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Imaging of luciferase secretion from transformed Chinese hamster ovary cells

(bioluminescence/exocytosis/*Vargula hilgendorfii*/luciferin/brefeldin A)

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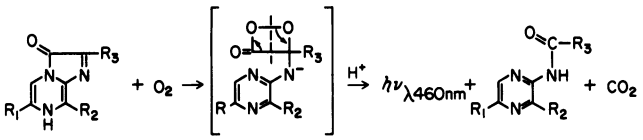
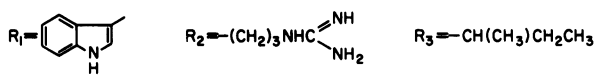
ABSTRACT **The blue luminescence characteristic of the marine ostracod crustacean *Vargula hilgendorfii* is from a simple, but highly specific, enzyme–substrate reaction. Light is emitted by the oxidation of *Vargula* luciferin (substrate) by molecular oxygen, a reaction catalyzed by luciferase. Stable transformants of Chinese hamster ovary cells carrying the *Vargula* luciferase gene secreted luciferase from discrete sites on the cell surface, and this secretion could be monitored in real time by the bioluminescence produced by the secreted luciferase in the presence of *Vargula* luciferin by using an image-intensifying technique. Addition of anti-*Vargula* luciferase IgG to the luminescing cells almost completely extinguished the luminescence, confirming that *Vargula* luciferase caused the luminescence.**

The small (≈3-mm long) marine ostracod crustacean, *Vargula* (formerly *Cypridina*) *hilgendorfii*, found in the shallow coastal waters of Japan, has been the subject of numerous studies since Harvey (1) first described the properties of the bioluminescence reaction of the animal. When tactilely stimulated, the ostracod ejects a bright-blue luminous secretion into sea water. The luminescence is from an enzyme–substrate reaction in which a small organic molecule, luciferin (M_r, 478), is oxidized by molecular oxygen in a reaction catalyzed by luciferase (1–3) (Scheme I). The products of the reaction are light, oxyluciferin, and carbon dioxide (4, 5). The excited-state oxyluciferin bound to luciferase is the emitter in the reaction (6).





Scheme I

Recently, the cDNA for *Vargula* luciferase has been cloned, and its primary structure has been deduced from the nucleotide sequence (7). *Vargula* luciferase consists of 555-amino acid residues in a single polypeptide chain with two potential N-glycosylation sites (amino acid residues 186–188 and 408–410). The expressed enzyme also possesses a secretion signal, and mammalian cells transfected with cDNA for *Vargula* luciferase secrete the enzyme (8). The activity of luciferase in the culture medium can be readily assayed by mixing an aliquot of the medium with luciferin and measuring the light intensity. Thus, luciferase may be used as a convenient reporter enzyme for studying gene expression in mammalian cells. The light-emitting capability of the enzyme has now led us to look for the site of secretion of luciferase from a transformed mammalian cell. The secretion of a protein follows a pathway from the endoplasmic reticulum through the Golgi complex into storage and transport vesicles (9). The vesicles then migrate to the cell surface and fuse with the cell membrane, whereupon the protein is released into the extracellular space through an opening formed at the site of fusion. To observe this secretory process, Chinese hamster ovary (CHO) cells were transfected with cDNA for *Vargula* luciferase, and stable transformants were isolated. In culture, the cells secreted luciferase, and the secretory process could be monitored in real time from individual cells in the presence of luciferin by using an image-intensification procedure.

