Quick Detection of Firefly Luciferase Gene Expression in Live Developing Bovine Embryos by Photoncounting

A. Nakamura, J. Okumura and T. Muramatsu[1]
Department of Biological Resources and Environmental Sciences, School of Agricultural Sciences, Nagoya University, Chikusa-ku Nagoya-shi 464-0814, Japan

ABSTRACT : The present study was designed, first to develop the new methodology to measure the bioluminescence activity easily in live developing bovine embryos by photoncounting, and secondly to compare the expression efficiency of four luciferase reporter genes in bovine embryos at four- to 16-cell stages. In experiment 1, equimolar pSVlacZ and pSVEluc were microinjected into the pronucleus of fertilized bovine oocytes. At 2 days after microinjection, bioluminescence activity of these embryos was measured by photoncounting with a luminometer for 1 min, and lacZ gene expression in the same embryos was assayed by X-gal staining. All the luciferase-positive oocytes showed some bacterial β-galactosidase activity irrespective of the intensity. In experiment 2, four firefly luciferase genes (pTKEluc, pTK6WEluc, pSVEluc and pMiwluc) were introduced by microinjection, and the injected embryos were cultured for the following 2 days. Detection of the luciferase gene expression was done by photoncounting at 5 to 55 min. Over the measurement period, the luciferase activity was almost constant irrespective of the transgenes microinjected. The luciferase activity and expression efficiency at 2 days after microinjection were not significantly affected by the difference in the microinjected transgenes. The present results demonstrated that the bioluminescence activity in live developing bovine embryos could be measured quickly by photoncounting.
(Key Words: Bovine Embryo, Gene Expression, Microinjection, Firefly Luciferase, Luminometer)

INTRODUCTION

Production of foreign proteins in milk using genetically engineered livestock has been considered to be a suitable system for making therapeutic recombinant proteins (Harris et al., 1990; Muramatsu and Nakamura, 1997). However, the production efficiency of transgenic cattle by using microinjection is very poor due to a low rate of embryos carrying transgenes in total transplantable ones (Roschlau et al., 1989; McEvoy and Sreenan, 1990; Krimpenfort et al., 1991; Hill et al., 1992). Therefore, selection of transplantable bovine embryos carrying transgenes is of crucial importance to improve its production. For this purpose, we have developed a qualitative screening method in which the transgene expression is visualized by using photonimaging in microinjected bovine embryos (Muramatsu and Nakamura, 1997).

So far, quantitative analyses of gene expression in preimplantation embryos have been done with bacterial chloramphenicol acetyltransferase (Majumder et al., 1993), bacterial β-galactosidase (Ueno et al., 1987) and firefly luciferase (Majumder et al., 1993; Mélin et al., 1993; Thompson et al., 1994, 1995b) as reporter proteins. In these quantitative assays, however, fertilized oocytes have to be sacrificed and their cell extracts are to be assayed. During such procedures, some, if not all, of the enzyme activity derived from the reporter gene may be lost. Moreover, once sacrificed, the embryos can no longer be usable for examining the luciferase activity at later developmental stages nor for transplantation to recipient cattle. These problem could be solved by developing a noninvasive monitoring system of gene expression in live developing embryos.

The present study was conducted to establish a new methodology for measuring bioluminescence activity easily in live developing bovine embryos, and to compare expression efficiency of four luciferase genes at four- to 16-cell stages of embryos.

MATERIALS AND METHODS

Transgene constructs for microinjection

The transgenes used in the present study are given in

[1] Address reprint requests to T. Muramatsu.
Received August 25, 1997; Accepted March 10, 1998

figure 1. The pSVlacZ (pSV β-Galactosidase Control) and pSVEluc (pGL2-Control) containing the SV40 early promoter and enhancer were obtained commercially (Promega, Madison, WI, USA). The plasmid pTKEluc was constructed by replacing the SV40 promoter region of pSVEluc for the thymidine kinase (TK) promoter sequences of p6WTKlacZ (Schöler et al., 1989). The plasmid pTK6WEluc was constructed from pSVEluc by replacing the SV40 promoter region for the fragment of 6W (a hexamer of 1W) enhancer and the TK promoter.

