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Two forms of secreted and thermostable luciferases from the marine copepod crustacean, *Metridia pacifica*

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ABSTRACT

We cloned two forms of the secreted and thermostable luciferase genes, MpLuc1 and MpLuc2, from the marine copepod, *Metridia pacifica*. The 840-bp MpLuc1 cDNA comprised a 630-bp open reading frame encoding a 210-amino acid polypeptide (22.7 kDa). MpLuc1 had the closest homology with *Metridia longa* luciferase. The 753-bp MpLuc2 cDNA consisted of a 567-bp open reading frame (20.3 kDa), and it had the closest homology with *Gaussia princeps* luciferase. Single-specimen genomic PCR confirmed the presence of two luciferase genes in *M. pacifica*, and single-specimen RT-PCR revealed that both luciferase mRNAs were expressed. Both MpLuc1 and MpLuc2 (MpLucs) specifically reacted with the substrate coelenterazine producing identical bioluminescent spectra (λmax, 485 nm), but with different kinetics. Adding salt such as $MgCl_2$ and $CaCl_2$ to the reaction mixture significantly enhanced MpLuc1 and MpLuc2 activities. Wild-type MpLucs were remarkably thermostable; MpLuc1 retained about 60% of the original activity even after incubation at 90 °C for 30 min. MpLucs expressed in NIH-3T3 and HeLa cells were largely secreted into the culture medium. Continuous monitoring of secreted MpLuc1 driven by the c-*fos* promoter demonstrated the potential usefulness of MpLuc1 in nondisruptive reporter assays.

1. Introduction

Nondisruptive reporter assays using a secreted luciferase is essential for the continuous, automated or high-throughput monitoring of gene expression in live cells since preparation of cell lysates is not required to measure bioluminescence. In the previous studies, the cloning and sequence analysis of secreted luciferases from marine organisms have been reported. These proteins have signal sequences at their N-termini, and they are efficiently secreted into culture medium, even when expressed in mammalian cells. Thus, the gene expression can be continuously monitored by measuring luciferase activity in a portion of culture medium without disrupting cells when it is expressed under the control of a promoter of interest. *Vargula* and *Cypridina* luciferases have been isolated from the marine ostracods *Vargula hilgendorfii* (Thompson et al., 1989) and *Cypridina noctiluca* (Nakajima et al., 2004), respectively, and expressed in mammalian cells (Thompson et al., 1990; Inouye et al., 1992; Nakajima et al., 2004), which demonstrated their advantages as secreted reporters in the real-time monitoring of gene expression. The luciferase of the deep-sea shrimp *Oplophorus gracilirostris* is also a secretory enzyme which is composed of 35 kDa and catalytic 19 kDa proteins (Inouye et al., 2000).

Secreted luciferases from the copepod crustaceans *Gaussia princeps* (Verhaegen and Christopoulos, 2002) and *Metridia longa* (Markova et al., 2004) have also been cloned, expressed in mammalian cells (Markova et al., 2004; Tannous et al., 2005) and used to continuously monitor gene expression in live cells (Verhaegen and Christopoulos, 2002; Markova et al., 2004; Wu et al., 2007). The advantages of copepod luciferases are as follows. Firstly, light emission simply depends on the presence of the substrate, coelenterazine, and no other co-factors are required. Secondly, copepod luciferases are

Abbreviations: Cluc, *Cypridina* luciferase; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MpLuc, *M. pacifica* luciferase; Rluc, *Renilla* luciferase.

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relatively small proteins. The molecular masses of the *M. longa* and *G. princeps* luciferases are 24 and 20 kDa, respectively, whereas those of the *Vargula*, *Cypridina*, and firefly luciferases are 61.7 (Thompson et al., 1989), 61.5 (Nakajima et al., 2004), and 60.7 (de Wet et al., 1987) kDa, respectively. Therefore, we believe that the further identification and characterization of novel copepod luciferases would have potential for the development of more advanced bioluminescent assays. Here we describe the cloning of the secreted and thermostable luciferases, Mpluc1 and MpLuc2, from the marine copepod, *Metridia pacifica*, family Metridinidae.

2. Materials and methods

2.1. PCR cloning of M. pacifica luciferase cDNAs

Zooplankton samples were collected from seawater pumped from a depth of 321 m in Toyama Bay and distributed through pipes to holding tanks in the Hotaruika ("firefly squid") Museum, located on the shore at Namerikawa, Japan (see Supplementary information). Total RNA was prepared from about 400 specimens in 4 ml of TRIzol reagent (Invitrogen), and then 5.8 μg of mRNA was purified from 179 μg of total RNA using an Oligotex-dT30 mRNA Purification Kit (Takara Bio). We synthesized cDNA from 1 μg of mRNA using the SMART RACE cDNA Synthesis Kit (Takara Bio). Degenerate primers were designed based on the *M. longa* and *G. princeps* luciferases. The 3′-end of *M. pacifica* luciferase cDNA was amplified using the White luc UP1 primer, 5′-GGC TGC ACY AGG GGA TGY CTK ATM TG-3′, and Universal Primer Mix (UPM) following the manufacturer's instructions. After sequencing the 3′-RACE products and homology identification with known luciferases by a BLAST search, the 5′-end of the luciferase cDNA was amplified using White luc LP4, 5′-TCA GCG CAA AGA TCA ACT TGA GCA ATG AAC-3′, and UPM. The amino acid sequences of the five copepod luciferases were aligned using the program ClustalW with default parameters.

2.2. Genomic PCR and RT-PCR analyses of a single M. pacifica

The bioluminescence emitted by a single specimen of *M. pacifica* was monitored by flicking each tube with a finger in a dark room. The specimens were fixed in 100% ethanol, and stored at −80 °C until DNA extraction. After complete removal of ethanol, the specimens were immersed in 200 μl of Buffer ATL (DNeasy Tissue Kit, Qiagen) containing 2 μg/μl of proteinase K, and then homogenized by passage through a 25G needle. Genomic DNA was purified using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. The primers for MpLuc1 gene and MpLuc2 gene amplification were as follows: (1) MpLuc1: White luc1 5′-UP2, 5′-AAA AGG AAA GGA GCT AAA TCT ACA GTC TA-3′ and White luc1 3′-LP2, 5′-CAT GAT CCA GTT ATC TCT TGT TCT GTT CT-3′; (2) MpLuc2: White luc2 5′-UP1, 5′-GAG TCC AAA CTG AAA GGT ACT CAA AAA TGG GAG TCA A-3′ and White luc2 3′-LP2, 5′-TAA CAT CAT TGG GCT AAA GTA TCA TCA TC-3′. Thermal cycling proceeded in a Mastercycler gradient PCR machine (Eppendorf) at 96 °C for 1 min, followed by 30 cycles of 96 °C for 5 s, 60 °C for 10 s and 68 °C for 50 s with Advantage 2 DNA polymerase (Takara Bio). For single-plankton RT-PCR, *M. pacifica* was lysed in 150 μl of Buffer RLT (Qiagen) and disrupted by homogenization as described above. Total RNA was isolated using an RNeasy Micro Kit (Qiagen), and reverse-transcribed using a Sensiscript RT Kit (Qiagen) with an oligo-dT primer. The MpLuc1 and MpLuc2 cDNAs were amplified using the same primer sets as those used in the genomic PCR, respectively.

