in FG293 cells transfected with lentiviral vectors encoding either the wild-type (**a**) or mutant (**b**) luciferase fusion product, as a function of aminoglycoside concentration. Luciferase activity is measured in relative light units (RLU); percent of levels of luciferase activity achieved with wild-type luciferase fusion product are indicated for each drug concentration. (**c**) Kinetics of induction of luciferase gene expression in FG293 cells transfected with the lentiviral vector encoding the mutant luciferase fusion product. We exposed cells to a constant concentration of G418 (200 μg/ml) and assayed for luciferase expression at hourly intervals. Percent of wild-type luciferase activity is indicated for each time point and represents the average of five independent determinations. (**d**) Induction of luciferase expression in various cell lines infected with lentiviral vectors encoding the mutant luciferase fusion product. Induction is shown after exposure of cells to 300 μg/ml G418 for 48 h. Luciferase activity is measured in RLU; percent of levels of luciferase activity achieved with wild-type luciferase fusion product are indicated for each cell line and represent the average of three independent determinations. (**e**) Induction of ZS-Green DR in cells infected with lentiviral vectors encoding an inducible ZS-Green DR gene product. The left panel is a histogram of fluorescence before (red line) or after (blue line) induction of reporter expression via G418 administration (300 μg/ml). The right panels show photographs of cells before (top panels) and after (bottom panels) induction. (**f**) Regulated expression of hGH. The expression of hGH in FG293 cells infected with a lentiviral vector encoding an inducible form of the gene encoding hGH as a function of aminoglycoside concentration. Percent of levels of hGH production achieved with a constitutively expressed hGH gene product are indicated for each drug concentration. Error bars represent standard deviation.

Other small molecules can regulate gene expression

Based on the activity of G418, we also surveyed the ability of other aminoglycoside antibiotics to induce reporter expression in the human FG293 cells used above. Each of the other antibiotics tested (gentamicin, amakacin, tobramycin and paromomycin) were capable of inducing luciferase gene expression, albeit to different extents (**Fig. 3a**). Consistent with previous studies of the ability of antibiotics to suppress nonsense mutations in endogenous genes[11], G418 was more effective at suppressing nonsense mutations than any of the other aminoglycosides tested.

In addition to aminoglycosides, a number of nonantibiotic small molecules capable of suppressing nonsense mutations in endogenous genes in mammalian cells have recently been identified by high-throughput screening methods[17]. To establish whether such molecules might also serve as inducers of gene regulation in translation-based gene regulation systems, we evaluated two compounds reported to possess the ability to moderately suppress nonsense mutations[17], using the transduced FG293 cells described above. Both of the compounds tested were capable of inducing gene expression in a dose-dependent fashion, and in the case of one compound, greater than 20-fold induction of expression could be achieved (**Fig. 3b,c**). Notably, at least one of the compounds, 3-{2-[4-(1,1-dimethyl-propyl)-phenoxy] acetylamino}-benzoic acid, displayed activity at concentrations (for example, 5 μg/ml) substantially lower than the concentration of G418 needed to achieve a comparable level of induction. Although we have

not extensively characterized these compounds for their effects on cells, they were not acutely toxic to cells except at concentrations (100 μg/ml) well above the concentrations needed for maximal induction (data not shown).

