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Testing transgenic regulatory elements through live mouse imaging

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Abstract To overcome positional and methylation effects on transgene expression, we developed a universal cloning cassette for in vivo assessment of regulatory elements using the luciferase reporter gene and the CCCD camera. Monitoring luciferase expression pattern in live mice enables screening of large numbers of transgenic founders quickly and inexpensively. We demonstrate that in the engineered transgenic mice, the chicken β-globin 5′HS4 insulator did not always provide the desirable expression pattern, and the Island Element, responsible for the demethylation of the surrounding DNA region, was not beneficial. Both tested liver-specific and developmentally regulated promoters exhibited the expected expression pattern in most transgenic founders.

Keywords: CCCD camera; Transgene; Insulator; In vivo imaging; Promoter; Liver

1. Introduction

Genetically modified mice, including transgenic animals, serve as important models of human diseases. However, stable transgene expression in transgenic mice depends on many factors and is difficult to achieve in some cases [1,2]. Unstable transgene expression can be a result of a 'position effect', which is caused by the influence of the genomic environment on the transgene expression in the site of transgene integration [3,4]. Different host factors may also silence transgene expression by hypermethylation [1].

The goal of this study was to develop tools for generation of transgenic mice and for testing regulatory elements that should enhance the efficiency of transgenic mouse production. We generated expression cassettes carrying regulatory elements that would drive methylation resistant, position-independent transgene expression. In this work, the expression was also designed to be liver-specific and developmentally regulated.

In order to overcome the 'position effect', we flanked the desirable transgene by the chicken β-globin 5′HS4 insulators. Chromatin insulators, also known as boundary elements, are DNA sequences capable of suppressing the position effect by blocking the action of distant enhancers and other regulatory elements; moreover, they can shield a locus from repressive effects of flanking chromatin. The presence of insulators effectively increases the yield of phenotypically desirable transgenic mice obtained from each integration session [3–5]. The chicken β-globin 5′HS4 element has been shown to function as a typical insulator, shielding transgenes from position effects [6].

In order to prevent transgene methylation, we used the Island Element (IE), which was found to protect itself and flanking sequences from de novo methylation, when placed upstream of a promoter [7,8]. We inserted the IE between the promoter and transgene, in order to obtain protection from methylation for both of them.

To obtain liver-specific expression, two liver-specific promoters were applied: the human serum amyloid protein (SAP) promoter [2,9] or the mouse major urinary protein (MUP) promoter [10,11]. Both of these promoters are expressed only after birth, thus preventing transgene expression at the pre-natal stages. This approach helps avoid potential transgene interference with embryonic developmental programs and reduce immune tolerance to the transgene.

Using the luciferase gene as a reporter enabled monitoring of its expression in vitro, in vivo, and in transgenic mice. In vivo activity and expression pattern of the transgenic lines were monitored by the CCCD camera [12], which provides quantitative bioluminescence imaging of live mice [13,14]. This quick and inexpensive method also enabled us to screen large numbers of transgenic founders for the desirable expression pattern. We confirmed the postnatal expression of the reporter transgene and determined liver specificity, duration and intensity of its expression in different founders. This study demonstrates a non-invasive method of monitoring tissue-specific expression of a transgene, as well as utility of the tested regulatory elements for generation of transgenic mice.

2. Materials and methods

2.1. Plasmid construction

The pNH12 (Fig. 1A) was derived from pJC-13 (provided by Prof. Felsenfeld), which contains two duplicates of the 1.2 kbp chicken β-globin insulator [6]. First, we removed the Neo gene and the enhancer sequences to create the **pISUL**. Second, we inserted artificially synthesized DNA fragments containing sites for rare restriction enzymes (AscI, PacI, FseI, SfiI) flanking the insulator repeats in the pINSUL plasmid; the resulting construct (pNH12) was used as a general cassette for insertion of specific genes at BamHI site between duplicate copies of the chicken β-globin insulator.

