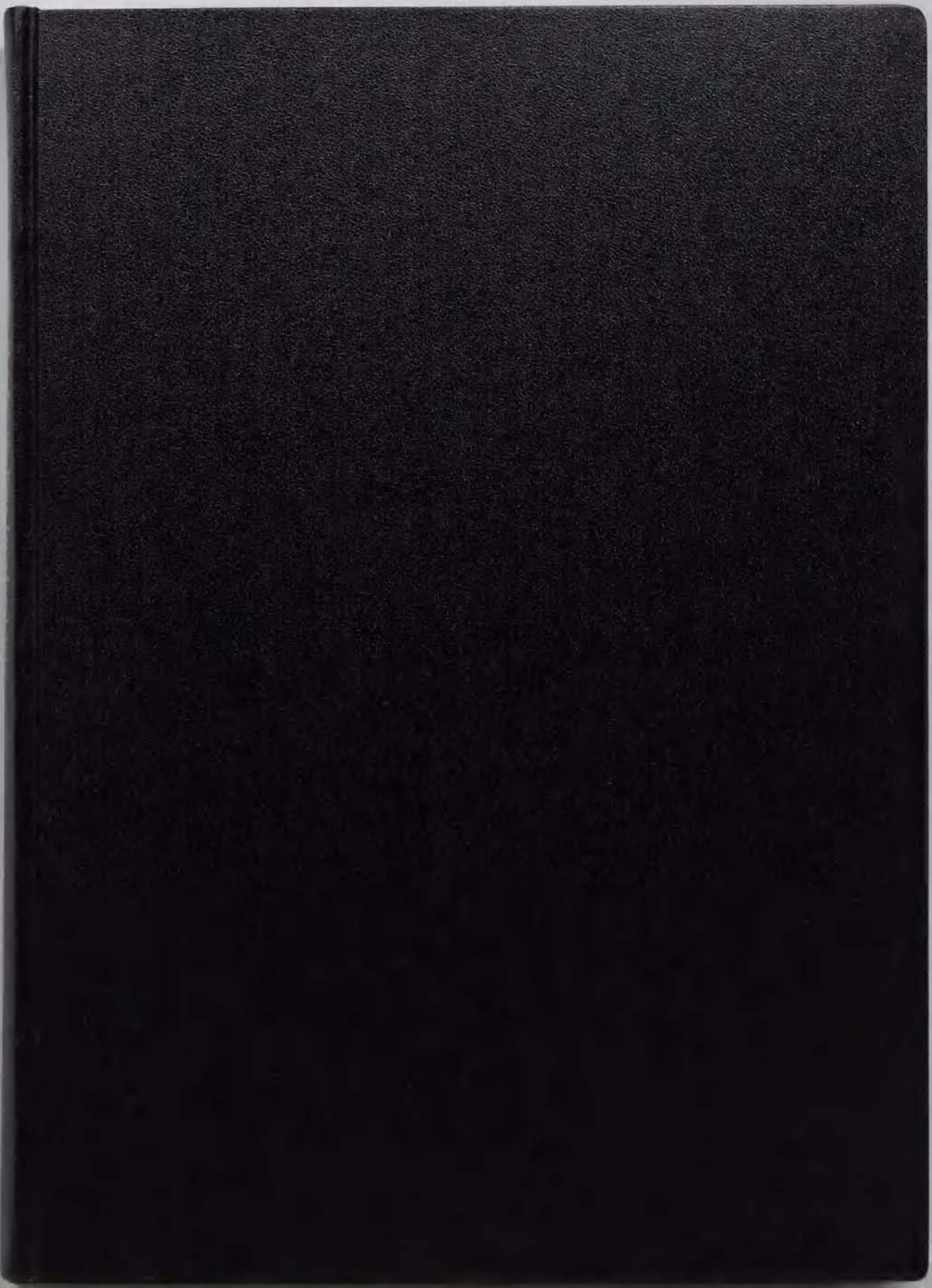

Studies on the Expression of a Foreign Gene Introduced into Bovine Embryos at Early Stages of Development

（ウシ胚発生初期における外来遺伝子の発現に関する研究）

Thesis submitted in accordance with the requirements of the
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by