2.3. Expression and purification of MpLuc1 and MpLuc2 in Escherichia coli

The coding regions of MpLuc1 and MpLuc2 were amplified from plasmid DNA harboring the cloned cDNA using *Pyrobest* DNA Polymerase (Takara Bio) and subcloned into the pET101/D-TOPO vector (Invitrogen). MpLuc proteins were expressed in BL21-CodonPlus®(DE3)-RIL *E. coli* host cells (Stratagene) that were vigorously shaken in 400 ml of LB medium containing 100 μg/ml carbenicillin at 18 °C. At the end of a 24-h induction by 1 mM isopropyl-β-D-thiogalactopyranoside, the cells were sedimented by centrifugation at 5000 ×*g* for 30 min at 4 °C, resuspended in 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, and sonicated using a Bioruptor UCD-200 (Cosmo bio) for 15 min at 4 °C. The soluble protein fraction was purified using DEAE Sepharose FF (column volume, 150 ml), HiTrap Butyl FF (5 ml×2) and HiLoad 16/60 Superdex 200 pg columns installed on an AKTAexplorer 10 S (GE Healthcare). Luciferase activities were assayed by mixing 20 μl fractions with 20 μl of coelenterazine (1 ng/μl) which was diluted in 10 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$. The initial light intensity counted for 3 s was recorded in relative luminescence units (rlu) using a Model AB-2200 luminometer (Atto). Protein concentrations were determined using a Protein Assay kit (Bio-Rad) with bovine serum albumin as the standard.

2.4. Assays of MpLuc1 and MpLuc2 bioluminescence

Luminescent spectra of recombinant MpLuc1, MpLuc2 and a crude extract of *M. pacifica* were measured using an AB-1850 S spectrofluorometer (Atto) (slit width, 1 mm; spectral resolution, 0.5 nm) for 20 s immediately after mixing 50 μl of MpLuc proteins and 200 ng of coelenterazine in a 0.2-ml PCR tube. To study the kinetics of a crude extract of *M. pacifica* and recombinant MpLuc proteins, a small portion of homogenate was placed in a reaction vial positioned in the sample compartment of a Mitchell–Hastings photomultiplier photometer, and then 500 μl of 20 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$ containing 500 ng of coelenterazine was injected. The initial maximal light intensity was recorded as rlu using a Panasonic VP-6712A chart recorder. Bioluminescence in all experiments, except for analyses of the spectra and kinetics, was measured using a MiniLumat LB 9506 luminometer (Berthold). To investigate substrate specificity, 50 μl of chemically synthesized *Vargula* luciferin (1 ng/μl) and coelenterazine (1 ng/μl) were each mixed with 20 μl of partially purified MpLuc1 and MpLuc2 recombinant proteins. In the analyses of ionic effects on bioluminescence, recombinant MpLuc1 or MpLuc2 was dialyzed against 2 changes of 20 mM Tris–HCl (pH 8.0) at 4 °C, then diluted at 1:100 with buffers containing 0.01 to 250 mM of $MgCl_2$, $CaCl_2$, NaCl, or KCl. The amount of bioluminescence was measured by mixing diluted MpLucs and 50 μl of coelenterazine (2 ng/μl) in buffers containing same concentration of salt ions as luciferase solutions. To determine MpLuc thermostability, 3.5 μl of partially purified MpLuc1 (0.62 mg/ml) or MpLuc2 (0.13 mg/ml) was diluted 30-fold with 20 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$, then incubated in a block-heater over a range of 4–90 °C for 30 min and cooled on ice for 5 min. Bioluminescent activity was measured by adding 50 μl of coelenterazine (1 ng/μl) to 20 μl of heated MpLucs in a luminometer cell at room temperature. The following buffers were used to measure bioluminescent spectra and pH sensitivity: 0.1 M acetate buffer (pH 4.5–5.5), 0.1 M MES-NaOH (pH 6.0 and 6.5), 0.1 M HEPES-NaOH (pH 6.7–7.5), 0.1 M Tris–HCl (pH 7.8–9.0) and 0.1 M carbonate buffer (pH 10.0). All buffers contained 50 mM $MgCl_2$.

2.5. Construction of mammalian expression vectors

The coding regions of the *Cypridina* and *Renilla* luciferases were amplified by *Pyrobest* DNA Polymerase (Takara Bio) from the template plasmids pCL-sv (Atto) and phRL-TK (Promega), respectively, and cloned into pcDNA3.2/V5/GW/D-TOPO (Invitrogen) to generate mammalian expression constructs. MpLuc1 and MpLuc2 expression vectors were also constructed as described above. The 0.86-kb fragment of the c-*fos* promoter (from positions −707 to +155) was amplified from human genomic DNA using *Pyrobest* DNA Polymerase, and introduced

into the Spe I–Not I sites of the MpLuc1- (pcDNA3.2-MpLuc1) and *Renilla* luciferase- (pcDNA3.2-Rluc) expression vectors to exchange the CMV promoter. All expression vectors were purified using an EndoFree Plasmid Maxi Kit (Qiagen).

2.6. Assay of the c-fos promoter

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and transfected in 6-well culture dishes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 10 h after co-transfection of pcDNA3.2-c-*fos*-MpLuc1 or pcDNA3.2-c-*fos*-Rluc with pGL3-Control into HeLa cells, the medium was replaced with fresh DMEM containing 10% FBS. The medium was removed on the following day, and serum-free DMEM was added. The cells were incubated for 24 h at 37 °C, and then provided with 2 ml of fresh DMEM with or without 10% FBS (time point, 0 h). Portions (50 μl) of the culture medium were collected from the serum-stimulated or unstimulated cells at 0, 2, and 6 h, and then 10-μl aliquots of the medium were assayed with 50 μl of 1 ng/μl of coelenterazine. Firefly and *Renilla* luciferase activities were assayed using the PicaGene Dual SeaPansy luminescence kit (Toyo Ink) according to the manufacturer's instructions.