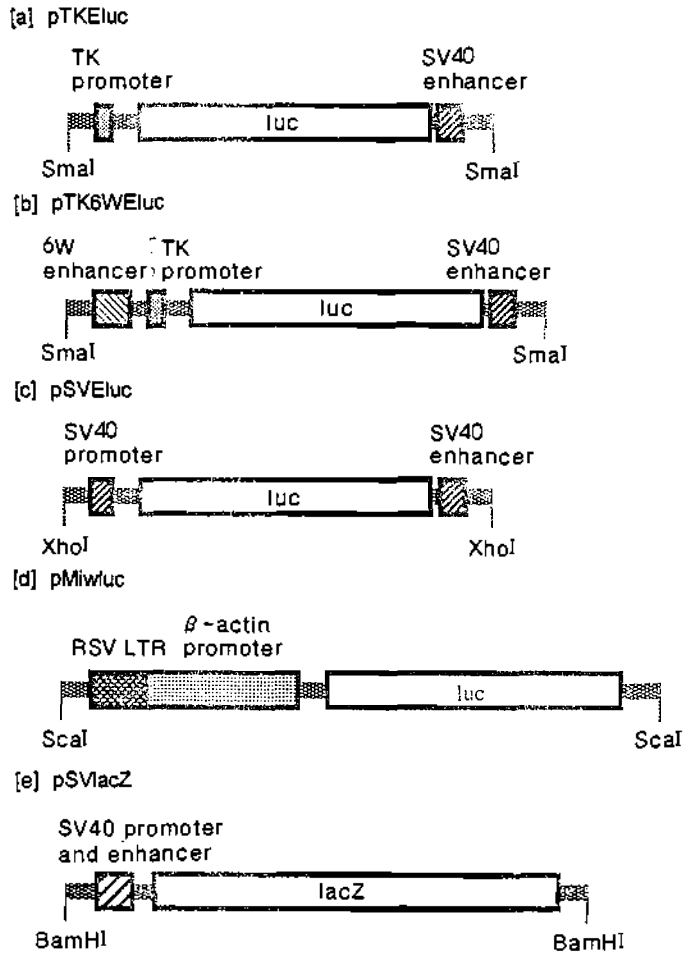

Figure 1. Structures of microinjected transgenes: [a] pTKEluc, [b] pTK6WEluc, [c] pSVEluc, [d] pMiwluc and [e] pSVlacZ. These plasmid DNAs were linearized with unique restriction endonucleases and dissolved in TE buffer (10 mM Tris-HCl (pH 7.5)/0.1 mM EDTA) to give an equimolar concentration at 3.2 μg/ml (pTKEluc), 3.4 μg/ml (pTK6WEluc), 3.2 μg/ml (pSVEluc), 3.8 μg/ml (pMiwluc) and 3.6 μg/ml (pSVlacZ). Abbreviation: luc, firefly luciferase cDNA; TK, thymidine kinase; 6W, hexamer of the 1W fragment containing an octmer motif; SV40, simian virus 40; RSV, Rous sarcoma virus; LTR, long terminal repeat; β-actin, chicken β-actin; lacZ, bacterial lacZ gene encoding β-galactosidase.

The 1W fragment was the oligonucleotides containing the μE4 and Oct binding sites (Schöler et al., 1989). The pMiwluc was constructed by fusing the regulatory region of pMiwZ (Suemori et al., 1990) upstream to the firefly luciferase cDNA and the SV40 polyadenylylation signal sequences. The transgenes were linearized by unique restriction endonuclease digestion, and gel-purified with SUPREC-01 (TaKaRa, Kyoto, Japan). The linearized gene constructs of pSVlacZ, pSVEluc, pTKEluc, pTK6WEluc and pMiwluc were resuspended in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA, and filtered through a 0.20 μm filter.