The SAP (human serum amyloid protein) promoter was first cloned from the pSAP5-3V (provided by Dr. Yamamura [2] into the pBS-ssp plasmid (which was constructed by deleting the F1 fragment between SspI sites in the pBS-SK$^-$ (Stratagene, Cedar Creek, TX, USA))) at EcoRI–SalI sites. Then it was excised at the XbaI–XhoI sites and cloned upstream the luciferase gene in the pGL3 basic plasmid (Promega, Madison, WI, USA) at NheI–XhoI sites. The resulting plasmid was called **pSAP-LUC** (**pSL**). The IE, kindly provided by Prof. Cedar [8], was inserted between the SAP promoter

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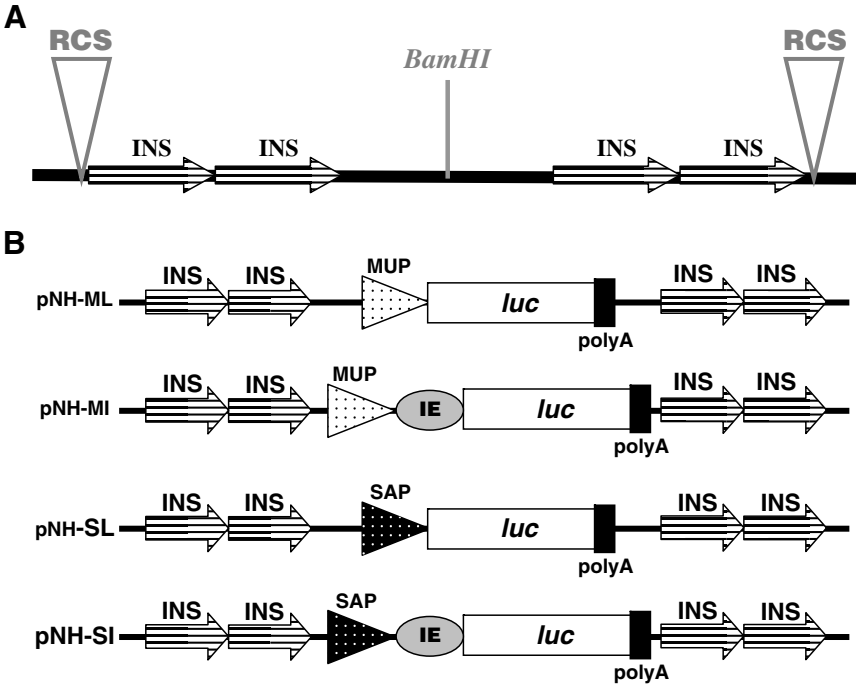

Fig. 1. Generation of expression cassettes. (A) The universal cloning cassette for transgene expression. INS – chicken β-globin 5′HS4 insulator (1.2 kbp), RCS – rare cleavage sites (AscI, PacI, FseI, SfiI). BamHI – cloning site for transgene insulators. (B) The four constructed expression cassettes: firefly luciferase reporter gene (*luc*) under the control of liver-specific developmentally regulated SAP (serum amyloid protein) or MUP (major urinary protein) promoters, polyA – SV40 polyA, IE – Island element sequence, INS – chicken β-globin 5′HS4 insulator (1.2 kbp).

and the luciferase gene at the XhoI–HindIII sites to create the **pSLIE** plasmid. The MUP (mouse major urinary protein) promoter was cloned from the p11AS-SV40-7 (provided by Prof. Rogler [10]) into pBS-ssp at PstI–XbaI sites, then cut by EcoRI–NotI sites and inserted into pCDNA3.1 (Invitrogen, Frederick, MD, USA) to create **pcDNA-MUP**. The **pMUP-LUC** (**pML**) was created by cutting the MUP promoter from pcDNA-MUP at KpnI–XhoI sites and its cloning upstream the luciferase gene into the pGL3 basic plasmid (Promega, Madison, WI, USA). The **pMLIE** was created by replacing the SAP promoter from pSLIE with the MUP promoter at KpnI–XhoI sites. The BamHI fragments from all four plasmids – pML, pMLIE, pSL, pSLIE – contained the promoter and the luciferase gene, with or without IE, and were inserted into the pNH12 cassette to create: pNH-ML, pNH-MI, pNH-SL, pNH-SI, respectively (Fig. 1B). All plasmids were propagated in either JM109 or XL1B strains, and purified using the Qiagen® plasmid purification kits (Hilden, Germany).