MATERIALS AND METHODS

Expression of *Vargula* Luciferase by CHO Cells. The method used to express *Vargula* luciferase in CHO cells was essentially the same as earlier described (10, 11). A dihydrofolate reductase (dhfr)-deficient CHO cell line (CHO-DG44) (12) was cotransfected with expression plasmids pSV2-v1 (8) and pSV2-dhfr as a selectable marker (13) by using the calcium phosphate method (14). The construction of plasmid pSV2-v1 is shown in Fig. 1. The plasmids pSV2-v1 (20 μg) and pSV2-dhfr (1 μg), precipitated with calcium phosphate, were used to transfect CHO cells at a density of 5×10^5 cells per 10-cm dish in 10 ml of α minimum essential medium (αMEM, GIBCO/BRL, 410-1900)/10% (vol/vol) heat-inactivated fetal calf serum (HyClone Labs, Logan, UT)/penicillin at 100 units per ml/streptomycin at 100 μg/ml (GIBCO/BRL). The cells were cultured at 37°C in a humidified 5% CO_2 incubator. At 48 hr after transfection, the cells were subcultured in nucleotide-free αMEM (GIBCO/BRL, 410-2000)/10% (vol/vol) heat-inactivated, dialyzed fetal calf serum (GIBCO/BRL, 200-6300). After 10 days the transformants that had survived the nucleotide-free medium were isolated and cultured in a 24-well culture plate (Falcon–Becton Dickinson). After 5 days of incubation, the culture supernatant was assayed for luciferase activity, and immunologic dot-blot analysis was done with anti-*Vargula* luciferase to select stable transformants. In the time-course experiments, where the cell number and luciferase expression were followed, transformed cells were grown (37°C, 5% CO_2) in a 6-well culture plate (Sumitomo Bakelite, Osaka) at an initial concentration of 2.3×10^4 cells per well in 2.5 ml of nucleotide-free αMEM/10% (vol/vol) heat-inactivated, dia-

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Abbreviation: αMEM, α minimal essential medium.

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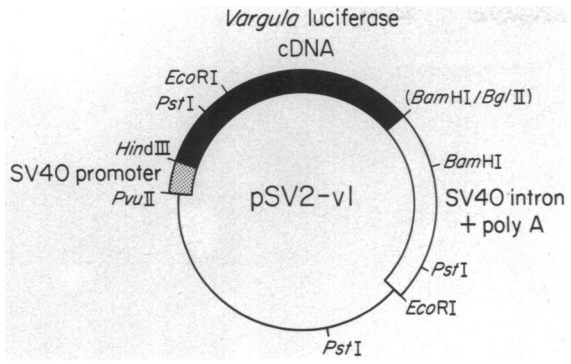

FIG. 1. Structure of luciferase expression vector used to transform CHO cells. The vector is the same as that described (8). SV40, simian virus 40.

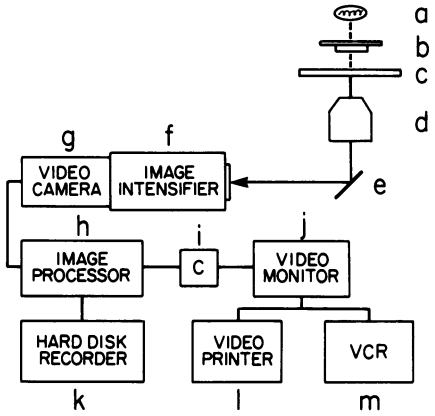

FIG. 2. Block diagram showing arrangement of equipment used for imaging. Nikon Diaphot TMD model EF2 microscope, consisting of 12-V/50-W halogen lamp (*a*); phase-contrast condenser (*b*); stage (*c*); Fluor/UV-F 20× objective (*d*); dichroic mirror (*e*); and Argus 100/Vim 3 imaging system (Hamamatsu Photonics, Hamamatsu, Japan), consisting of image intensifier (*f*); video camera (*g*), 22×; image processor (*h*); video camera controller (*i*); Sony model PVM 14429 color video monitor (*j*); hard disk image and data storage unit (*k*); Japan Radio (Tokyo) model NJW-950 Videofix II color photo printer (*l*); Victor BR-S611 video camera recorder (VCR) (*m*). According to the manufacturer, the sensitivity range of the Vim 3 system is from 10–10^7 quanta per mm^2-sec at 550 mm, and the resolution is 512 pixels × 512 pixels × 16 bits.

lyzed fetal calf serum. For the imaging experiments, the transformed cells were grown (37°C, 5% CO_2) in a 96-well culture plate (Sumitomo Bakelite) for 2 days starting from an initial concentration of ≈100 cells per well in 100 μl of nucleotide-free αMEM/10% (vol/vol) heat-inactivated, dialyzed fetal calf serum. All molecular biology procedure were done according to Sambrook *et al.* (15), and reagent-grade chemicals were used throughout.