Modulation of basal and inducible levels of transgene expression

Previous studies have shown that different translational termination codons are recognized at different efficiencies and that nucleotides directly adjacent to translation termination sequences can affect termination efficiencies and the extent of suppression of termination that can be achieved[7,8,10,18,19]. Because the ability to vary basal and inducible levels of expression might be useful for different experimental applications, we next evaluated several additional configurations of termination codons and adjacent sequences for both their efficiency of translational termination and their ability to be suppressed by addition of specific compounds (**Table 1**). Of all the configurations tested, sequences derived from the $APOC2_{Paris2}$ (TGAC) gene exhibited the greatest spontaneous read-through (1.2%) and the largest extent of 'induction' with aminoglycoside treatment (61-fold induction, with 73% of wild-type expression achieved). Although as expected[10], the ATAAA configuration displayed a substantially lower level of spontaneous read-through (0.24%), the levels of induction that could be achieved (42-fold) were quite comparable to those achieved with the TGAC configuration. Whereas the placement of two UGA codons directly adjacent to each other
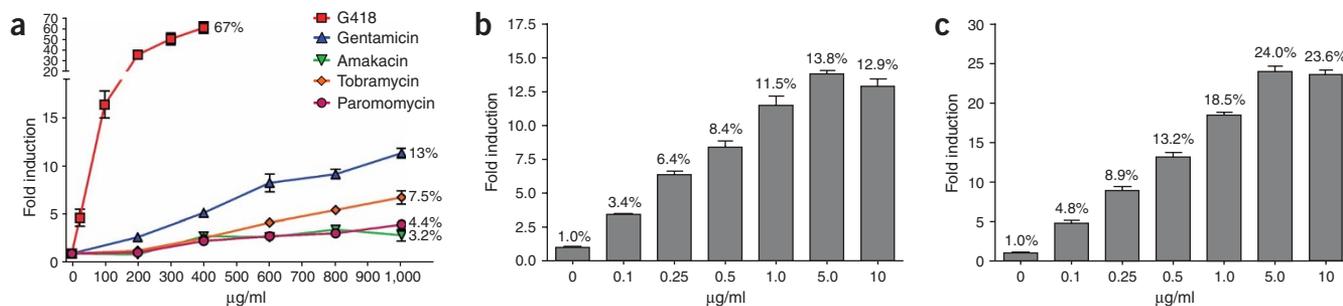
Figure 3 Other aminoglycosides and nonantibiotic compounds can be used to regulate gene expression via suppression of nonsense mutations. (**a**) We tested aminoglycosides *in vitro* using FG293 cells infected with virus encoding the mutant luciferase fusion product. We exposed cells to drugs at the indicated concentrations for 48 h before being assayed for luciferase expression, and the results represent the average of three independent experiments. Percent of levels of luciferase activity achieved with wild-type luciferase fusion product are indicated for each drug concentration. (**b,c**) Expression of luciferase in FG293 cells infected with lentiviral vectors encoding the mutant luciferase fusion product as a function of the concentration of the nonantibiotic compounds 3-[2-(4-tert-butyl-phenoxy)-acetylamino]-benzoic acid (**b**) and 3-{2-[4-(1,1-dimethyl-propyl)-phenoxy]acetylamino}-benzoic acid (**c**). Luciferase activity is measured in relative light units and percent of levels of luciferase activity achieved with wild-type luciferase fusion product is indicated for each drug concentration. The results shown for **b** and **c** each represent the average of three independent experiments. Error bars represent standard deviation.

decreased the basal levels of expression (0.39–0.43% of wild-type expression), the level of induction achieved was only moderate (26–28-fold). Introduction of sequences between the two UGA codons to provide a +4 nucleotide previously shown to be permissive to read-through[19] actually led to a further decrease in basal levels (0.11%) and an 18-fold induction. Lastly, the juxtaposition of two UAA codons led to a low basal level (0.22%) but a poor level of induction (fivefold). These studies indicate that specific configurations of termination codons and adjacent nucleotide sequences can indeed be used to provide for different levels of basal and induced gene expression.

Translation-based gene regulation *in vivo*

To further establish the general applicability of the gene regulation system, we asked whether gene regulation at the level of translation could be readily accomplished in the *in vivo* setting. In a first animal model, intratracheal delivery (see Methods) of a lentiviral vector carrying wild-type or mutant *APOC2*-luciferase sequences was used to assess the ability to regulate luciferase expression in mouse lung tissue by administration of aminoglycoside. Five days after transduction, before administration of aminoglycoside, we imaged mice for luciferase expression using the Xenogen IVIS noninvasive bioluminescent imager[20]. In contrast to mice infected with the vector encoding

