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Expression of the *Renilla reniformis* Luciferase Gene in Mammalian Cells

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A cDNA encoding the *Renilla reniformis* luciferase was expressed in simian and murine cells in a transient and stable manner, respectively. Light emission catalyzed by luciferase was detected from transfected cells both *in vitro* and *in vivo*. This work establishes the *Renilla* luciferase gene as a new efficient marker of gene expression in mammalian cells.

Keywords: Renilla luciferase; bioluminescence; coelenterazine; gene expression

INTRODUCTION

Light emission from the soft coral *Renilla reniformis* (Order Cnidaria) is catalyzed by a luciferase. Oxidation of the luciferin substrate, coelenterazine, by this luciferase leads to an excited state product (oxyluciferin) from which energy is transferred to the acceptor green fluorescent protein (GFP). GFP emits the green light ($\lambda_{max} = 509$) seen in the living coral (1). In the absence of GFP, luciferase-catalyzed oxidation of coelenterazine yields blue light ($\lambda_{max} = 480$ nm) (2).

A *Renilla* luciferase cDNA was cloned previously and expressed in *Escherichia coli* (3) and in plants (4). This cDNA was judged to be full length based on: (i) the deduced amino acid sequence; (ii) the apparent molecular size of the recombinant protein relative to native luciferase as determined from SDS-PAGE and Western blot analyses; and (iii) the catalytic and kinetic characteristics of the recombinant protein (3,5).

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The use of bioluminescence- and chemiluminescence-based reporter gene assays and immunoassays has increased dramatically in the past 10 years (6,7,8). Luciferases and photoproteins are especially useful since light emission can be measured and quantified easily, and they are non-toxic. A number of bacterial (9) and beetle (10,11) luciferase genes have been isolated and their uses have been well characterized. The bacterial *lux* genes have been used extensively as reporters, although the applications are primarily in procaryotic and plant hosts. *Lux* genes have a demonstrated utility as reporters of environmental pollutants and toxins, temporal and spatial developmental events, promoter strength and bacterial detection (see reviews 9,12,13). Similarly, the beetle luciferase *luc* genes have a well documented history as markers of transfection and transformation (14,15,16), as reporters of transcriptional regulation of genetic elements (17), and for determination of ATP concentrations *in vivo* and *in vitro* (18,19).

Other bioluminescence reporter genes include the *phot* genes which encode the calcium activated photoproteins aequorin (20) and obelin (21). The aequorin gene has been used to report Ca^{2+} flux in plants (22) and mammalian cells (23). Membrane-based assays using biotinylated recombinant aequorin have been developed for detection of nucleic and amino acids (24), and diagnostic immunoassays have been developed for a number of target molecules (25,26). The *luc* gene from the crustacean *Vargula hilgendorfii* has been isolated (27) and tested as a reporter gene (28); however, the substrate for this secreted luciferase is not readily available. Most recently, the gene encoding GFP from *Aequorea victoria* and has been used as a reporter of gene expression (29). It is not likely that any single reporter gene can offer the versatility required in the expanding number of applications that can be found for bioluminescence; each gene may have a particular advantage or disadvantage, depending on the assay and the conditions under which it is performed. Here, we report transient and stable expression of the *Renilla* luciferase r*luc* gene in COS-7 cells and C5 mouse fibroblasts, respectively, and demonstrate that this gene can be used both *in vitro* and *in vivo* as a marker gene in mammalian cells.

MATERIALS AND METHODS

Enzymes and media

Restriction enzymes, polymerases and ligases were obtained from New England Biolabs (Beverly, MA) and Promega (Madison, WI). PCR reagents and Taq polymerase were purchased from Perkin Elmer (Norwalk, CT). Media and chemicals required for the growth and maintenance of mammalian cell cultures were purchased from Gibco-BRL (Green Island, NY) and Sigma (St Louis, MO).