Assay and Imaging of Luciferase Secretion. To assay for luciferase activity, the medium in each well was aspirated and centrifuged to remove contaminating cells; the supernatant was then saved. Cells adhering to the wall were washed once with 10 mM sodium phosphate buffer/137 mM NaCl/2.7 mM KCl, pH 7.4, treated with 300 μl of trypsin solution (GIBCO/BRL, 610-5300) to release the cells, and the mixture was diluted with 700 μl of serum-free αMEM. Fifty microliters of the suspension was removed for cell count, and the remaining 950 μl was sonicated in a model W-385 sonicator (Heat Systems/Ultrasonics) to disrupt the cells. The sonicated suspension was centrifuged, and the supernatant was saved. Each of these supernatant solutions was assayed by transferring 300 μl into a reaction vessel and injecting a saturating concentration of luciferin (740 μl of 0.043 μM luciferin, prepared in 0.2 M Tris·HCl, pH 7.5/0.17 M NaCl). Supernatants with high luciferase activity were diluted with Tris·HCl buffer. The initial maximal light intensity was read with a Labo Science (Tokyo) model TD-8000 photometer, calibrated with a ^{14}C light source (16). In excess luciferin concentration, light intensity is directly proportional to luciferase concentration (17). The number of cells in each well was calculated after adding 50 μl of 0.2% trypan blue to 50 μl of the cell suspension and counting with a hemacytometer.

In the imaging experiments, the culture medium (200 μl) was aspirated, the adhering cells were rinsed once with 10 mM sodium phosphate buffer/137 mM NaCl/2.7 mM KCl, pH 7.4, and layered with 100 μl of serum-free αMEM. To begin the imaging, which was done in a dark room at room temperature, 100 μl of 49 μM luciferin solution (20 μg of luciferin in 20 μl of 1-butanol plus 836 μl of 10 mM sodium phosphate buffer/137 mM NaCl/2.7 mM KCl, pH 7.4) was added to the well. The apparatus used to image the CHO cells is shown schematically in Fig. 2. *Vargula* luciferin was chemically synthesized (18–20) to a purity of >99%, as analyzed by TLC.

Immunoblot Analysis. The 2.5 ml of culture medium in each well of the 6-well culture plate was aspirated and centrifuged; then the supernatant was decanted. Fifty microliters of the supernatant was mixed with 50 μl of anti-bovine serum albumin (2.0–3.0 mg of IgG per ml, BM 1151, BioMakor, Rehovot, Israel) and allowed to incubate in an ice bath for 3 hr, after which 50 μl of 30% (vol/vol) polyethylene glycol 6000 (Wako Pure Chemical, Osaka 169-09125)/10 mM sodium phosphate buffer/137 mM NaCl/2.7 mM KCl, pH 7.4, was added, and the incubation was continued for another 3 hr in an ice bath. The mixture was centrifuged, the supernatant was decanted, and 2 μl of the supernatant was diluted with electrophoresis buffer. SDS/PAGE was done under reducing conditions by using a 12% polyacrylamide fractionating gel (1-mm thickness) and a current of 25 mA for 2 hr (21). The proteins in the gel were then transferred electrophoretically to a 0.45-μm-pore Immobilon-P [poly(vinylidene difluoride)] membrane (Millipore) (22). The membrane was blocked with casein/TBST (1% casein/50 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% Tween 20) for 1 hr at room temperature and incubated with rabbit anti-*Vargula* luciferase IgG (1:2000 dilution of a solution at 1 mg/ml in casein/TBST) for 2 hr at room temperature. The antibody was raised against purified native *Vargula* luciferase (7) in Japanese white rabbits and purified with a protein A–Cellulofine column (Seikagaku Kogyo, Osaka). After washing with TBST solution, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) for 2 hr at room temperature. To observe the presence of antibody to *Vargula* luciferase, the membrane was finally treated with a solution of nitro blue tetrazolium chloride (Sigma) (300 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt) (Sigma) (150 μg/ml) dissolved in 100 mM Tris·HCl, pH 9/100 mM NaCl/5 mM $MgCl_2$.