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Abbreviations and Symbols Used

CFTR; cystic fibrosis transmembrene conductance regulator

EDTA; ethylene diamine tetraacetic acid

ES cells; embryonic stem cells

exoIII; exonuclease III

FDG; fluorescein-di-β-D-galactopyranoside

GFP; green fluorescent protein

Hepes; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

IVF; *in vitro* fertilization

LCR; locus control region

LTR; long terminal repeat

Luc; firefly luciferase

MAR; matrix attachment region

MI; microinjection

PBS; phosphate-buffered saline

PCR; polymerase chain reaction

PG cells; primordial germ cells

RSV; Rous sarcoma virus

SEM; standard error of mean

SV40; simian virus 40

TK; thymidine kinase

X-gal; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

YAC; yeast artificial chromosome

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Chapter 1

GENERAL INTRODUCTION

1.1. Transgenic Cattle

1.1.1. Utilization of Transgenic Cattle as an Animal Bioreactor

One of the promising fields to utilize transgenic livestock is its application to an animal bioreactor for producing pharmaceutical proteins as shown in Table 1. This would be of particular value because some biomedical proteins can be synthesized in their active forms not by microorganisms but only by animal cells and tissues due to the need for post-translational modifications. From a view point of large scale production and easy harvest, advantage may lie in the secretion of these pharmaceutical proteins into milk under the control of regulatory elements from a gene that is expressed in the mammary gland.

To date, several groups of scientists have successfully demonstrated the feasibility of targeting transgene expression to the mammary gland of sheep, goats and pigs. In transgenic sheep, for example, human anti-hemophilic factor IX and human α1-antitrypsin have been produced and secreted into the milk (Clark *et al.*, 1989; Wright *et al.*, 1991). In transgenic goats, human tissue-type plasminogen activator has been expressed in their mammary gland (Ebert *et al.*, 1991; Denman *et al.*, 1991). Similarly, a successful generation of transgenic swine with high level expression of human protein C in their milk has been reported (Velander *et al.*, 1992). Furthermore, in the milk of transgenic mice, production of cystic fibrosis transmembrene conductance regulator (CFTR) has been reported (DiTullio *et al.*, 1992). The finding that CFTR can be sequestered by apocrine secretion into the milk fat globules suggests that other human membrane-associated proteins such as receptors, transporters, and channels that are confined at least in part to the apical surface may be produced by this means.

Scaling up the production of these biomedical proteins through larger transgenic animals is presently being explored. In this sense, dairy cows would seem to be an optimal animal species to be chosen, since a cow can produce over 10,000 liter of milk, hence 350 kg of milk protein per year. So far, however, expression of foreign genes in the mammary gland of cows has not been reported yet. Krimpenfort *et al.* (1991) have demonstrated the generation of two transgenic calves harboring the human lactoferrin gene governed by

bovine αS1-casein regulatory sequences. Unfortunately, the transgene was detected only in the placental tissue of the heifer calf, and therefore it is unlikely that she will transmit the gene to progeny or produce human lactoferrin when she lactates. It will take several years until the progeny of the transgenic bull calf can be tested for the presence of human lactoferrin in milk. If transgenic cattle can be generated more efficiently, this could provide an inexpensive means of producing medically important pharmaceutical proteins.

1.1.2. Problems of Generating Transgenic Cattle

At present, most promising application of transgenic cattle may be to produce pharmaceutical proteins that are relatively low value, but required in large quantity such as human lactoferrin (Strijker *et al.*, 1992) and blood proteins. As summarized by Eystone (1994), the production of transgenic cattle presents a unique set of challenges relative to other species. Those problems are : low frequency of generating transgenic offspring from microinjected embryos, poor embryo and fetal survival; low transgene integration frequency, and; many recipient herds needed. To overcome these obstacles, a variety of supporting technologies summarized in Figure 1 are to be applied. Although so far, the logical and financial burden associated has discouraged the establishment of sustained, large-scale transgenic cattle programmes, resolution of the major problems at each step should allow easy and efficient production. In the following sections, a couple of important steps of transgenic cattle production are briefly discussed.