Modification of the luciferase cDNA

Plasmid pBSRLuc-1 was constructed by ligation of the 2.2 kbp SstI/EcoRI fragment isolated from pTZRLuc-1 (3) into plasmid Bluescript (Stratagene, La Jolla, CA) and transformed into *E. coli* DH5α cells. pBSRLuc-1 DNA was purified using a Qiagen tip-100 kit (Qiagen Inc, Chatsworth, CA) according to the manufacturer's instructions, linearized by digestion with SstI, and desalted in a G-25 spin column (Boheringer Mannheim Biochemicals) according to the manufacturer's instructions. Oligonucleotide primers were synthesized at the University of Georgia Molecular Genetics Sequencing Facility. The first oligo (GGCTGCAGATGACTTCGAAAGTTTAT) contained a PstI site immediately 5′ to the ATG start codon, followed by 18 nucleotides identical to the 5′ end coding sequence; the second (GGCTGCAGACATTTATATTATTAAACCC) also contained a PstI site and 20 nucleotides identical to the 3′ end immediately adjacent to an endogenous SmaI site located in the non-coding region. PCR amplification was performed using a programmable thermal cycler (MJ Research Inc, Watertown, MA). The PCR product was filled in with T-4 polymerase, isolated from a low melting temperature agarose gel (NuSeive; FMC, Rockland, ME) (30), and blunt-end ligated into alkaline phosphatase-treated SmaI digested pUC18 resulting in the plasmid pRLuc-4.1. *E. coli* DH5α cells were transformed and recombinant plasmid DNA was purified with a Qiagen tip-100. PstI and PstI–SmaI digests were performed to ensure correct orientation and size of the fragments.

Plasmid constructions

pRLuc-6 & pRLuc-6.1. Plasmid pRLuc-4.1 was digested with PstI and the insert was ligated into a unique PstI site contained on the mammalian expression vector pXM (31). Recombinants in pXM can be selected for by ampicillin resistance;

the luciferase gene is placed under the transcriptional control of the adenovirus major late promoter. *E. coli* DH5α cells were transformed and plasmid screens were performed to determine size and orientation of the insert DNA. Two clones, pRLuc-6.1 and pRLuc-6.1R, were isolated and contained the luciferase gene in the forward and reverse orientation, respectively, with respect to the promoter region.

pMCT-RUC. Plasmid pMCT-RUC (14 kbp) was constructed for site-specific targeting of the *Renilla* luciferase gene to a mammalian chromosome (details of the plasmid construction will be presented elsewhere). The relevant features of this plasmid are the *Renilla* luciferase gene under transcriptional control of the human cytomegalovirus immediate-early gene enhancer/promoter; the hygromycin gene under the transcriptional control of the thymidine kinase promoter; and a unique HpaI site is used to linearize the plasmid.

Mammalian cell culture

COS-7 cells. COS-7 cells were grown on 100 mm Costar plates as described previously (32). Cultures were trypsinized and split 1/20 2 days before use. Cell monolayers were 70–80% confluent at the time of transfection and were washed once in DME immediately prior to transfection. To each plate 3 mL DME was added containing 10 μg of plasmid DNA and 1.5 μL/μg Transfectam (Promega). After 5 h the transfection media was removed and the plates were washed once in DME. Fresh growth media was added to the cells, which were incubated for another 60 h. Cells were washed gently in PBS and then collected by scraping in 5 mL PBS/plate. Cells from one plate were centrifuged at 500 $\times$ *g* for 10 min, then resuspended in 0.5 mL 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.6. Cells were incubated on ice for 10 min followed by sonication with a Branson Cell Disruptor using several 1 s bursts. Half of the cell extract was clarified by centrifugation at 15,000 $\times$ *g*, and luciferase activity was determined in the crude and clarified cell extracts.

C5 cells. C5 mouse fibroblasts were maintained as a monolayer as previously described (33). Cells at 50% confluency in 100 mm Petri dishes were used for calcium phosphate transfection (34) using 10 μg of linearized pMCT-RUC per plate. Colonies originating from single transfected cells were isolated and maintained in F-12 medium containing hygromycin (300 μg/mL) and 10% fetal bovine serum. Cells were grown in 100 mm Petri dishes prior to the *Renilla* luciferase assay.