wild-type *APOC2*-luciferase sequences (wild-type luciferase mice), which showed a strong photon signal indicative of luciferase activity, mice that were infected with the vector encoding mutant *APOC2*-luciferase sequences (mutant luciferase mice) showed no detectable signal (data not shown). We then injected mice once daily for 4 d, with either G418 (1.5 mg per injection) or PBS, except on the fourth day when we injected the mice twice, once in the morning and then again 60 min before luciferase imaging. As expected, upon imaging, wild-type luciferase mice showed strong luciferase expression, whether or not G418 had been administered (**Fig. 4a,b**). In contrast, whereas mutant luciferase mice that were injected with PBS continued to show no detectable luciferase activity (**Fig. 4c**), mutant luciferase mice that were subsequently given G418 showed substantial induction of luciferase activity (**Fig. 4d**). The five mice treated in this manner had substantial luciferase expression, resulting in an average of 27% of the level of expression observed in wild-type luciferase mice. Because of the lack of detectable luciferase expression in uninduced mice, it was not possible to determine the extent of induction.

To further evaluate the potential *in vivo* utility of the gene regulation system, we used a standard mouse bone marrow transplantation model to assess the ability to regulate genes introduced into hematopoietic cells with lentiviral vectors. For these studies, we transduced 500 purified C57/Bl6 hematopoietic stem cells (SP cells)[21] with lentiviral vectors encoding either wild-type or mutant reporters and then we introduced them into lethally irradiated recipients. Four weeks after bone marrow transplantation, we imaged reconstituted mice for luciferase expression and then subsequently treated them with PBS or G418 daily for 3.5 d (1.5 mg/day). Before the administration of PBS or G418, mice reconstituted with cells transduced by virus encoding mutant *APOC2*-luciferase sequences (mutant luciferase mice) showed negligible luciferase expression, whereas mice reconstituted with cells transduced by viruses encoding wild-type *APOC2*-luciferase sequences (wild-type luciferase mice) showed robust luciferase expression (data not shown). After administration of PBS or G418, wild-type luciferase mice continued to show strong luciferase expression (**Fig. 4e,f**). Although administration of PBS had no effect on luciferase expression in mutant luciferase mice (**Fig. 4g**), treatment of those mice with antibiotic led to the strong induction of luciferase (**Fig. 4h**). Quantification of luciferase activity in four mutant luciferase mice before and after drug administration showed an average 65-fold induction of luciferase expression, with induced levels

Table 1 Basal and inducible levels of transgene expression are dependent upon the choice of termination codon and adjacent sequences

	Fold induction	Induced	Uninduced
A **TGA** C	61	73	1.2
A **TAA** A	42	10	0.24
A **TGA TGA** C	28	11	0.39
A **TGA TGA** G	26	11	0.43
A **TGA** CTC **TGA** CTC	18	2.0	0.11
A **TAA** ATC **TAA** ATC	5.0	1.1	0.22