Egg manipulation and microinjection

Bovine ovaries were collected at an abattoir and transported to the laboratory at 37℃ in physiological saline (0.85% (w/v) sodium chloride). Cumulus-oocyte complexes collected from ovarian follicles were matured, fertilized, microinjected with DNAs, and cultured as described previously (Nakamura et al., 1995).

In experiment 1, the mixture of 1.6 μg/ml pSVEluc and 1.8 μg/ml pSVlacZ (1 : 1) was microinjected at 1 to 3 pl into the pronucleus of the fertilized eggs. The microinjected eggs were cultured for 2 days, and assayed for bioluminescence intensity and histochemical staining. In experiment 2, microinjection of four luciferase reporter genes, pTKEluc, pTK6WEluc, pSVEluc and pMiwluc, was performed as in experiment 1 at an equimolar concentration of 500 copies of the transgene in 1 pl, i.e. 3.2, 3.4, 3.2 and 3.8 μg/ml, respectively. The fertilized and microinjected oocytes were similarly cultured for 2 days, and analysed for bioluminescence intensity.

Detection of transgene expression

In experiments 1 and 2, the measurement of bioluminescence intensity was conducted by photoncounting with a luminometer (AutoLumat LB953, EG & G Berthold, Bad Wildbad, Germany). Each bovine oocyte cultured for 2 days was transferred into 50 μl of the fresh culture medium held in a plastic bute, to which an aliquot of 100 μl modified phosphate-buffered saline (PBS) (Whittingham, 1971) supplemented with 500 μM D-luciferin and 150 μl 40% (w/v) sucrose was added. The solution was mixed gently to bring the embryo up to a bioluminescence detectable level of the apparatus, i.e. 6 mm above the bottom of tubes. Luciferase activity was measured for 1 min at 5 min (experiment 1), and at 5, 15, 20, 25, 30, 35, 40, 45, 50 and 55 min (experiment 2) after the addition of D-luciferin and sucrose.

In experiment 1, following the luciferase assay, detection of the lacZ gene expression in the same

embryos was also performed by histochemical X-gal staining for bacterial β-galactosidase as reported by Nakamura et al. (1995).

Statistical analyses

The χ^2 test was used to examine the significance of differences in the expression efficiency of bovine embryos injected with the four firefly luciferase genes in experiment 2. The quantitative data of luciferase activity in experiment 2 were treated statistically by analysis of variance, and significance of differences between means was assessed by a Duncan's multiple range test by using General Linear Model Procedures of SAS (SAS Institute, 1985).

RESULTS

Gene expression of bovine oocytes injected with both pSVlacZ and pSVEluc in experiment 1 is presented in table 1. All the luciferase-expressing oocytes showed some bacterial β-galactosidase activity irrespective of the intensity. In two out of 30 (6.7%) luciferase-negative embryos, β-galactosidase activity was detected.

Table 1. Gene expression at four- to 16-cell stages of bovine embryos microinjected with both the lacZ and firefly luciferase genes (experiment 1)

	No. of embryos microinjected
Luciferase-positive	7
lacZ-positive	7
lacZ-negative	0
Luciferase-negative	30
lacZ-positive	2
lacZ-negative	28

The mixture of 1.6 μg/ml pSVEluc and 1.8 μg/ml pSVlacZ (1 : 1) was microinjected into the pronucleus of the bovine zygotes. The DNA solution contains 250 copies of each transgene.
The embryos that cleaved normally and developed to four- to 16-cell stage were analysed at 2 days following gene transfer.

Time course of luciferase activity in bovine embryos microinjected with pSVEluc in experiment 2 is represented in figure 2. The luciferase activity was almost stable throughout the measurement period from 5 to 55 min, and there was no significant difference in the luciferase level detected at each time ($p > 0.05$). Similarly, the luciferase activity in embryos microinjected with pTKEluc, pTK6WEluc of pMiwluc, was not significantly ($p > 0.05$) influenced by the detection time (data not shown).