Luciferase assays

COS-7 cells. The *Renilla* luciferase assay has been described previously (2). Assays of COS-7 cell extracts were performed in a luminometer (Turner model Td-20e) equipped with a sample chamber aperture designed to hold 12 $\times$ 75 mm tubes. The light path from the sample cell to the photomultiplier tube was restricted such that only the light passing through a 5 mm hole in base of the sample aperture was quantified. This design offered a geometry more suitable for reproducible measurements and calibration. The instrument was calibrated with a ^{14}C phosphor light standard for determination of quanta per second (35). For this instrument, 1 LU (light unit) = 6.4×10^6 hv/s. 10 μL of crude or clarified extracts were diluted into 1 mL luciferase assay buffer and injected rapidly into the sample tube containing 10 μL of 2.5 mmol/L coelenterazine in 1 mol/L HCl/MeOH. Integration time of the signal was 5 s.

C5 cells. Hygromycin-resistant cell lines obtained after transfection of mouse fibroblasts with linearized plasmid pMCT-RUC ("B" cell lines) were grown to 100% confluency for measurements of light emission both *in vivo* and *in vitro*. Light emission was measured *in vivo* after about 30 generations as follows: growth medium was removed and replaced by 1 mL RPMI 1640 containing coelenterazine (1 mmol/L final concentration). Light emission from cells was then visualized by placing the Petri dishes in a low light video image analyzer (Hamamatsu Argus-100). An image was formed after 5 min of photon accumulation using 100% sensitivity of the photon counting tube. For measuring light emission *in vitro*, cells were trypsinized and harvested from one Petri dish, pelleted, resuspended in 1 mL assay buffer (0.5 mol/L NACl, 1 mmol/L EDTA, 0.1 mol/L potassium phosphate, pH 7.4) and sonicated on ice for 10 s. Lysates were than assayed in a Turner TD-20e luminometer for 10 s after rapid injection of 0.5 mL of 1 mmol/L coelenterazine, and the average value of light

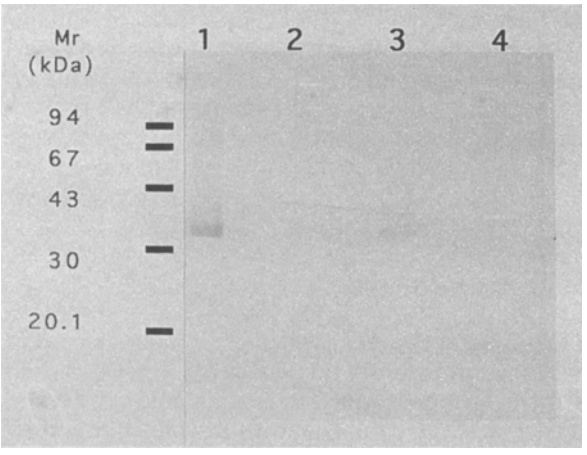

Figure 1. Western analysis of COS-7 cells transfected with the *Renilla* luciferase gene. Protein extract was fractionated with 12.5% SDS-PAGE before transfer to nitrocellulose as described in Methods. Lane 1, *Renilla* luciferase; Lane 2, pRLuc-6.1R transfected cells; Lane 3, pRLuc-6.1 transfected cells; Lane 4, pXM transfected cells

emission was recorded as LU (1 LU = 1.6×10^6 hν/s for this instrument).

SDS PAGE/Western blot analysis of recombinant luciferase

The protein concentration in lysates of cells expressing *Renilla* luciferase was determined by the method of Bradford (36). Clarified, crude extracts (50 μg) and native *Renilla* luciferase (0.5 μg) were fractionated on 12.5% SDS-PAGE gels (37). Western blot analysis was performed by transfer to nitrocellulose (Schleicher and Schnell, Keene, NH) (38). Luciferase detection was performed with a rabbit polyclonal anti-*Renilla* luciferase antibody (1/1000 dilution) and horseradish peroxidase conjugated goat anti-rabbit IgG second antibody (1/5000 dilution; Bio-Rad, Hercules, CA) as described previously (3).