Levels of luciferase reporter gene expression obtained with additional configurations of nonsense codon sequences and adjacent sequences. Uninduced and induced refer to levels of luciferase achieved in absence and presence of aminoglycoside, respectively; the values are expressed as the percentage of luciferase activity achieved in cells carrying the luciferase construct possessing no nonsense mutations and not exposed to drug and represent the average of five independent determinations. Fold induction refers to the ratio of induced to uninduced levels of activity.
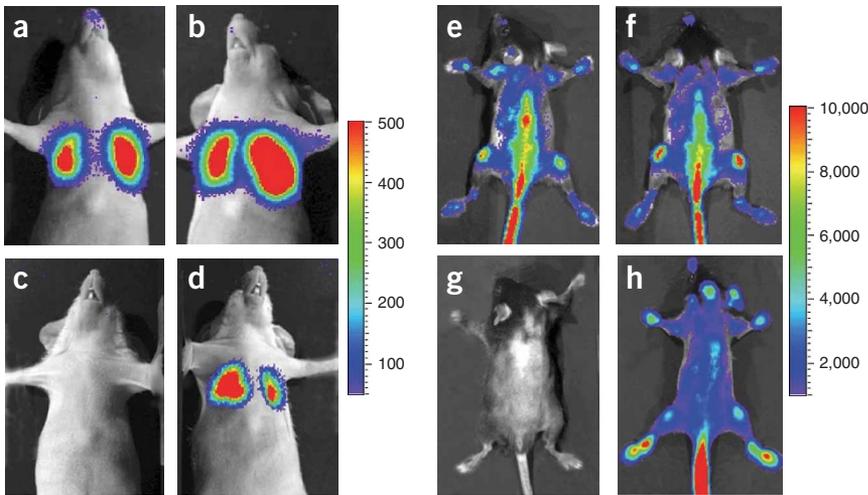
Figure 4 *In vivo* regulation of gene expression in mouse lungs and mouse hematopoietic cells. (**a**–**d**) After intratracheal administration of virus encoding a regulatable luciferase reporter, luciferase expression was measured in lungs of mice infected with virus encoding wild-type luciferase fusion product and exposed to PBS (**a**) or G418 (**b**), or in mice infected with virus encoding mutant luciferase products and exposed to PBS (**c**) or G418 (**d**). We conducted imaging as described in Methods. Experiments involved the use of five mice per experimental variable. Mice shown are representative of the entire cohort. (**e**–**h**) *In vivo* regulation of gene expression in mouse hematopoietic cells. Luciferase expression in mice reconstituted with hematopoietic stem cells transduced with viruses encoding wild-type (**e**,**f**) or mutant (**g**,**h**) luciferase products and subsequently treated with PBS (**e**,**g**) or G418 (**f**,**h**). Experiments involved the use of four mice per experimental variable. Mice shown are representative of the entire cohort.

approaching an average of 51% of the expression observed in wild-type luciferase mice.

DISCUSSION

Here, we show the feasibility of using translation-based systems for the control of gene expression in mammalian cells both *in vitro* and *in vivo*. Perhaps the most important design feature of this gene regulation strategy is its simplicity: the only 'genetic element' required is a three-nucleotide DNA sequence encoding a translational termination codon (and in some cases, additional sequences that influence the spontaneous suppression of termination). Because the regulatory element is placed within transgene coding sequences, it should be possible to achieve regulation in the context of virtually any expression vector, and to provide for the regulation of expression of protein coding sequences within the context of their normal endogenous control elements. Although the range of inducibility obtainable with the system (in some cases more than 60-fold) is considerably more limited than that of gene regulation systems based on the control of transcription or RNA self-cleavage, the ability to fine-tune the basal and induced levels of gene expression through the use of specific termination codons and adjacent sequences may mitigate this limitation in many experimental situations.

One inherent limitation of the gene regulation system as described is that the induced transgene product will in many cases possess an altered amino acid sequence because of the amino acid introduced as a consequence of misreading of the nonsense codon. Although this may limit application of the technology, it should be possible in some cases to design codon substitutions that lead to the production of a fully wild-type gene product, by taking account of knowledge regarding the amino acids that are inserted when specific nonsense codons are misread[22]. Furthermore, in the special case of regulating the expression of secreted gene products, exemplified by our experiments with

hGH, the mature gene product that is secreted is generated by co-translational cleavage of the preprotein[23], and therefore modifications of sequences adjacent to the initiator AUG will not lead to alterations in the structure of the mature gene product. A second, more speculative limitation of the system relates to the possibility that insertion of a nonsense codon within transgene coding sequences may engender the 'reinitiation' of translation at downstream AUGs, a phenomenon previously shown to be influenced by both the presence of translation termination codons and the distance between those codons and the next downstream AUG[24]. Future experiments will be necessary to determine whether this issue would ever practically limit the application of the gene regulation strategy.