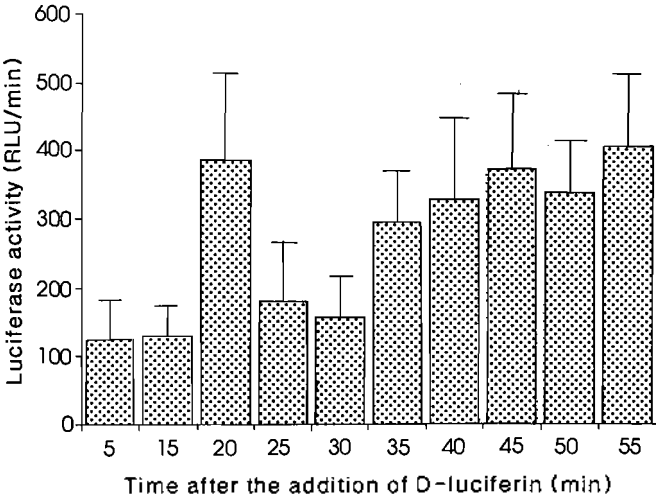

Figure 2. Time course of luciferase activity in bovine embryos microinjected with pSVEluc (experiment 2). The zygotes that have developed normally up to 16-cell stages were assayed. Each value represents mean ± SEM of 15 to 18 replicates. There was no significantly difference in the luciferase activity between the measurement time points ($p > 0.05$).

Expression efficiency of bovine embryos injected with the four firefly luciferase genes in experiment 2 is shown in table 2. The efficiency was not significantly affected by the difference in the transgenes microinjected according to the χ^2 test ($p > 0.05$). Values for bioluminescence intensity analysed by photoncounting are also represented in figure 3. All embryos microinjected with the luciferase gene showed significantly higher luciferase activity than did non-injected embryos ($p < 0.05$), although there was

Table 2. Expression efficiency of firefly luciferase in bovine embryos microinjected with the firefly luciferase genes driven by four different promoter constructs (experiment 2)

	Transgene			
	pTKEluc	pTK6WEluc	pSVEluc	pMiwluc
1-cell[a]	70	94	92	71
> 4-cell[b]	45	68	62	46
Luciferase-positive(%)[c]	15 (33.3%)	17 (25.0%)	19 (30.7%)	13 (28.3%)

[a] No. of oocytes survived after microinjection.
[b] No. of oocytes developed to four- to 16-cell stage at 2 days after microinjection.
[c] No. of oocytes expressing luciferase gene at 2 days after microinjection/No. of oocytes survived after microinjection.
There was no significant difference between the genes microinjected in expression efficiency of the luciferase gene according to the χ^2 test ($\chi^2 = 1.03$, df = 3, $p > 0.05$).

no significant difference in luciferase activity between four different promoters ($p > 0.05$).

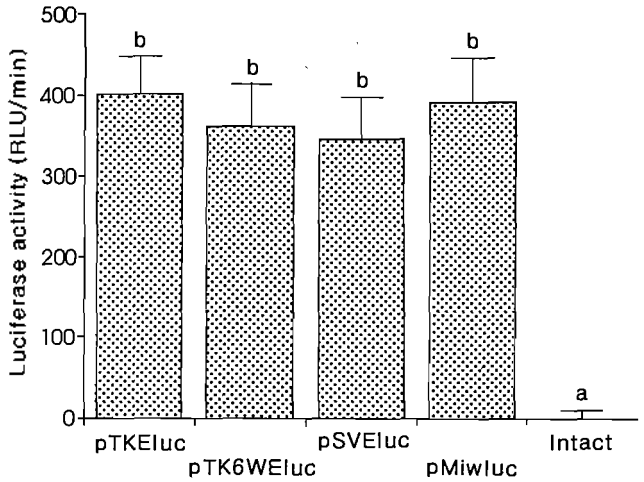

Figure 3. Comparison of the luciferase gene expression directed by four different promoter constructs in bovine embryos at 2 days after microinjection (experiment 2). The zygotes that have developed normally to four- to 16-cell stages were assayed. The values were presented as mean ± SEM with no. of replicates as: pTKEluc, n = 14; pTK6WEluc, n = 17; pSVEluc, n = 18; pMiwluc, n = 13; Intact, n = 54, ^{a,b} Means not having the same letter are significantly different at $p < 0.05$.