RESULTS

COS-7 cells. Restriction analysis of the modified luciferase gene showed that: (i) it contained all the endogenous restriction sites of the template DNA as well as the additional PstI sites; and (ii) pRLuc-6.1 contained a single insert in the correct orientation with respect to the promoter and that pRLuc-6.1R contained a single insert in the reverse orientation (data not shown). An integrated value of 3708 LU was obtained for 10 μL of cell sonicate from pRLuc-6.1 transfected cells. This corresponds to approximately 2.3×10^{10} hν/s generated from 2% of the total protein released from the plate (Table 1). Sonicates clarified by centrifugation had a value of 3315 LU. No detectable light was measured from cells transfected with pXM or pRLuc-6.1R. It is important to note that the light intensity values reported above represent minimal ones, since the luciferase assays performed on these same samples with an unrestricted aperture (having a 25 mm bore) in the photometer sample chamber led to light emissions which exceeded the full scale limit of the instrument by a factor of 10^3 when at the lowest gain setting. Crude extracts from cells transfected with the above plasmids were analyzed by Western blotting (Fig. 1). A single protein band was seen in the pRLuc-6.1 transfected cell extracts (lane 3) but not in the pRLuc-6.1R (lane 2) or pXM (lane 4) cell extracts. Native *Renilla* luciferase (lane 1) is shown as a control. An identical blot incubated with preimmune serum failed to give a detectable signal (data not shown).

C5 cells. Independent cell lines of mouse fibroblasts transfected with linearized plasmid pMCT-RUC

Table 1. *In vitro* light emission from COS-7 cells transfected with plasmid pXM, pRLuc-6,1R, amd pRLuc-6.1. Values are an average of five measurements.

Plasmid	pXM	pRLuc-6.1R	pRLuc 6.1
L.U. (total crude extract)	0.0	0.0	3708 + / − 375
L.U. (clarified crude extract)	0.0	0.0	3315 + / − 540