3. Results

3.1. PCR cloning of luciferase genes, cDNA structures and alignment of amino acid sequences

While collecting fluorescent plankton from deep-sea water harvested at Toyama bay (Masuda et al., 2006), we found and isolated bioluminescent copepod, *Metridia pacifica*, which is transparent in seawater but visible as white spots on a black screen (Supplementary Fig.). Three luciferase sequences from copepod genera of the Metridinidae family, *G. princeps*, *Pleuromamma* sp. CSG-2001, and *Metridia longa* (GenBank accession numbers AY015993, AY015994, and AY364164, respectively) have been deposited in the database. Amino acid sequence alignment revealed greater homology between the *G. princeps* and *M. longa* luciferases at their internal and C-terminal regions than between the *Pleuromamma* luciferase and the others (Fig. 1). Since *M. pacifica* is classified in the same genus as *M. longa*, their luciferases should have high homology. We thus designed degenerate primers based on the homologous region between the *G. princeps* and *M. longa* luciferases. The 3′-end of cDNA was amplified from *M. pacifica* cDNA using degenerate and adapter primers. The deduced peptide sequence of the ~600-bp 3′-RACE product was homologous with those of the *G. princeps* and *M. longa* luciferases. We used this sequence to design a reverse primer with which we amplified the 5′-end of the *M. pacifica* luciferase cDNA. Sequence analysis of eight 5′-RACE products revealed that they could be categorized into two types. Full-length cDNAs of both types were then amplified using an adapter primer and two gene-specific primers that were individually designed based on the putative 5′-end sequence of each cDNA. Type I cDNA (MpLuc1) was 840-bp long plus polyA and contained a 630-bp open reading frame encoding a protein of 210 amino acids (22.7 kDa; p*I*, 5.03). Type II cDNA (MpLuc2) comprised 723-bp with an open reading frame that encoded a 189-amino acid polypeptide (20.3 kDa; p*I*, 7.39). The complete sequences of the MpLuc1 and MpLuc2 cDNAs were deposited in the DDBJ/EMBL/GenBank database under the accession numbers AB195233 and AB195234, respectively. A homology search using the BLAST program revealed that MpLuc1 was the most homologous with *M. longa* luciferase (nucleotide and amino acid sequence identity, 90 and 77%, respectively). In contrast, the identity of MpLuc2 was the highest to the *G. princeps* luciferase (84 and 68%, respectively)(Fig. 1). The amino acid sequence identity of MpLuc1 relative to MpLuc2 was 61%.

Both PSORT II (http://psort.nibb.ac.jp/) and SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) predicted the N-terminal signal peptides and the signal cleavage sites between Ala-18 and Asn-19 (VQA-NP) in the MpLuc1 sequence, and Ala-17 and Ala-18 (AQA-AT) in the MpLuc2 sequence with a maximal probability of 98 and 94%, respectively (Fig. 1, arrowhead). These features resemble that of *M. longa* luciferase, in which the N-terminal 17 amino acids comprised a predicted signal peptide (Markova et al., 2004).

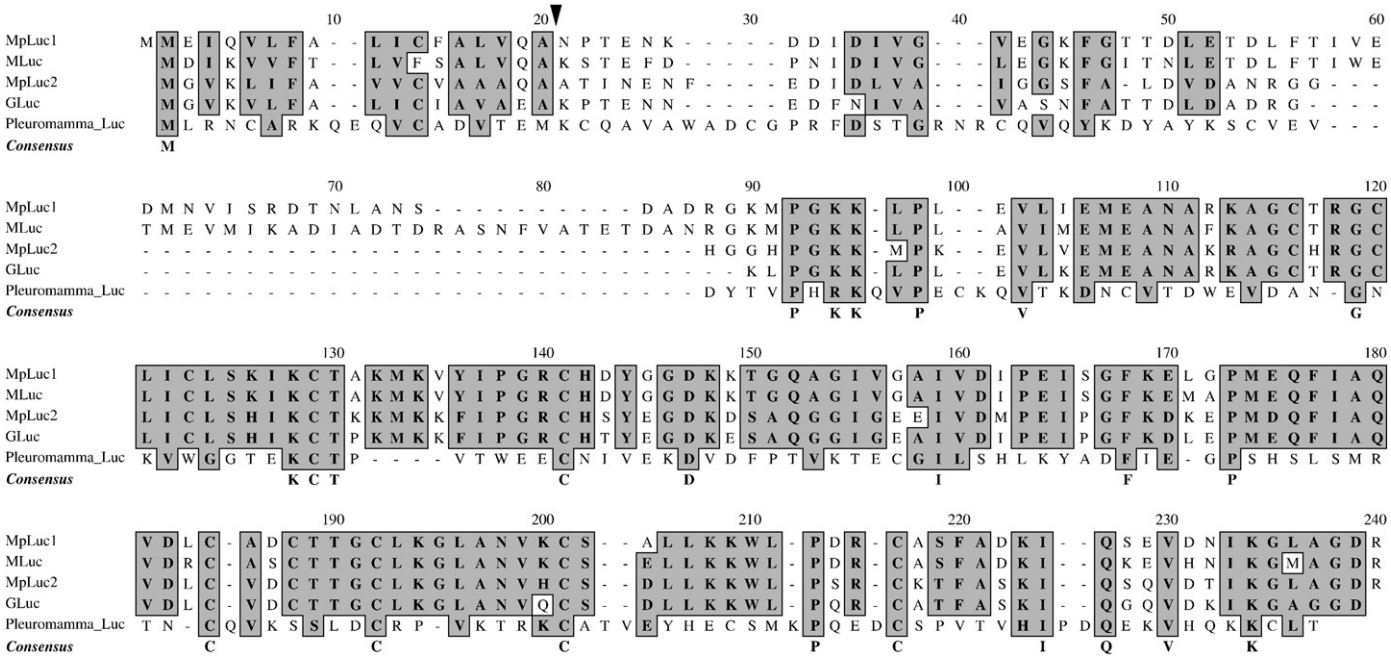

Fig. 1. Multiple amino acid sequence alignment of five copepod luciferases. Shaded and outlined residues represent at least 80% identical or similar amino acids. Organisms are *M. pacifica* (MpLuc1, AB195233 and MpLuc2, AB195234), *M. longa* (MLuc, AAR17541), *G. princeps* (GLuc, AAG54095) and *Pleuromamma* sp. CSG-2001 (*Pleuromamma*_Luc, AAG54096). Arrowhead indicates putative cleavage sites of N-terminal secretion signals of MpLuc1 and MpLuc2.