DISCUSSION

In the present study, an attempt was made first to develop a new method for measuring firefly luciferase acitivity in living bovine embryos by photoncounting with a luminometer. The bioluminescence activity of living zygotes was found to be assayed rapidly and easily by using the photonimaging system (Matsumoto et al., 1994; Thompson et al., 1995a; Muramatsu and Nakamura et al., 1997). Because the imaging apparatus is very expensive and its analysis is qualitative, we examined the possibility of the use of photoncounting with a luminometer. So far, it has been reported that by using a luminometer the firefly lucifrase gene expression has been analysed quantiatively in the cell extract of mouse preimplantation embryos (Majumder et al., 1993; Mélin et al., 1993; Thompson et al., 1994, 1995b). However, the embryos can no longer be used for examining the luciferase activity at later developmental stages nor for transplantation to recipient cattle. To overcome this shortcoming, we demonstrated in the present study a noninvasive monitoring system of firefly luciferase gene expression in live bovine embryos.

To the substrate solution for photoncounting, ATP, which is essential for the bioluminescence reaction of the firefly luciferase, was not added. If the embryo is alive, the bioluminescence reaction would proceed by consuming endogenous ATP. On the other hand, if the embryo is dead and hence endogenous ATP is depleted, then the embryo would show no or little bioluminescence irrespective of the remaining firefly luciferase activity. Thus, with this system, only luciferase-expressing, and at the same time presumably viable embryos can be selected.

As seen in table 1, all the luciferase-expressing oocytes exhibited β-galactosidase signals, demonstrating that even a trace of luciferase activity in live oocytes could be assayed easily and rapidly by photoncounting with a luminometer. However, two out of 30 (6.7%) luciferase-negative embryos showed bacterial β-galactosidase activity. Most likely reason for this may be that these two embryos were already dead, and therefore the luciferase was not detected due to the lack of endogenous ATP or diminished luciferase activity. As the half-life of the frefly luciferase is short, only about 3 hrs (Thompson et al., 1991), the enzyme would be rapidly degraded if the synthesis is ceased. In contrast, the β-galactosidase is more stable and therefore its remaining activity could confer lacZ-positive signals even if the embryos were already dead.

In experiment 2, to examine the stability of luciferase activity, bioluminescence intensity of bovine embryos injected with the four transgenes was measured during the period from 5 to 55 min after the addition of D-luciferin. So long as the bioluminescence measurement is made within 55 min, the values for luciferase activity should be almost constant as demonstrated in figure 2. It was suggested, therefore, that the measurement of luciferase activity in live bovine embryos could be performed any time within 55 min after the addition of substrate.

In experiment 2, an attempt was also made to compare the promoter activity in the hope to construct a good reporter gene usable for the embryo selection method. The results showed no significant difference among the four different promoters used in the present study, although the embryos microinjected with the luciferase reporter genes had significantly higher values than did the non-injected control embryos ($p < 0.05$). We reported recently that the activity of Miw promoter was significantly higher than those of TK and TK6W promoters (Nakamura et al., 1997, 1998). However, the luciferase activities of pTKEluc and pTK6WEluc harboring the SV40 enhancer showed the same reporter activity as that of pMiwluc (see figure 3). Thus, the SV40 enhancer would activate the transcriptional activity of TK

and TK6W promoters in bovine embryos at four- to 16-cell stages.

ACKNOWLEDGEMENTS

This work was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (No. 0589) and in part by the Research Foundation for the Electrotechnology of Chubu (No. 06190).

REFERENCES

Harris, S., M. McClenaghan, J. P. Simons, S. Ali and A. J. Clark. 1990. Gene expression in the mammary gland. J. Reprod. Fertil., 88:707-715.

Hill, K. G., J. Curry, F. J. DeMayo, K. Jones-Diller, J. R. Slapak and K. R. Bondioli. 1992. Production of transgenic cattle by pronuclear injection. Theriogenology, 37:222 (Abstr.).