For immediate experimental application of this translation-based gene regulation system, practical, useful inducers of gene expression are crucial. In this regard, we have shown that G418 and other aminoglycosides can be effectively used to regulate gene expression in a number of cell lines and in animals at concentrations that are not acutely toxic. Similarly, others have shown that G418 can be used at relatively nontoxic doses to suppress nonsense mutations in cell lines and in animal models of inherited disease[11,25]. Therefore, in spite of the well-known toxicities of G418 and other aminoglycosides *in vitro* and *in vivo*[26], we believe the gene regulation system as described here will have immediate experimental application. Nevertheless, it will still be important in the future to identify additional small molecules capable of suppressing translation termination. Recent animal studies, which have suggested that the suppression of nonsense mutations could provide a new therapeutic approach to the treatment of certain inherited diseases[9–11], have fueled efforts to identify nontoxic molecules that efficiently suppress nonsense mutations in endogenous chromosomal genes. As we have shown, at least some of the molecules identified through those studies can indeed serve to control the expression of vector-encoded genes as well. The availability of new small molecules with improved suppression activity and novel pharmacokinetic properties should further expand the utility of translation-based gene regulation strategies.

Lastly, in addition to experimental applications, we believe that the system described here could also have significant near-term clinical applications as well, in the setting of gene therapy. Recently, there has been a heightened awareness of the need to rapidly incorporate gene regulation technology into gene therapy strategies, based on the serious adverse events observed in a clinical gene therapy trial[27]. One very important technical capability related to vector safety well suited to the system described here is the ability to conditionally kill transduced cells possessing abnormal growth properties. Such an application would require only the acute administration of inducer, and depending upon the conditionally toxic marker used, may not require high levels of induction for effective cell killing to occur. With regard to the feasibility of using currently available inducers in a clinical setting, it is noteworthy that a number of clinical studies designed to examine the ability of conventional aminoglycosides and

other novel nonantibiotics to suppress nonsense mutations responsible for several different inherited diseases have already been initiated[2,12,28]. More broad clinical application of the gene regulation technology to the regulated delivery of protein therapeutics is also an exciting possibility.

METHODS

Compounds and cell culture. We used G418 (Geneticin) in powder form (Invitrogen), reconstituted in sterile deionized water to a stock concentration of 80 mg/ml and frozen at –20 °C. We obtained final concentrations by diluting the stock directly into tissue culture medium. We obtained the novel non-antibiotic compounds 3-[2-(4-tert-butyl-phenoxy)-acetylamino]-benzoic acid and 3-{2-[4-(1,1-dimethyl-propyl)-phenoxy]acetylamino}-benzoic acid in powder form (ChemBridge). We reconstituted them first in DMSO and then diluted them in sterile deionized water to a stock concentration of 10 mg/ml and froze them at –20 °C. We obtained final concentrations by diluting the stock directly into tissue culture medium. We obtained all cell lines from the American Type Culture Collection (ATCC) and maintained them in DMEM (4.5 g/l glucose) supplemented with 10% FCS, antibiotics and L-glutamine (all from Invitrogen).

Vectors, viral production and *in vivo* transduction. We created the replication-defective lentiviruses used in the gene transfer experiments using a five-plasmid transfection procedure. Briefly, we transfected 293T cells using TransIT 293 (Mirus) according to the manufacturer's instructions using the pHAGE backbone lentiviral vector together with four expression vectors encoding the packaging proteins *gagpol*, *rev*, *tat* and the G-protein of the vesicular stomatitis virus. The pHAGE lentiviral backbone used in the experiments was an optimized self-inactivating nonreplicative vector derived from the pHR'CMV-lacZ vector and will be described elsewhere (A.B. Balazs *et al.*, unpublished data). The *gagpol* helper plasmid has been codon-optimized for efficient mammalian expression and modified to severely reduce the homology with the *gag* sequences present in the vector packaging signal (J. Gray, J. Lee & R.C.M., unpublished results). All of the expression helper plasmids contain only the coding sequences, with minimal 5′ or 3′ untranslated sequences and no introns. In addition, the backbone contains the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)[29] to enhance levels of transcription and gene expression. We collected viral supernatants starting 24 h after transfection, for four consecutive collections every 12 h, pooled and filtered through a 0.45 μm filter. We then concentrated viral supernatants ∼100-fold by ultracentrifugation in a centrifuge (Beckman) for 1.5 h at 16,500 r.p.m. Using this protocol, we achieved titers of ∼5 × 10^8 to 1 × 10^9 transducing units/ml.