2.2. Transfections and in vitro activity assays

AML12 cells (non-transformed mouse hepatocytes) [15] were transiently transfected with luciferase expressing plasmids. Cells were seeded at 0.5×10^5 cells/ml in 12-well plates; growing medium: 1:1 DMEM/HAM's F-12 medium + 10% FCS, 1% pen/strep, 1% ITS-G (insulin–transferrin–selenium; all reagents – from Biological Industries Co., Beit Haemek, Israel), and 40 ng/ml dexamethasone (Sigma, St. Louis, MO, USA). After 24 h, cells were transfected with 0.27 μg of each plasmid: pNH-ML, pNH-MI, pNH-SL, pNH-SI or pGL3p (Promega, Madison, WI, USA) as a control. Each transfection mixture contained also 0.03 μg of pRL plasmid (Promega, Madison, WI, USA) that expresses Renilla Luciferase, to normalize the results. The transfections were performed using the Fugene 6® transfection reagent (Roche Diagnostics, Basel, Switzerland).

Luciferase activity *in vitro*: Firefly luciferase activity was measured 24 h after transfection. Cell lysis and luciferase reactions were carried out using the Dual-Luciferase® Reporter Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The activity was normalized to the Renilla luciferase activity from pRL plasmid.

2.3. Mice

Mice were maintained at the Animal Facility of the Hebrew University Medicine School in a specific pathogen-free unit, under a 12 h light/dark cycle, and were provided with food and water ad libidum. The Institutional Animal Care and Use Committee approved all procedures. DNA purification from mouse tails was performed using either the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) or the Purgene® DNA isolation kit (Gentra systems, Minneapolis, MN, USA).

2.4. Hydrodynamic tail-vein injections

In each experiment, 50 μg/1.5 ml PBS of plasmid DNA was tail vein injected at high pressure into three Balb/c mice. The total volume was injected rapidly, over 5–8 s, into the tail-vein of a mouse, using a 27-gauge needle. Luciferase activity was monitored by CCCD camera after 24 h, as described below.

2.5. Generation of luciferase transgenic mice

Transgenic animals were produced at the Transgenic Unit of the Animal Facility of the Hebrew University Medical School.

Preparation of DNA for microinjection. The appropriate transgene DNA fragments were excised from the vectors (pNH-ML, pNH-MI, pNH-SL, pNH-SI) with three restriction enzymes: AscI, PacI, SfiI, electrophoresed on 1% agarose gel and the isolated band (purified by with the Qiagen® gel extraction kit (Hilden,Germany)) was next purified on an ion exchange column (Elutip-D® column, Schleicher & Schuell, Germany) with Millex®-HV 0.45μm filter (Millipore, Billerica, MA, USA). The purified fragment was ethanol precipitated, dissolved in the injection buffer (7.5 mM Tris, 0.2 mM EDTA, pH 7.5) to a final concentration of 15 ng/μl and injected into a pronucleus of a fertilized egg derived from (C57Black × BALB/c) F1 mice. Embryos at the two-cell stage were transferred into the oviducts of pseudopregnant F1 females. Founder animals were identified by PCR analysis of tail DNA and confirmed by Southern blot analysis. Primers for PCR: luciferase sense: 5′-gagaattacacggcgatctttc, luciferase antisense: 5′-gaggttccatctgccaggta, IE sense: 5′-cgccgctcgagatccttagggagcgatcca, IE antisense: 5′-ccctaaggaagcttccagcaaatgcgttac. When the luciferase PCR fragment (650 bp) was used as a hybridization probe, it was