3.2. Identification of two forms of luciferases in a single M. pacifica

Although we isolated a substantial number of both the MpLuc1 and MpLuc2 cDNAs, we could not rule out the possibility that one of the two luciferases was derived from other luminous organisms contaminating the collection of *M. pacifica*. Another possibility was heterogeneous MpLuc expression, in which some *M. pacifica* express only MpLuc1 mRNA whereas others express only MpLuc2. To determine whether both luciferases were derived from *M. pacifica*, we performed single-specimen genomic PCR using MpLuc1- and MpLuc2-specific primers. Fig. 2A shows that both MpLuc1 and MpLuc2 genes were amplified from genomic DNA isolated from a single *M. pacifica* among all 4 tested specimens. All bands were at positions corresponding to higher molecular masses (~1.2 kb and 1.1 kb for MpLuc1 and MpLuc2, respectively) than those predicted (706 and 647 bp, respectively). We then cloned and sequenced both PCR products from 2 specimens. A comparison of the sequences of the genomic PCR products (GenBank accession numbers AB371096 and AB371097) with those of the MpLuc cDNAs revealed several introns in both genes at different positions (Fig. 2C). We found 5 introns in the MpLuc1 gene and 3 in the MpLuc2 gene, and all of them had consensus 5′ and 3′ splice site sequences (5′-GT….AG-3′) (Fig. 2D), ranging in length from 62 to 232 bp. These findings indicated that the mRNAs for the MpLucs were not alternatively spliced, but were transcribed from genes at different loci. We addressed the second question by analyzing the mRNA expression of both MpLuc1 and MpLuc2 in *M. pacifica* using single-specimen RT-PCR. We amplified the MpLuc cDNAs with the same primer pairs used in genomic PCR, followed by isolation and reverse transcription of mRNA from *M. pacifica*. The PCR products migrated at the predicted size (Fig. 2B) were cloned and sequenced to confirm MpLuc amplification (data not shown). Both MpLuc1 and MpLuc2 mRNAs were expressed in a single *M. pacifica* (Fig. 2B). Plasmid DNAs harboring MpLuc1 or MpLuc2 cDNA served as positive and negative controls for the PCR templates to confirm the primer specificity and the equivalent efficiency of MpLuc1 and MpLuc2 amplification by PCR (Fig. 2B, V1 and V2).

3.3. Bioluminescence spectra, kinetics, and substrate specificity of MpLucs

MpLuc1 and MpLuc2 were each overexpressed in *E. coli* without purification tags at their N- or C-termini because the effect of tagging on the activity or stability was unknown. The protein expression levels of both MpLuc1 and MpLuc2 were very low, although they were slightly improved by inducing protein expression at low temperature (18 °C) for a longer period (24 h). To date, we have not achieved completely pure proteins. Further improvements of expression and purification, including tag-fusion, are underway for more detailed characterization of these proteins. They were partially purified using sequential anion-exchange, hydrophobic interaction and gel-filtration column chromatography. The final concentrations of partially purified

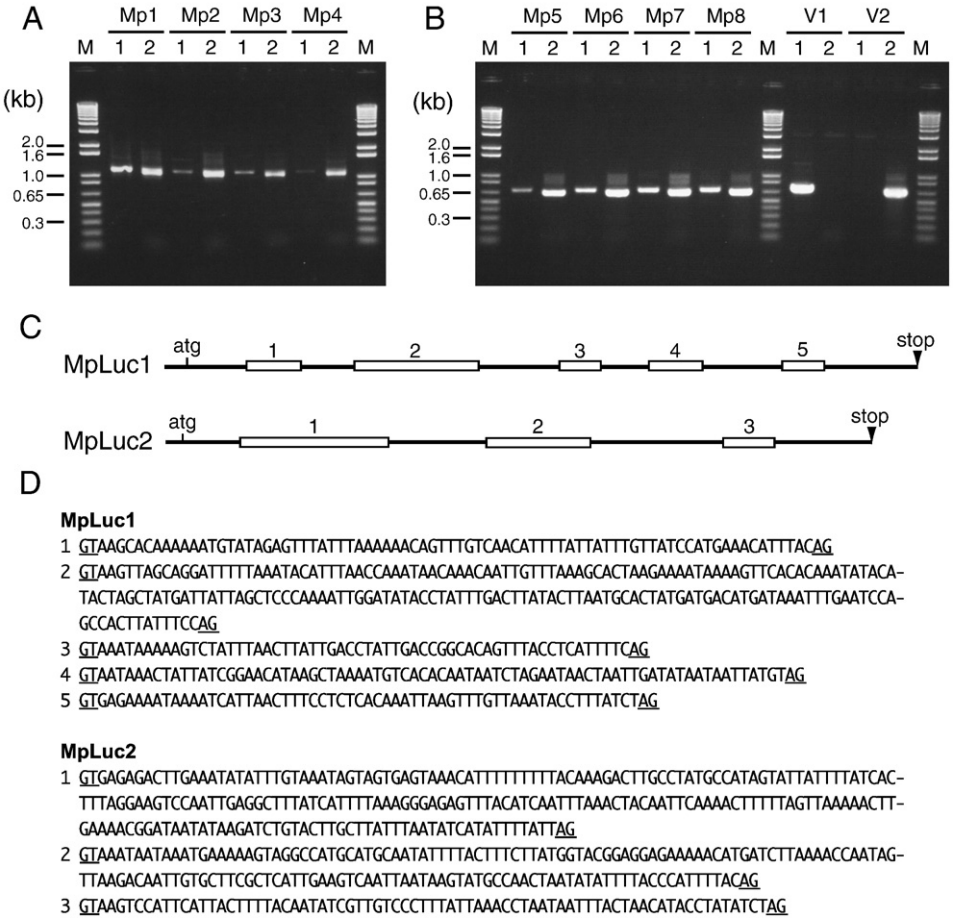

Fig. 2. Genomic and cDNA amplification of MpLuc1 and MpLuc2 from a single *M. pacifica*. (A) Genomic DNA isolated from four ethanol-fixed *M. pacifica* specimens (Mp1–4) was amplified with MpLuc1- or MpLuc2-specific primers. M, 1 kb plus DNA ladder; 1, PCR products using MpLuc1 primers (1.2 kb); 2, MpLuc2 primers (1.1 kb). (B) RT-PCR analyses of four *M. pacifica* specimens (Mp5-8) with MpLuc1- or MpLuc2-specific primers. PCR amplification also proceeded using plasmid vectors harboring MpLuc1 (V1) or MpLuc2 (V2) cDNA as templates with primers used in genomic and RT-PCR analyses under identical conditions. (C) Structures of MpLuc1 and MpLuc2 genes. Numbered open boxes indicate introns in MpLuc1 and MpLuc2 genes. (D) Sequences of introns in MpLuc genes. Consensus 5′ and 3′ splice site sequences are underlined. Numbers correspond to those indicated in (C).