For intratracheal infections, we anesthetized nude mice and then delivered 100 μl of concentrated virus upon normal inhalation using a blunt-ended 16-gauge needle inserted in such a way as to depress the tongue.

HSC purification, viral transduction and bone marrow transplantation. We obtained purified hematopoietic stem cells (HSCs) by isolating bone marrow SP cells using fluorescence-activated cell sorting after Hoechst staining, as previously described[21]. Viral transduction of sorted HSCs has been optimized by our laboratory and described elsewhere[30]. We purchased all mice used in bone marrow transplants from the Jackson Laboratories and maintained them in a specific pathogen-free animal facility at Harvard Medical School. We lethally irradiated Ly5.2 recipient mice with two doses of 7 Gy, 3 h apart, 1 d before bone marrow transplant and maintained them with antibiotic-supplemented water for 15 d. We injected transduced SP cells from Ly5.1 donors retro-orbitally into recipient mice under isofluorane anesthesia. All animal procedures were approved by the Standing Committee on Animals of Harvard Medical School.

***In vitro* reporter assays.** We infected FG 293, A549, B16 or C2C12 cells with viral supernatants at a multiplicity of infection (MOI) of 10. After 72 h, we split cells into six-well plates, exposed them to various concentrations of G418 or nonaminoglycoside inducer for 48–72 h and lysed them. We then assayed protein extracts for luciferase expression using the Promega Luciferase Assay System.

Noninvasive bioluminescent imaging. Before imaging, we anesthetized mice and injected them with 150 μl luciferin (30 mg/ml, Xenogen). We then took a series of bioluminescent images for up to 30 min using the Xenogen IVIS imager. We quantified photon output at the plateau of the time course using the Living Image software. We calculated relative induction based on the photon output in the mice before and after drug treatment.

Production and quantification of hGH. We created wild-type and mutant (TGA) cDNA sequences using PCR, cloned them into the abovementioned lentiviral vector and used them to make high-titer virus as described above. We infected FG293 cells with viral supernatants at an MOI of 10, and 72 h later, we split the cells into six-well plates and exposed them to various concentrations of G418 for 48–72 h. We collected the supernatants, filtered them through 0.45 μm syringe filters and quantified them using a commercially available sandwich ELISA (Calbiotech).

1. Gossen, M., Bonin, A.L., Freundlieb, S. & Bujard, H. Inducible gene expression systems for higher eukaryotic cells. *Curr. Opin. Biotechnol.* **5**, 516–520 (1994).
2. Wang, Y., O'Malley, B.W., Jr., Tsai, S.Y. & O'Malley, B.W. A regulatory system for use in gene transfer. *Proc. Natl. Acad. Sci. USA* **91**, 8180–8184 (1994).
3. Rivera, V.M. *et al.* Long-term pharmacologically regulated expression of erythropoietin in primates following AAV-mediated gene transfer. *Blood* **105**, 1424–1430 (2005).
4. Suhr, S.T., Gil, E.B., Senut, M.C. & Gage, F.H. High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl. Acad. Sci. USA* **95**, 7999–8004 (1998).
5. McManus, M.T. & Sharp, P.A. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* **3**, 737–747 (2002).
6. Yen, L. *et al.* Exogenous control of mammalian gene expression through modulation of RNA self-cleavage. *Nature* **431**, 471–476 (2004).
7. Burke, J.F. & Mogg, A.E. Suppression of a nonsense mutation in mammalian cells *in vivo* by the aminoglycoside antibiotics G-418 and paromomycin. *Nucleic Acids Res.* **13**, 6265–6272 (1985).
8. Martin, R., Mogg, A.E., Heywood, L.A., Nitschke, L. & Burke, J.F. Aminoglycoside suppression at UAG, UAA and UGA codons in *Escherichia coli* and human tissue culture cells. *Mol. Gen. Genet.* **217**, 411–418 (1989).
9. Barton-Davis, E.R., Cordier, L., Shoturma, D.I., Leland, S.E. & Sweeney, H.L. Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice. *J. Clin. Invest.* **104**, 375–381 (1999).
10. Howard, M.T. *et al.* Sequence specificity of aminoglycoside-induced stop codon readthrough: potential implications for treatment of Duchenne muscular dystrophy. *Ann. Neurol.* **48**, 164–169 (2000).
11. Sangkuhl, K. *et al.* Aminoglycoside-mediated rescue of a disease-causing nonsense mutation in the V2 vasopressin receptor gene *in vitro* and *in vivo*. *Hum. Mol. Genet.* **13**, 893–903 (2004).
12. Wilschanski, M. *et al.* Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. *N. Engl. J. Med.* **349**, 1433–1441 (2003).
13. Loebenberg, D., Counels, M. & Waitz, J.A. Antibiotic G418, a new micrommomospora-produced aminglycoside with activity against protozoa and helminths: antiparasitic activity. *Antimicrob. Agents Chemother.* **7**, 811–815 (1975).
14. Rammensee, H.G., Falk, K. & Rotzschke, O. Peptides naturally presented by MHC class I molecules. *Annu. Rev. Immunol.* **11**, 213–244 (1993).
15. Parrott, C.L., Alsayed, N., Rebourcet, R. & Santamarina-Fojo, S. ApoC-IIParis2: a premature termination mutation in the signal peptide of apoC-II resulting in the familial chylomicronemia syndrome. *J. Lipid Res.* **33**, 361–367 (1992).
16. Ory, D.S., Neugeboren, B.A. & Mulligan, R.C. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA* **93**, 11400–11406 (1996).
17. International patent application WO 2004/009533 A1.