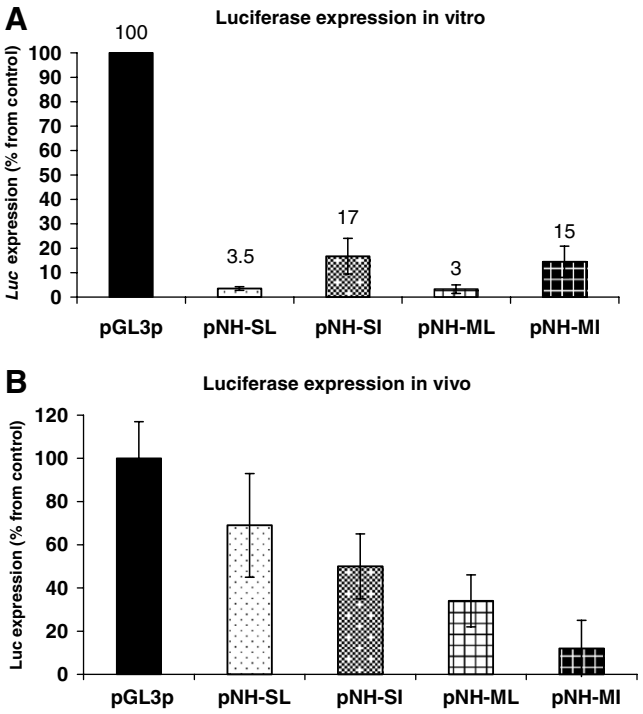

Fig. 2. Assessment of constructed expression cassettes using the luciferase reporter gene. (A) In vitro assessment of expression cassettes after transient transfection to cells: AML12 cells were transiently transfected with the four expression cassettes and luciferase activity was measured after 24 h. The graph represents results (±S.D.) of three separate experiments, each one made in triplicates relative to the control plasmid, pGL3p. The luciferase activity was normalized to internal pRL control plasmid (see Section 2). The designated plasmids are described in Fig. 1B. (B) In vivo assessment of expression cassettes: transient in vivo expression after tail vein injection of different cassettes: plasmid DNA was tail vein injected at high pressure into three Balb/c mice. Luciferase activity was monitored by CCCD camera after 24 h, as described in Section 2. The graph represents results from one experiment done in triplicates normalized to control plasmid, pGL3p. The designated plasmids are described in Fig. 1B.

radioactively labeled using the random DNA labeling kit (Biological Industries Co., Beit Haemek, Israel), according to manufacturer's instructions.

2.6. In vivo transgenic monitoring by the CCCD camera

Mice that were PCR positive for the presence of luciferase transgene were screened by the CCCD camera to evaluate and monitor luciferase expression, as described previously [12]. A pseudocolor image (Fig. 3A and B) represents light-intensity (blue being the least intense, and red being the most intense). The integration light was, in all cases, the result of a 2-min exposure and acquisition. Recorded measurements were the total sum of integrated signals, subtracted from the background area of equal size. In all experiments, mice were anesthetized before light detection, with 0.2–0.3 ml of 4% chloral hydrate (Sigma–Aldrich, Steinheim, Switzerland). Five minutes before monitoring light emission, the animals were injected IP with Beetle luciferin (Promega, Madison, WI, USA) in PBS (126 mg/kg body weight).

3. Results and discussion

3.1. Generation of a universal gene expression cassette

First, we constructed a universal cassette for transgene expression (pNH12) which enabled cloning of any desired gene (at BamHI site) between duplicates of chicken β-globin insulator [6] (Fig. 1A). Two identical sequences of four rare restrictions enzymes sites (AscI, PacI, FseI, SfiI) were inserted to flank the insulators duplicates, in order to alleviate cut off of the whole construct with the desired transgene, for its injection into fertilized egg.

3.2. Cloning the luciferase reporter gene and the liver-specific promoters into the universal expression cassette

The four different variants containing the luciferase reporter gene under the control of two different promoters, each with or without IE, were constructed (Fig. 1B). The luciferase gene from pGL3 basic plasmid was cloned under the regulation of one of the two liver-specific promoters: the human serum amyloid protein (SAP) promoter [2,9] or the mouse major urinary protein (MUP) promoter [10,11]; the products were designated pNH-SL and pNH-ML, respectively. Their variants which carried also the IE [8] between the promoter and the reporter gene were designated pNH-SI and pNH-MI, respectively. All four constructs were inserted into the universal expression cassette pNH12 under the same names (Fig. 1B).

3.3. In vitro assessment of the expression vectors

Measurements of luciferase activity of the generated expression cassettes in vitro following transient transfection of AML12 cells demonstrated that plasmids carrying the IE exhibited higher activity (Fig. 2A). Thus, insertion of the IE did not only interfere with transient expression in vitro, but even enhanced it. Both tested liver-specific promoters provided similar expression levels.