MpLuc1 and MpLuc2 were 0.62 and 0.35 μg/μl, respectively (24.2- and 35.1-fold purification).

Crude extracts of *M. pacifica* mixed with coelenterazine emitted blue light (λmax, 485 nm), which corresponded to the fluorescence emission maximum of coelenteramide, the oxidized product of coelenterazine (Fig. 3A). The bioluminescent spectra of recombinant MpLuc1 and MpLuc2 were similar to that of *M. pacifica* extracts and their spectral maxima were pH-insensitive (Fig. 3B and C). However, the intensity of light emission from these luciferases appears highly pH-dependent (Fig. 4). The bioluminescent activities of both luciferases rapidly decreased in basic buffers at pH>8.4 and were completely lost at pH 10. In contrast, MpLuc1 in acidic buffers (pH 5–7) sustained about 40% of the intensity relative to the highest intensity at pH 8.0, and was still detectable even at pH 4.5 (10%).

The bioluminescent kinetics of MpLuc1 and MpLuc2 were monitored in a Mitchell–Hastings photomultiplier photometer starting immediately before injecting substrate coelenterazine, their results were evidently different. Upon coelenterazine injection, both the *M. pacifica* extract and recombinant MpLuc1 rapidly emitted light (Fig. 3D and E), whereas the bioluminescence of MpLuc2 increased and decayed more slowly (Fig. 3F). Both the *M. longa* and *G. princeps* luciferases oxidize coelenterazine as a substrate and emit luminescence. To analyze the substrate specificity of MpLuc1 and MpLuc2, we mixed the same amount of chemically synthesized coelenterazine or *Vargula* luciferin with the MpLuc1 or MpLuc2 recombinant protein. MpLuc1 and MpLuc2 emitted bright light only upon the addition of coelenterazine (Fig. 3G and H, respectively). These results are in good agreement with the substrate specificity of the crude *M. pacifica* extract (data not shown).

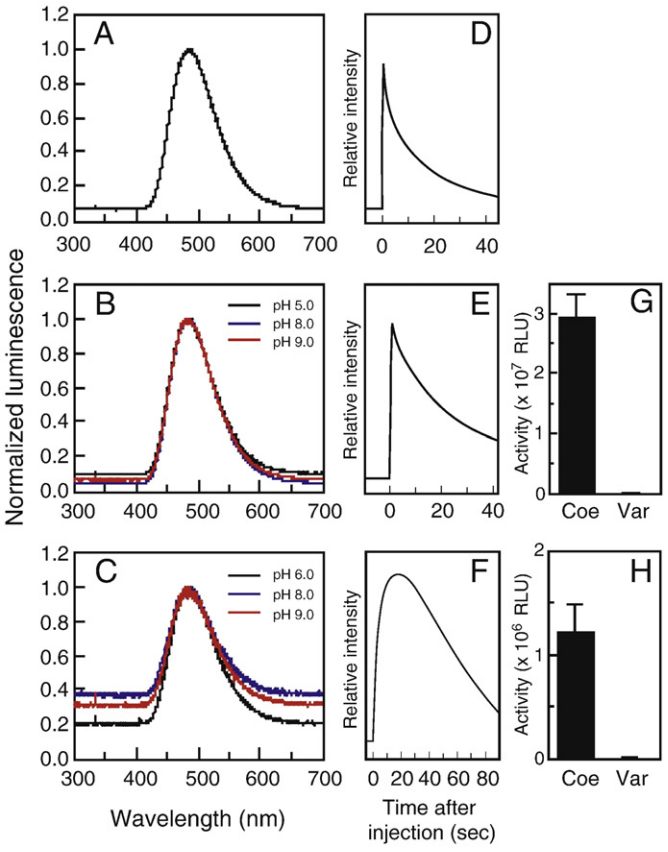

Fig. 3. Bioluminescence spectra and kinetics. Spectral curves of luminescent reactions of a *M. pacifica* crude extract (A), recombinant MpLuc1 (B), and MpLuc2 (C). Light emission spectra of MpLucs were measured at various pH values. Each curve was normalized against maximal light intensity at 485 nm as 1.0. Kinetics of bioluminescence of a *M. pacifica* crude extract (D), recombinant MpLuc1 (E), and MpLuc2 (F) were monitored using a Mitchell–Hastings photometer. Coelenterazine was injected into reaction vial at time zero. Substrate specificities of MpLuc1 (G) and MpLuc2 (H) were assayed with coelenterazine (Coe) and *Vargula* luciferin (Var). Results are shown as means±SD (n=3).

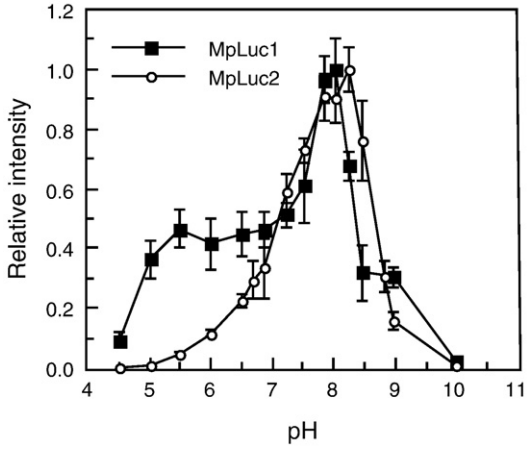

Fig. 4. Sensitivity of recombinant MpLucs to pH. Luminescence intensity of MpLuc1 (closed squares) or MpLuc2 (open circles) was measured in buffer of various pH values (n=5). Before measurement of bioluminescence, 1 μl of either MpLuc1 or MpLuc2 was mixed with 99 μl of dilution buffer to give final pH between 4.5 and 10.0. Luciferase activity was measured by mixing 10 μl of diluted MpLuc and 25-μl coelenterazine (4 ng/μl) in buffers at various pH values. Both curves were normalized against maximal intensity at pH 8.0 (MpLuc1) and 8.2 (MpLuc2), respectively.