RESULTS AND DISCUSSION

In a previous paper, it was reported that L929, NIH 3T3, PU5-1.8, COS-7, CHU2, and HeLa cells carrying the *Vargula* luciferase gene secrete luciferase (8). We now report that stable transformants of CHO cells secrete luciferase similarly and that the secretory process can be viewed from a single cell in real time by an imaging technique. Fig. 3 shows the time course of growth and expression of luciferase in a 6-well culture plate. The growth of the CHO cells is seen to be accompanied by an intracellular increase in luciferase and by an increase of luciferase concentration in the culture medium. The cells in the medium approached confluence at

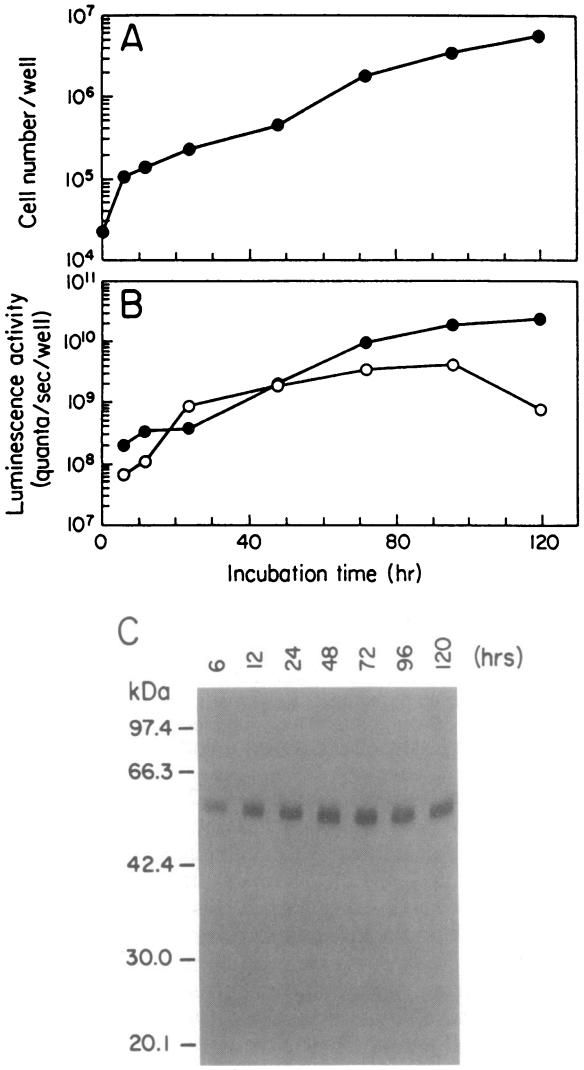

FIG. 3. Expression of luciferase by stable, transformed CHO cells. (*A*) Growth curve of CHO cells carrying pSV2-v1. (*B*) Luciferase activity in medium (●) and in cells (○). Three wells were assayed to obtain an average value. (*C*) Immunoblot analysis of luciferase in the culture medium at different incubation times.