Table 1
Pattern of transgene expression in 1st (A) and 2nd (B) generation transgenic mice

Name	Promoter	IE	# of Mice	Transgene presence (PCR)	Luciferase expression	Liver-specific expression
(A) DNA construct			*First generation*			
NHML	MUP	–	64	2 (3.1%)	2 (3.1%)	1 (1.5%)
NHMI	MUP	+	85	15 (17%)	2 (2.3%)	1 (1.2%)
NHSL	SAP	–	55	5 (9%)	3 (5.4%)	2 (3.6%)
(B) DNA construct			*Second generation*			
NHML	MUP	–	0	–	–	–
NHMI	MUP	+	29	NM	9 (31%)	2 (7%)
NHSL	SAP	–	25[a]	7 (28%)	7 (28%)	7 (28%)
			9[b]	4 (44%)	4 (44%)	2 (22%)

NM: not measured, mice were screened only by the CCCD camera.
[a]Progeny of NHSL transgenic male.
[b]Progeny of NHSL transgenic female.

3.4. In vivo monitoring of luciferase activity

Prior to the generation of the transgenic mice, we decided to test the expression of the generated expression cassettes in vivo in the mouse liver using the hydrodynamic tail vein injection method [16]. Luciferase activity was measured after luciferin injection and animal exposure to the CCCD camera (Fig. 2B). The results of transient in vivo expression demonstrated that: (a) the difference between control plasmid pGL3p and the tested vectors became less significant and (b) IE decreased the expression level.

3.5. Generation of transgenic mice and assessment of transgene expression

Transgenic mice were generated as described in Methods. The data on generated animals are summarized in Table 1.

The NH-MI mice (see Fig. 1B): 85 first generation mice born after microinjection of the NH-MI fragment (see Fig. 1B) were overall analyzed. Verification of the transgene presence by PCR demonstrated that 15 mice were positive. These mice were screened for luciferase expression by the CCCD camera. Out of 15 tested mice, two males expressed luciferase, however, only one displayed liver-specific expression pattern (Fig. 3A). The presence of the luciferase gene in these two mice was confirmed also by Southern blot hybridization with a luciferase probe (data not shown). In order to verify the inheritance pattern of the transgene in the next generation, and to prove the absence of luciferase expression in embryos, the transgenic male was bred with CB6/F1 females. Pregnant mothers were photographed with the CCCD camera in the second week of pregnancy, and no light emission was detectable. Among the progeny of this male (19 mice), nine were PCR positive for the luciferase gene, among these, two demonstrated liver-specific postnatal luciferase expression.

The NH-ML mice (see Fig. 1B): 64 founders were analyzed; among these, two were positive by PCR. In both of these mice, although luciferase expression was also detected by the CCCD camera, only one mouse demonstrated liver-specific expression pattern; since this latter mouse was not reproductive, we could not study its inheritance pattern.

The NH-SL mice (see Fig. 1B): 55 mice were generated in the first generation; among these, five were positive by PCR. These five mice were screened for luciferase expression by the CCCD camera: only three of them expressed luciferase, and only in two mice (male and female) was this expression liver-specific. For the second generation, these two mice were bred with CB6/F1 partners. The females that were bred with a transgenic male were screened for luciferase expression in the second week of pregnancy: no luciferase expression was detected. Among his progeny (25 mice): seven were PCR positive for the luciferase transgene, and all seven demonstrated liver-specific luciferase expression (Table 1B). Among the transgenic female's progeny (nine mice): four were PCR positive for the luciferase gene; all four expressed luciferase, however, in only two was its expression liver-specific (Table 1B).

The NH-SI mice (see Fig. 1B): After comparing the results from the two lines expressing luciferase under the MUP promoter with or without IE (NH-ML vs. NH-MI), we concluded that the insertion of the IE did not confer any advantage for gene expression pattern in the transgenic mice. As a result, we did not generate transgenic mice with luciferase under the SAP promoter and the IE.