3.4. Ionic effects on MpLuc bioluminescence

Firefly luciferase requires magnesium ions to exert enzymatic activity (Deluca, 1976) but is inhibited by excessive ionic strength (Denburg and McElroy, 1970). To clarify the effect of salt ions on MpLucs activity, we diluted MpLuc1 or MpLuc2 in buffers containing various concentrations of $MgCl_2$, $CaCl_2$, NaCl, or KCl, and then assayed their bioluminescence. Both MpLuc1 and MpLuc2 were significantly sensitive to all tested salts but with different degrees. Light emission by MpLuc1 and MpLuc2 linearly increased in $MgCl_2$ concentrations up to 2.5 mM (29-fold relative to activity at 0 mM) and 5 mM (4.7-fold), respectively (Fig. 5A). Fig. 5B shows that the bioluminescence of MpLuc1 and MpLuc2 also increased with increasing $CaCl_2$ concentrations. MpLuc1 activity was the highest at 1 mM $CaCl_2$ (9.3-fold) and decreased thereafter, whereas MpLuc2 activity increased up to 50 mM. By contrast, the activity of both MpLucs gradually increased with increasing NaCl or KCl concentrations up to 250 mM (Fig. 5C and D). Because $MgCl_2$ activated both MpLucs most at the lowest concentrations, MpLucs were purified in the buffer containing 10 mM $MgCl_2$ for sensitive detection of MpLuc activity.

3.5. Thermostability

We examined the thermostability of MpLuc1 and MpLuc2. Fig. 6A shows the relative bioluminescence of MpLucs plotted against increasing preincubation temperatures. Both MpLucs retained almost total activities after a 30-min incubation between 4 and 50 °C, but were gradually inactivated over 60 °C. Surprisingly, the bioluminescence of both proteins remained detectable even after incubation at 90 °C for 30 min (59 and 14% for MpLuc1 and MpLuc2, respectively). Therefore, we extended the incubation period of MpLucs at 95 °C up to 3 h (Fig. 6B). The bioluminescence intensity of MpLuc2 gradually decreased over time, whereas the curve of MpLuc1 flattened between 15 and 60 min of incubation. Even after 3 h-incubation at 95 °C, MpLuc1 and MpLuc2 sustained 15 and 2% of the bioluminescence, respectively, relative to their starting activities. Autoclaving MpLucs at 120 °C for 20 min resulted in the complete inactivation of MpLuc2, but in 2.4% retention (7.0×10^5 rlu/10 s) of the MpLuc1 activity (untreated protein, 2.9×10^7 rlu/10 s), indicating its robust thermostability.

3.6. Secretion of MpLuc1 and MpLuc2 expressed in mammalian cells

To evaluate MpLucs as possible reporter proteins, we constructed the mammalian expression vectors, pcDNA3.2-MpLuc1 and pcDNA3.2-MpLuc2, to express MpLucs under the control of the CMV promoter. NIH-3T3 and HeLa cells were transiently transfected with the vectors and incubated at 37 °C for 48 h. Thereafter, we assayed the bioluminescence of the cell lysates and the culture media. The morphology and the growth of cells transfected with these constructs were normal, indicating that the overexpression of MpLucs was not toxic to these mammalian cells. Both MpLuc1 and MpLuc2 were mostly secreted into the culture medium of the transfected cells. The percentages of secreted MpLuc1 activity relative to total activity detected in cellular lysate and medium were 94.0±6.1 and 97.9±3.5% for NIH-3T3 and HeLa cells, respectively, and those of MpLuc2 were 97.5±0.1 and 99.3±2.4%, respectively, which were equivalent to or higher than those of the *Cypridina* luciferase (CLuc) and *Vargula hilgendorfii* luciferase (Nakajima et al., 2004). We next continuously monitored the luciferase activities in the culture medium of cells expressing MpLuc1, MpLuc2 or CLuc, immediately after replacing the culture medium with fresh DMEM. The luciferase activities of MpLuc1, MpLuc2 and CLuc in the media were detectable as early as 15 min after media replacement and increased 30.3- (n=3, P<0.01), 3.6- (n=3, P<0.01) and 5.8-fold (n=3, P<0.01), respectively, as compared with those at 5 s after media replacement. The activities of MpLucs and CLuc in the culture media continuously increased for >24 h, indicating the stable accumulation of secreted luciferases in the media (Fig. 7).

3.7. Secreted reporter assay of the c-fos promoter

The c-fos protein functions in cell differentiation and proliferation as a crucial transacting factor for various genes. The c-*fos* gene is expressed in response to a wide variety of stimuli, such as platelet-derived growth factor, nerve growth factor, 12-*O*-tetradecanoylphorbol-13-acetate and ras p21 in many cell types. The c-*fos* gene enhancer contains serum- and cAMP-response elements. To demonstrate the capability of MpLuc1 as a secreted reporter protein, we created the expression construct, pcDNA3.2-c-*fos*-MpLuc1, in which MpLuc1 mRNA is transcribed under the control of the c-*fos* promoter. Furthermore, the pGL3-Control vector was transiently co-transfected to express firefly luciferase as an internal standard among transfected cells in different plates. HeLa cells transfected with pcDNA3.2-c-*fos*-MpLuc1 and pGL3-Control were incubated in serum-free medium for 24 h, washed twice with phosphate-buffered saline, and replenished with fresh DMEM with or without 10% FBS at the start of the time course experiment (0 h). We also constructed pcDNA3.2-c-*fos*-Rluc, which comprised Rluc (*Renilla* luciferase) under the control of the c-*fos* promoter, and co-transfected it with pGL3-Control into HeLa cells under the same conditions as the c-*fos*-MpLuc1 construct to compare the secreted and non-secreted reporters. The MpLuc1