Krimpenfort, P., A. Rademakers, W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper, R. Strijker and H. de Boer. 1991. Generation of transgenic dairy cattle using '*in vitro*' embryo production. Bio/Technology, 9:844-847.

Majumder, S., M. Miranda and M. L. DePamphilis. 1993. Analysis of gene expression in mouse preimplantation embryos demonstrates that the primary role of enhancers is to relieve repression of promoters. EMBO J., 12:1131-1140.

Matsumoto, K., M. Anzai, N. Nakagawa, A. Takahashi, Y. Takahashi and K. Miyata. 1994. Onset of paternal gene activation in early mouse embryos fertilized with transgenic mouse sperm. Mol. Reprod. Dev. 39:141-146.

McEvoy, T. G. and J. M. Sreenan. 1990. The efficiency of production, centrifugation, microinjection and transfer of one- and two-cell bovine ova in a gene transfer program. Theriogenology, 33:819-828.

Mélin, F., M. Miranda, N. Montreau, M. L. DePamphilis and D. Blangy. 1993. Transcription enhancer factor-1 (TEF-1) DNA binding sites can specifically enhance gene expression at the beginning of mouse development. EMBO J., 12:4657-4666.

Muramatsu, T. and A. Nakamura. 1997. Production of transgenic cattle as an animal bioreactor: Current status and perspective. Trends Comp. Biochem. Physiol., 3:9-21.

Nakamura, A., J. Okumura and T. Muramatsu. 1995. Early screening of gene expression of SV40 driven lacZ introduced into bovine embryos. Asian-Australasian J. Anim. Sci., 8:449-454.

Nakamura, A., J. Okumura and T. Muramatsu. 1997. LacZ gene expression driven by four promoters in bovine preimplantation embryos. Anim. Sci. Technol. (Jpn.), 68:1023-1031.

Nakamura, A., J. Okumura and T. Muramatsu. 1998. Quantitative analysis of luciferase activity of viral and hybrid promoters in bovine preimplantation embryos. Mol. Reprod. Dev.

Roschlau, K., P. Rommel, L. Andreewa, M. Zackel, D. Roschlau, B. Zackel, M. Schwerin, R. Hühn and K. G. Gazarjan. 1989. Gene transfer experiments in cattle. J. Reprod. Fertil. (Suppl.), 38:153-160.

SAS Institute. 1985. SAS User's Guide: Statistics. Version 5 edition, SAS Institute, Cary, NC.

Schöler, H. R., R. Balling, A. K. Hatzopoulos, N. Suzuki and P. Gruss. 1989. Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. EMBO J., 8:2551-2557.

Suemori, H., Y. Kadodawa, K. Goto, I. Araki, H. Kondoh and N. Nakatsuji. 1990. A mouse embryonic stem cell line showing pluripotency of differentation in early embryos and ubiquitous β-galactosidase expression. Cell Differ. Dev., 29:181-186.

Thompson, E. M., P. Adenot, F. *I. Tsuji and J. P. Renard. 1995a.* Real time imaging of transcriptional activity in live mouse preimplantation embryos using a secreted luciferase. Proc. Natl. Acad. Sci. USA, 92:1317-1321.

Thompson, E. M., E. Christians, M. G. *Stinnakre and J. P.* Renard. 1994. Scaffold attachment regions stimulate HSP 70.1 expression in mouse preimplantation embryos but not in differentiated tissue. Mol. Cell. Biol., 14:4694-4703.

Thompson, E. M., *E. Legouy, E. Christians and J. P. Renard.* 1995b. Progressive maturation of chromatin structure regulates HSP70.1 gene expression in the preimplantation mouse embryo. Development, 121:3425-3437.

Thompson, J. F., L. S. Hayes and D. B. Lloyd. 1991. Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene, 103:171-177.

Ueno, K., Y. Hiramoto, S. Hayashi and H. Kondoh. 1987. Introduction and expression of recombinant β-galactosidase genes in cleavage stage mouse embryos. Develop. Growth & Differ., 30:61-73.

Whittingham, D. G. 1971. Survival of mouse embryos after freezing and thawing. Nature, 233:125-126.