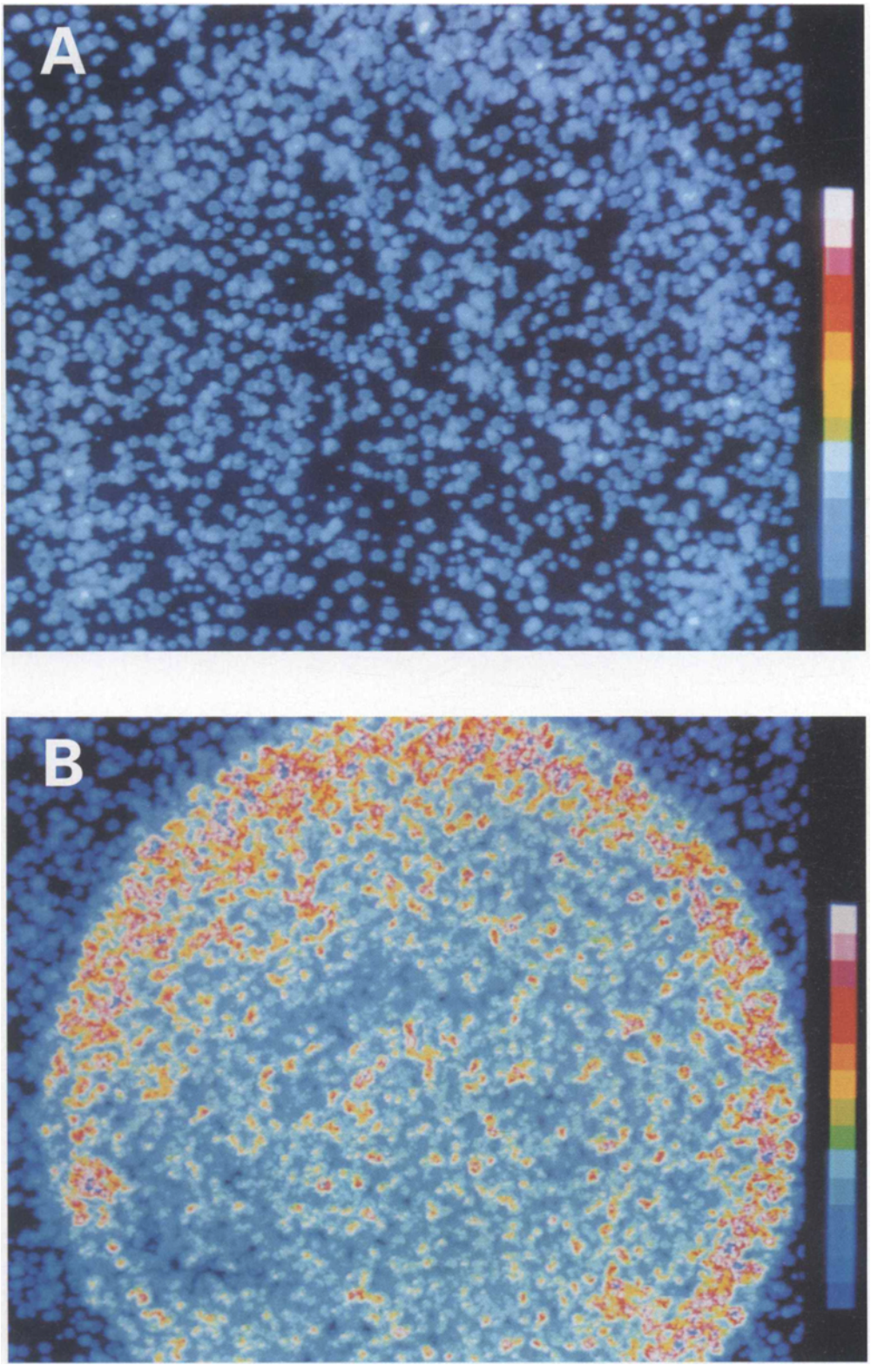

Plate 1. Low light video image analysis of Petri dishes (100mm) containing hyromycin-resistant mouse fibroblast cell lines ("B" cell lines) transfected with plasmid pMCT-RUC. a, cell line B3; b, cell line B6; c, cell line B9; d, cell line C5 (negative control)