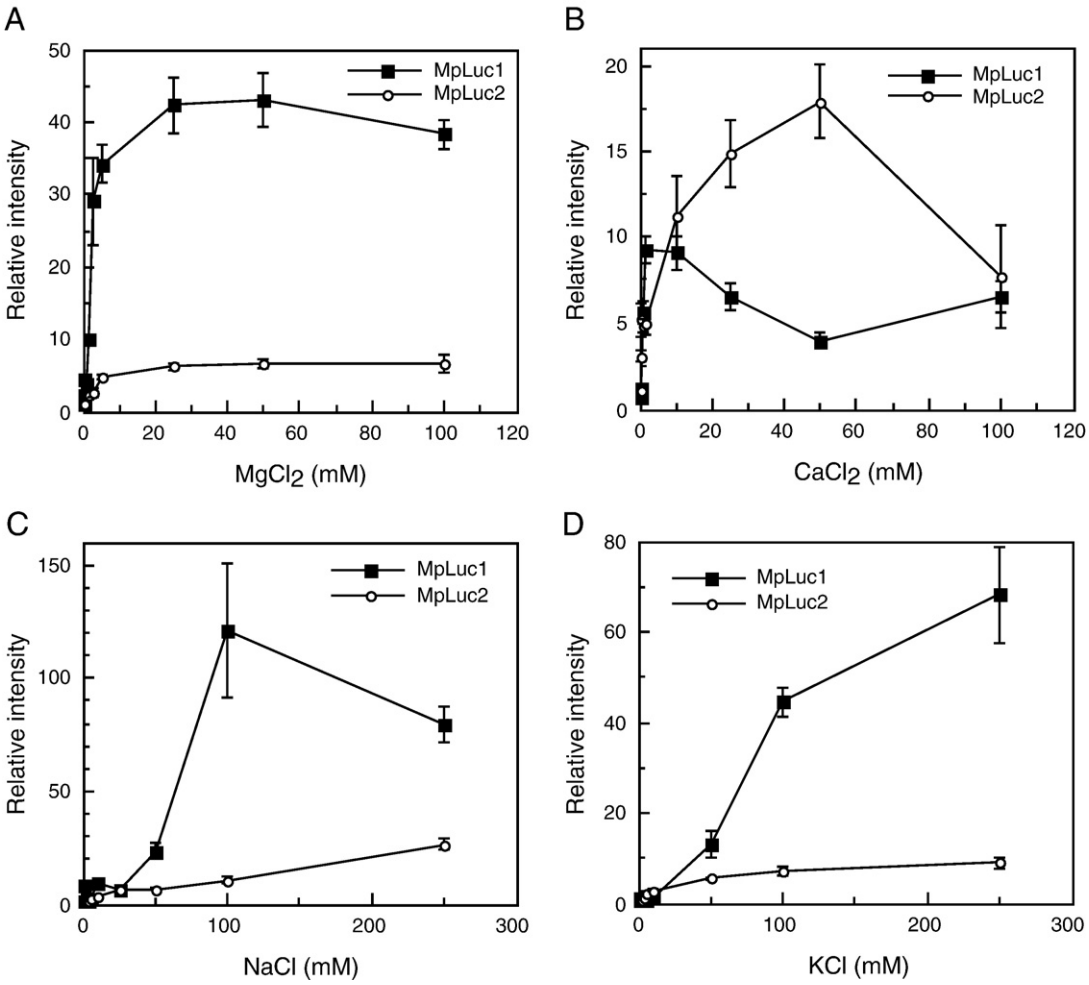

Fig. 5. Effect of salt ions on MpLuc1 and MpLuc2 bioluminescence. Recombinant MpLuc1 (closed squares) and MpLuc2 (open circles) were diluted in 20 mM Tris–HCl (pH 8.0) containing various concentrations of $MgCl_2$ (A), $CaCl_2$ (B), NaCl (C), or KCl (D), then luciferase activity was assayed three times to obtain an average value. Luminescent intensities are expressed as fold increase, in which the value without salt (nearly 0 mM) is defined as 1.0.

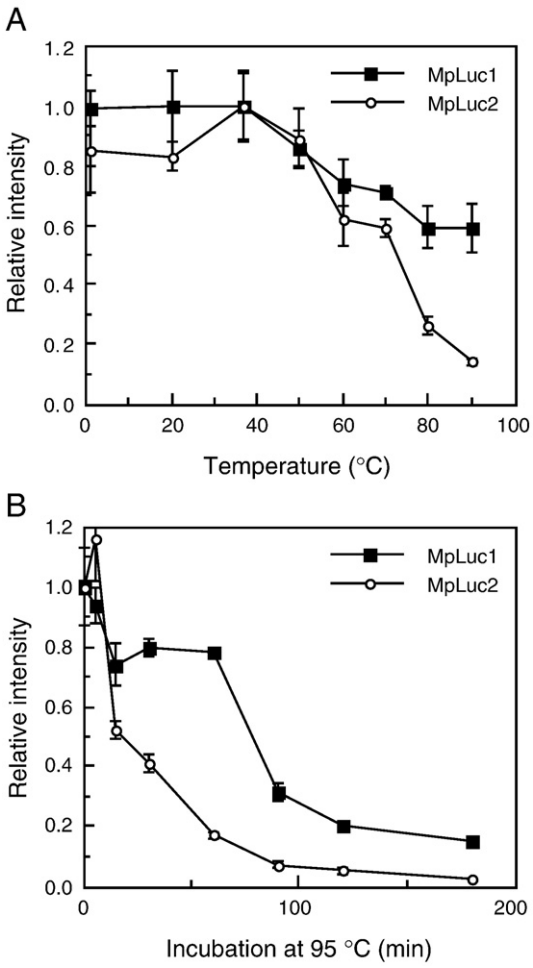

Fig. 6. Thermostability of MpLucs. (A) MpLucs were continuously heated at temperatures ranging from 4–90 °C in 20 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$ for 30 min. Luminescence intensity was measured at room temperature after cooling samples at 4 °C for 5 min (n=5). Results are expressed as normalized curves. (B) Thermostability of MpLucs at 95 °C. Incubation period was extended up to 3 h.

activity was slightly higher in the culture medium of serum-stimulated, than non-stimulated cells at 2 h after media replacement, and more clearly differed at 6 h (Fig. 8). The serum stimulation of cells

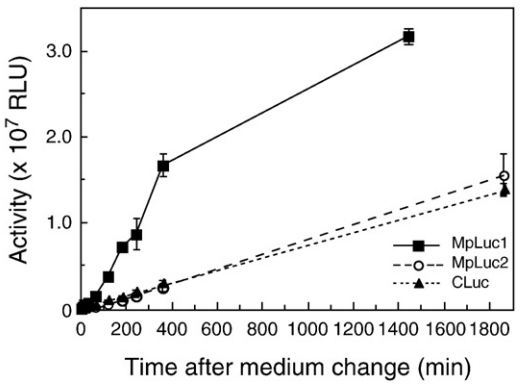

Fig. 7. Expression and secretion of MpLucs into culture media of HeLa cells. HeLa cells were transfected with pcDNA3.2-MpLuc1, pcDNA3.2-MpLuc2, or pcDNA3.2-CLuc, and incubated without medium changes. Luciferase activity of MpLuc1 (closed squares), MpLuc2 (open circles), or CLuc (closed triangles) in culture medium was continuously measured for 24 (MpLuc1) or 31 (MpLuc2 and CLuc) h. Results are shown as means ± SD (n=3).