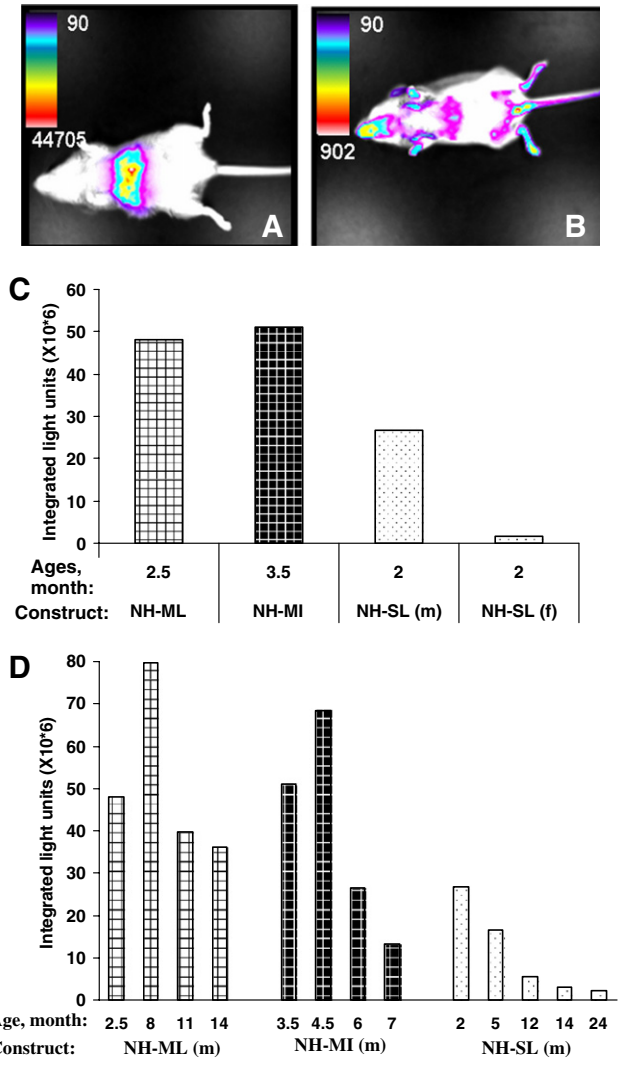

Fig. 3. Luciferase expression in transgenic mice. (A) an example for liver-specific luciferase expression (transgenic mouse with NH-ML cassette). (B) An example of non-specific luciferase expression (transgenic mouse with NH-MI cassette). Images (A,B) are overlaid on pictures from the CCCD camera. A pseudocolor scale represents light-intensity. (C) First measurements of Luciferase expression in the first generation of transgenic mice as determined by the CCCD camera, at ages as indicated ((f) stand for female and (m) for male). (D) Kinetics of luciferase expression in transgenic males. Luciferase expression in different transgenic lines was monitored at different time points, as indicated. Each color represents one mouse ((m) stands for male).

The expression level of luciferase in each transgenic mouse was measured at 2–3 months of age for the first time (Fig. 3C). The kinetics of luciferase expression in the first generation of transgenic mice was also followed during the animal's life (Fig. 3D). The age-dependent reduction of transgene expression may reflect the natural pattern of expression of the tested promoters: for example, such reduction of MUP2 mRNA expression in murine liver was reported previously [17].

In conclusion, we have constructed a universal cassette for transgene expression. In our constructs, insulators did not provide high uniformity of transgene expression (tissue specificity), or high frequency of desirable transgene expression

pattern (Fig. 3 and Table 1). These results are consistent with those of others indicating that the effect of insulators is not universal, but rather construct-dependent [18,19]. The IE located between promoter and the reporter did not confer any advantage to transgene expression in vivo. Although both tested promoters enabled a postnatal onset of luciferase expression in all transgenic lines, liver specificity was not always achieved (Fig. 3C). All tested constructs demonstrated prolonged expression of the reporter transgene without changes in tissue distribution during animal life. The NH-SL transgene (SAP promoter without IE) showed the best pattern of expression and the highest efficiency of transgenesis; this construct may be the vector of choice for the generation of transgenic mice with developmentally regulated, liver-specific expression of a desired transgene.

The use of the CCCD camera allowed us to monitor the expression of the luciferase transgene in live transgenic mice, and to compare expression patterns, both qualitatively and quantitatively, in different mice with an easy, quick and inexpensive system. Remarkably, with the aid of the CCCD camera, we were able to detect non-specific transgene expression in those tissues which are usually not tested by standard methods (Fig. 3C). In addition, this approach enabled us to visualize ongoing monitoring of gene expression through repeated imaging of luciferase bioluminescence.

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