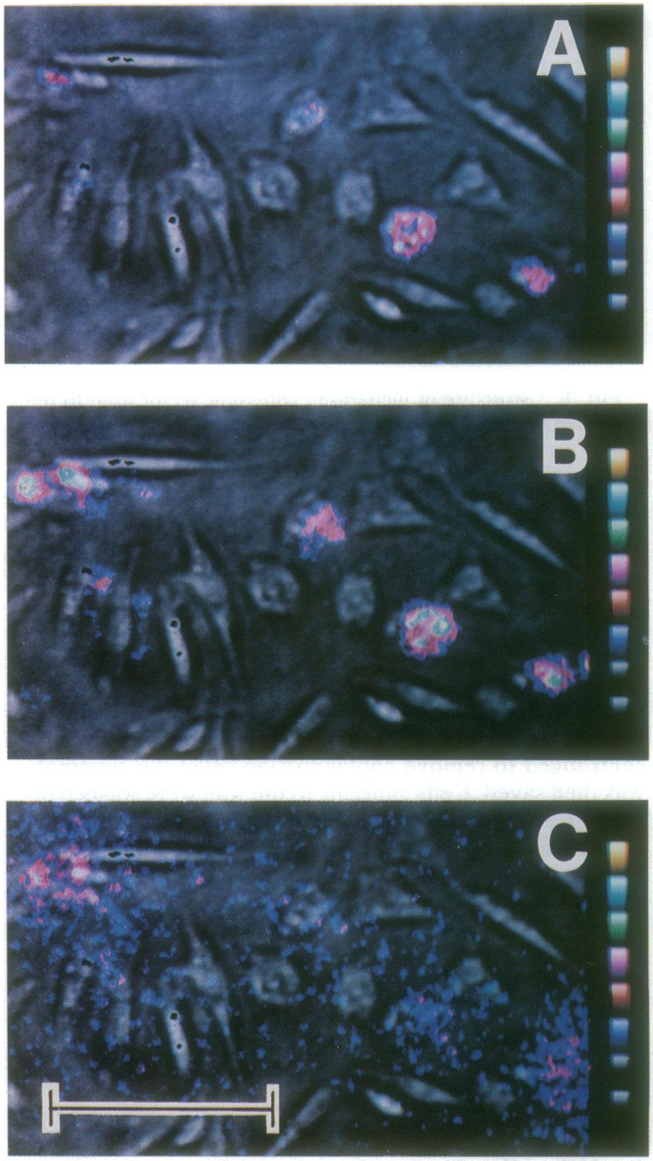

FIG. 4. Polaroid photographs of images of stable, transformed CHO cells incubated for 48 hr, showing luminescence originating at the site of luciferase secretion. The photographs were taken with a Videofix II color photo printer. The luminescence is due to the oxidation of luciferin by molecular oxygen, a reaction catalyzed by luciferase. A photon-counting image tube is used to detect the individual photons as bright points of light, which are then digitally accumulated over time to obtain the image. In the photographs, the image formed by luminescence alone is superimposed on the image of the cells (×290). The color code at right indicates the intensity profile of the accumulated photons, ranging from a low (blue) to a high (yellow). (*A*) Adherent CHO cells, layered with 100 μl of serum-free αMEM, 1 min after adding 100 μl of 49 μM luciferin solution to well. (*B*) Same CHO cells 5 min after luciferin addition. (*C*) Same CHO cells immediately after adding 20 μg of anti-*Vargula* luciferase IgG dissolved in 20 μl of 10 mM sodium phosphate buffer/137 mM NaCl/2.7 mM KCl, pH 7.4. (Bar = 100 μm.)

≈72 hr. Subsequently, the intracellular concentration of luciferase decreased (Fig. 3*B*), but the extracellular concentration of luciferase continued to increase, as shown by the luminescence-activity measurements and immunoblot analysis (Fig. 3 *B* and *C*).