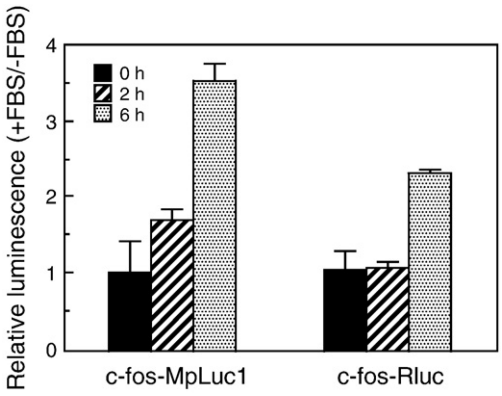

Fig. 8. Secreted luciferase assay using MpLuc1 as a reporter. HeLa cells expressing MpLuc1 (c-*fos*-MpLuc1) or Rluc (c-*fos*-Rluc) under control of the c-*fos* promoter were cultured in serum-depleted medium for 24 h, and then stimulated by DMEM with or without 10% FBS. Bioluminescence in culture medium was measured at 0 (black bars), 2 (striped bars) and 6 (gray bars) h after serum stimulation. Results are expressed as average fold increase ± SD (n=3) against values without FBS.

transfected with pcDNA3.2-c-*fos*-Rluc also resulted in an increase in the relative amount of light emission from the cell lysate at 6 h, implying that MpLuc1 is a potent reporter enzyme that can continuously monitor gene expression without destroying cells.

4. Discussion

Molecular cloning, sequencing and PCR analyses identified two forms of luciferases in *M. pacifica*. A single *Phrixothrix* railroad-worm has one luciferase that emits yellow-green light from lateral lanterns along the body, and another that emits red light through cephalic lanterns (Viviani et al., 1999). Another organism that possesses distinctly different types of luciferases is the bioluminescent click beetle, *Pyrophorus plagiophthalamus* (Wood et al., 1989). Unlike these luciferases that are distinguishable by the colors of their bioluminescence, the spectral properties of MpLuc1 and MpLuc2 were identical (Fig. 3B and C). One apparent difference between MpLuc1 and MpLuc2 was the nature of the bioluminescent kinetics under the same measurement conditions. Considering that the kinetics of the *M. pacifica* crude extract and MpLuc1 (Fig. 3D and E), but not MpLuc2 (Fig. 3F), were similar, more optimal reaction conditions for MpLuc2 might exist. MpLuc2 would rapidly respond to the addition of coelenterazine, as seen with the extract and MpLuc1. Another notable difference between the two luciferases was their specific activities when they were expressed in *E. coli* and mammalian cells. The MpLuc2 activity was always lower than that of MpLuc1 (data not shown) for reasons that remain obscure. We are presently investigating differences in protein expression levels and turnover rates between MpLuc1 and MpLuc2 in *E. coli* and mammalian cells. Further characterization of the MpLucs should explain why two luciferases are expressed in *M. pacifica*.

The luciferase activities of both MpLuc1 and MpLuc2 sharply increased with $MgCl_2$ and $CaCl_2$ concentrations at and below 10 mM, and were also enhanced at higher concentrations of NaCl and KCl (over 100 mM). Although the mechanism of enhancement is unknown, these results are consistent with the notion that MpLucs are secreted into and function in a marine environment that contains about 470 mM Na^+, 10 mM Ca^{2+} and 50 mM Mg^{2+}. A motif search of MpLuc1 and MpLuc2 indicated that their amino acid sequences did not include the EF-hand motif seen in aequorin (Inouye et al., 1985). Salt ions might affect on the protein conformation, association, or folding to modulate their activities. Analyses such as competitive effect and conformational change of MpLucs by salt ions remain to be clarified.

A striking feature of the MpLucs was thermostability. Among numerous thermal studies (Kajiyama and Nakano, 1993; White et al., 1996; Law et al., 2006; Branchini et al., 2007), to the best of our knowledge, none has described a luciferase with substantial activity after incubation at 95 °C for more than 30 min. We also confirmed the thermostability of MpLuc1 that was expressed in NIH-3T3 cells and secreted into the culture medium (65.5% of the initial activity upon incubation at 95 °C for 30 min). These results were completely unexpected for us because the *M. pacifica* specimens were collected from cold seawater, and their amino acid sequences contained no notable features. Therefore, the remarkable thermostability of the MpLucs might have a structural explanation. It is also noteworthy that the thermostability of recombinant MpLuc1 significantly decreased with the protein concentration (data not shown). We are further analyzing the thermal properties of MpLuc1 and MpLuc2 for their application to assays that require robust thermostability such as monitoring of gene expression in thermophilic microorganisms.

MpLuc1 and MpLuc2 were successfully expressed in mammalian cells. Both proteins were extensively secreted into culture media (Fig. 7), which allowed transcription activity to be monitored very sensitively without cell destruction (Fig. 8). Other potent properties of MpLuc1 and MpLuc2 as a secreted reporter were (i) smaller size (22.7 and 20.3 kDa, respectively) than those of *Vargula* (61.7 kDa) and *Cypridina* (61.5 kDa) luciferases, (ii) expression of active forms in *E. coli*, (iii) simple reaction in which luciferin and luciferases are the only two requirements for light emission, and (iv) specific reactions with coelenterazine that is not oxidized by any other proteins in the culture medium such as bovine serum albumin. Here we demonstrated that MpLuc1 and MpLuc2 have even more advantages such as remarkable thermostability, high salt tolerance and constant bioluminescent spectra in pH-independent manner. These features have not been reported in studies of *M. longa* and *G. princeps* luciferases. We consider that the application of bioluminescent MpLucs to virtually any detection system would expand their potential as reporter proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2008.07.041.

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