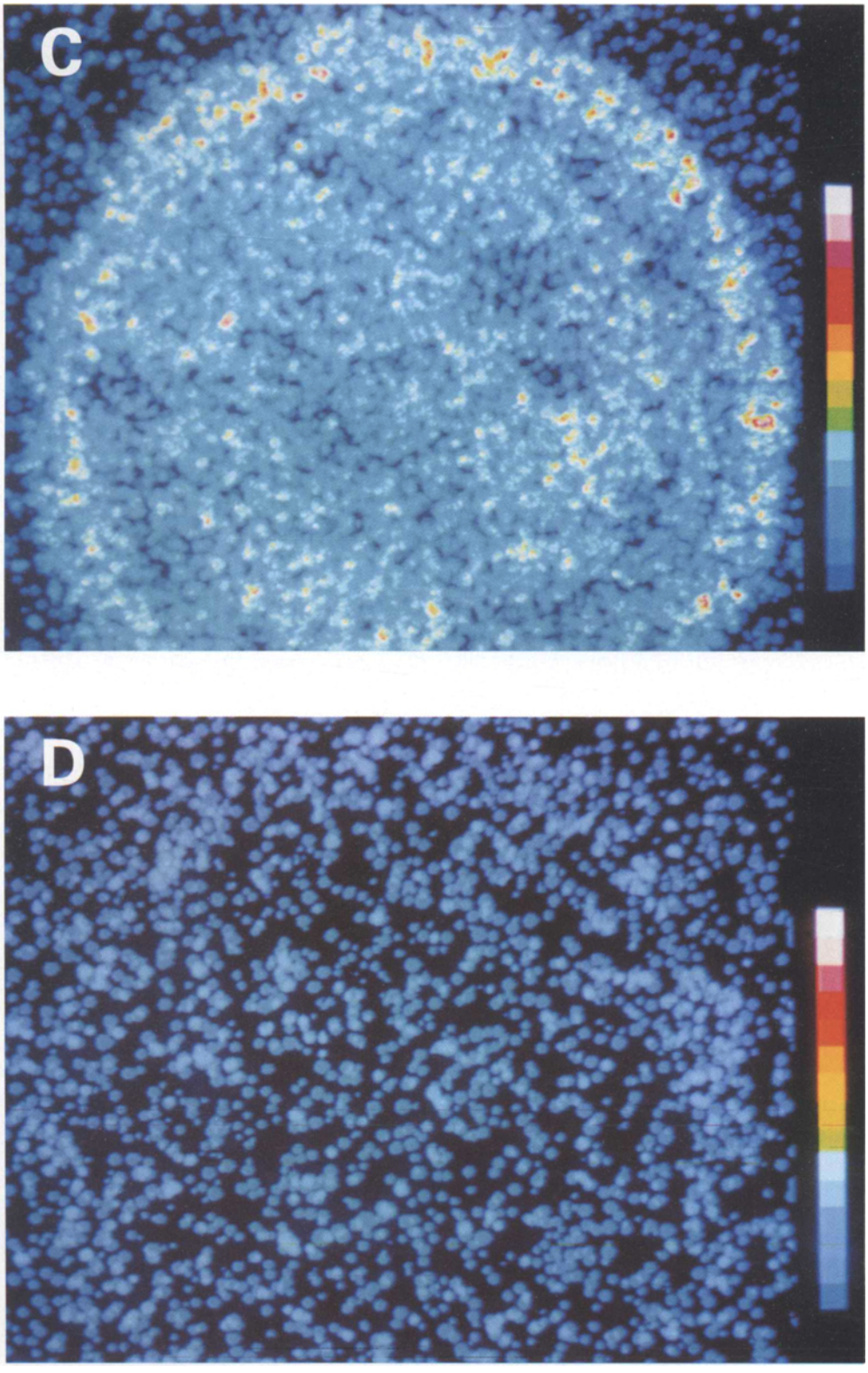

Plate 1. (cont.)

Table 2. *In vitro* light emission of C5 fibroblasts transfected with plasmid pMCT-RUC (B cell lines).

Cell Line	B3	B6	B9	C5
L.U. (clarified crude extract)	017.8	693.5	063.7	000.5

showed different levels of *Renilla* luciferase activity (Plate 1). Similar differences in light emission were observed when measurements were performed on lysates of the same cell lines (Table 2). This variation in light emission was probably due to a position effect resulting from the random integration of plasmid pMCT-RUC into the mouse genome, since enrichment for site targeting of the luciferase gene was not performed in this experiment.

DISCUSSION

The *Renilla* luciferase gene can be expressed and detected in a mammalian cell background. Transient expression in COS-7 cells showed that the *in vitro* bioluminescence emission from both total and clarified crude extracts exceeded 1×10^{10} hν/s. Also, the negligible difference between the light emissions from these two samples indicates that, as in expression in an *E. coli* host, recombinant *Renilla* luciferase exists as a soluble, cytoplasmic protein in transiently transfected mammalian cells. Analysis of these same extracts by Western blotting demonstrates that recombinant luciferase expressed in mammalian cells is essentially identical to the native protein, in both its molecular size and immunological reactivity. In addition, the stable expression of the *Renilla* luciferase gene in C5 fibroblasts demonstrates non-toxicity of the gene product in mammalian cells, and confirms that coelenterazine will readily permeate mammalian cell membranes (23). Unlike photoproteins, *Renilla* luciferase requires no time period for charging an apoprotein prior to assay and signal detection. The ease of detection of luciferase activity in transiently transfected cells makes the *Renilla* luciferase gene an ideal candidate as a marker of transfection, as well as a reporter gene of genetic events associated with transcription and translation.

Renilla luciferase requires only O_2 and the substrate coelenterazine, which is commercially available. The fact that the *Renilla* luciferase gene has been expressed at high levels in bacterial, plant and animal cells in a stable and transient fashion demonstrates its utility and versatility in the field of bioluminescence-based detection. Further testing of *Renilla* luciferase vectors may reveal that it is superior to other luciferases when used in some applications, because it requires no divalent cations as does aequorin, no ATP as do the beetle luciferases, and no long-chain aldehydes as do the bacterial luciferases. Also, mammalian membrane permeability to coelenterazine does not appear to pose a problem.

Assay and detection methods based on currently available bioluminescence genes offer sensitive and reliable alternatives to other isotopic and non-isotopic methods. There are, however, inherent problems, such as temperature instability (39,40), low turnover (41,42), rigid ionic strength/buffer constraints (43,44,45), lack of commercial availability of substrate (i.e. *Vargula* luciferin), ATP dependency (4,44), and susceptibility to proteolysis (46,47) which limit the usefulness of currently available luciferases and photoproteins. Preliminary evidence suggests that the expressed *Renilla* luciferase is stable with respect to elevated temperatures and a wide range of ionic strengths (5). Finally, when a dual marker system is needed, it should be noted that the *Renilla* luciferase gene used in conjunction with the firefly gene may fill this need, since the large difference in their peak light emissions (480 nm (2) and 562 nm (48), respectively) could be measured simultaneously.

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