The light emitted by the CHO cells in culture (10^{10}–10^{11} quanta per sec per well) (Fig. 3) is well within the visual sensitivity of the dark-adapted eye. The same cells viewed by image intensification are shown in Fig. 4. Not all cells are seen to be luminescing—that is, secreting luciferase. The most intense luminescence, corresponding to the highest concentration of luciferase being secreted, was associated with round-shaped cells. These cells were presumed to be in the still-developing, maturing stage because the cell culture had not reached confluence. Compared with cells in the 1-min photograph (Fig. 4*A*), the luminescing cells in the 5-min photograph (Fig. 4*B*) are seen to be luminescing more intensely, presumably as a result of the accumulation of luciferase. These cells also show a slight spreading in their luminous boundary, which is probably from diffusion of the enzyme. In contrast, mature elongated cells were found to be emitting only low levels of light. Thus, in a mixed population of CHO cells not dividing synchronously and in different stages of development, the cells secreting luciferase most actively appear to be those that have not yet reached maturity.

The *Vargula* reaction is extremely specific, and luciferin does not emit light in an aqueous medium without *Vargula* luciferase. Recombinant luciferase has a M_r of 62,171 (7), whereas native luciferase, which is glycosylated, has a M_r of 68,000 (23). The optimum pH of the reaction is 7.2 (24), and the turnover number (number of molecules of luciferin oxidized per molecule of luciferase) is 1600 per min (6). Luciferase is inhibited by EDTA and EGTA, suggesting that

Ca^{2+} may be involved in its activity (25). The diffusion constant is 7.4×10^{-7} cm^2/sec (26, 27), and the quantum yield is 0.28 ± 15% (28). *Vargula* luciferin is readily soluble in water, methyl alcohol, and 1-butyl alcohol. In the presence of a high concentration of serum albumin, *Vargula* luciferin emits a very weak chemiluminescence. The luminescence presumably results from the luciferin molecule becoming adsorbed nonspecifically to a hydrophobic site on the albumin molecule because *Vargula* luciferin chemiluminesces weakly in hydrophobic solvents, with, for example, a chemiluminescence quantum yield of 0.002 in dimethyl sulfoxide and 0.03 in diethylene glycol dimethyl ether (diglyme) (29). Therefore, serum-free αMEM was used for fetal calf serum αMEM in the imaging experiments. Control experiments also showed that the 20 μl of 1-butyl alcohol added with the luciferin did not affect the cellular luminescence.

Fig. 4 *A* and *B* shows two further characteristics of the actively secreting CHO cell: (*i*) secretion of luciferase takes place from a relatively small number of sites, as evidenced by their intense luminescence, and (*ii*) the sites are not evenly distributed over the cell surface, suggesting that domains may be involved in luciferase secretion. The sites remained the same during the entire observation period (30 min), even after the cells were washed with phosphate buffer/137 mM NaCl/2.7 mM KCl, pH 7.4, and treated with fresh luciferin (data not shown). It is problematical whether the amount of luciferase sequestered in a single vesicle is so large that the luciferase secretion can continue for >30 min. When adhering cells were released by treatment with trypsin, the cells ceased to luminesce. Further, when luminescing cells adhering to the well wall were washed with buffer, the cells stopped luminescing, but they immediately resumed luminescing when fresh luciferin was added; this result indicates that luciferin does not enter the cell for at least 30 min during exposure to luciferin. Cells exhibiting transient expression (48-hr incubation) emitted a weak luminescence from many points on the cell surface (data not shown). In both stable and transient CHO cells, however, luminescence was not seen between the adhering cell surface and the well wall.

When brefeldin A (Epicentre Technologies, Madison, WI), a fungal compound, was added to the culture medium at a concentration of 5 μg/ml, secretion of luciferase was >95% inhibited within ≈30 min. Brefeldin A is reported to have multiple targets, blocking the protein secretion pathway in mammalian cells (30–34). The observed effect of brefeldin A, therefore, suggests that a constitutive pathway may be involved in the exocytosis of luciferase.

Fig. 4*C* shows the effect of adding anti-*Vargula* IgG to the luminescing cells in Fig. 4*B*. The luminescence of the actively secreting cells is markedly reduced due to the inactivation of luciferase (17, 35–37), thus showing that the luminescence is associated with *Vargula* luciferase rather than with another protein.

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