18. Brown, C.M., Stockwell, P.A., Trotman, C.N. & Tate, W.P. Sequence analysis suggests that tetra-nucleotides signal the termination of protein synthesis in eukaryotes. *Nucleic Acids Res.* **18**, 6339–6345 (1990).
19. McCaughan, K.K., Brown, C.M., Dalphin, M.E., Berry, M.J. & Tate, W.P. Translational termination efficiency in mammals is influenced by the base following the stop codon. *Proc. Natl. Acad. Sci. USA* **92**, 5431–5435 (1995).
20. Contag, C.H. & Bachmann, M.H. Advances in *in vivo* bioluminescence imaging of gene expression. *Annu. Rev. Biomed. Eng.* **4**, 235–260 (2002).
21. Goodell, M.A., Brose, K., Paradis, G., Conner, A.S. & Mulligan, R.C. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J. Exp. Med.* **183**, 1797–1806 (1996).
22. Nilsson, M. & Ryden-Aulin, M. Glutamine is incorporated at the nonsense codons UAG and UAA in a suppressor-free *Escherichia coli* strain. *Biochim. Biophys. Acta* **1627**, 1–6 (2003).
23. Rothman, J.E. Mechanisms of intracellular protein transport. *Nature* **372**, 55–63 (1994).
24. Peabody, D.S. & Berg, P. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. *Mol. Cell. Biol.* **6**, 2695–2703 (1986).
25. Lai, C.H. *et al.* Correction of ATM gene function by aminoglycoside-induced read-through of premature termination codons. *Proc. Natl. Acad. Sci. USA* **101**, 15676–15681 (2004).
26. Kahlmeter, G. & Dahlager, J.I. Aminoglycoside toxicity - a review of clinical studies published between 1975 and 1982. *J. Antimicrob. Chemother.* **13** Suppl A, 9–22 (1984).
27. Hacein-Bey-Abina, S. *et al.* LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415–419 (2003).
28. Ainsworth, C. Nonsense mutations: running the red light. *Nature* **438**, 726–728 (2005).
29. Zufferey, R., Donello, J.E., Trono, D. & Hope, T.J. Woodchuck hepatitis virus post-transcriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73**, 2886–2892 (1999).
30. Mostoslavsky, G. *et al.* Efficiency of transduction of highly purified murine hematopoietic stem cells by lentiviral and oncoretroviral vectors under conditions of minimal *in vitro* manipulation. *Mol. Ther.* **11**, 932–940 (2005).