1.1.3. Gene Transfer

Microinjection

Currently applicable methods for transferring genes to bovine germ cells include microinjection (into pronuclei or cytoplasm), microparticle bombardment, retrovirus vectors, sperm-mediation, and nuclear transfer. Of these, the most widely employed method in cattle is, by far, microinjection of foreign DNA into the pronucleus of fertilized oocytes, mainly because the method has been successfully and routinely used in other animal species. However, in cattle, integration of microinjected transgenes occurs at particularly rare

frequency compared with that in other animal species. According to Eyestone (1994), proportion of transgenic cattle to the total zygotes injected is only at 0.09% compared with 0.8 to 1.2% for sheep, goats and pigs. Such poor integration frequency could possibly be mitigated by co-injecting carrier genomic DNA of bovine origin since Akasaka *et al.* (1995) reported that the co-introduction of a transgene with carrier DNA that was genomic origin and fragmented by restriction enzyme digestion resulted in accelerated integration of a reporter transgene into the chromosome.

In addition to the poor integration frequency, a reduced developmental potential of embryos after microinjection of transgenes has been recorded in cattle (Hyttinen *et al.*, 1992) as well as goats (Selgrath *et al.*, 1990) and sheep (Rexroad *et al.*, 1990). Handling of embryos mimicking gene transfer manipulation conditions as in mice was not in itself responsible for such decreased developmental potential. DNA microinjection conducted at the mid to late pronuclear stages did not decrease DNA detection frequency, but embryo development (Peura *et al.*, 1993a, 1993b; Krisher *et al.*, 1994a, b).

Because of the lipid content and opacity of the bovine embryo, DNA microinjection into pronuclei in cattle is more difficult than in mice. To ease the difficulties, cytoplasmic injection of DNA has been tested. Powell *et al.* (1992) found, for example, in cattle, sheep and pigs that the frequency of integrating DNA into the genome by cytoplasmic injection was lower than that by pronuclear injection. However, treating DNA with polylysine prior to the cytoplasmic injection restored the poor integration frequency to the level as high as that attained in the pronuclear DNA injection (Page *et al.*, 1995a). The presence of polylysine with construct DNA appeared to alter the *in vitro* activities of restriction endonuclease and DNA ligase on the construct DNA, and to protect injected DNA into cytoplasm by forming a complex of DNA/polylysine which, in turn, may act as a better substrate for transgenesis. If this were also applicable to farm livestock including cattle, the cytoplasmic injection would considerably alleviate the difficulties of pronuclear injection of DNA in cattle.

Viral-mediated gene transfer

Among the DNA transfer techniques not relying on direct injection with a fine-drawn

pipette, the most successful approach has been the use of viral vectors. By way of retroviral vectors, genes have been effectively transferred into the embryos of mice (Huszar *et al.*, 1985; van der Putten *et al.*, 1985; Yee *et al.*, 1994; Federspiel *et al.*, 1996), pigs (Jin *et al.*, 1991), chickens (Salter *et al.*, 1987; Bosselman *et al.*, 1989), and zebrafish (Lin *et al.*, 1994a). The first successful use of retroviral vectors in cattle was reported by Kim *et al.* (1993), who showed that not only trophectoderm but also inner cell mass retained the integrated transgene. As a variant of the retroviral vector technique, Haskell and Bowen (1995) microinjected retrovirus producer cells in the perivitelline space of one- to four-cell bovine embryos, resulting in transgenic fetuses that had identical patterns of integration in several tissues within each fetus.

Adenoviral vectors represent an alternative choice for virus-mediated transgenesis. It is generally believed that adenoviral vectors can deliver transgenes for non-dividing differentiated cells without integrating transgenes. Thus, the transgene delivered to the cell by this vector exists in the nucleus as an episomal form. However, a recent work by Tsukui *et al.* (1996) demonstrated that a replication-defective adenoviral vector, in fact, supported transgenesis in mice, resulting in about 10% transgenic pups when infected to zona-free embryos. It appears that the integration frequency by this method is higher than would be expected in cultured cells. Although the exact mechanism of this high integration frequency is unclarified, the adenoviral vector method may also provide a useful means of viral vectors for transgenesis.

Gene transfer using embryonic stem cells

Embryonic stem (ES) cells are now routinely used in gene transfer and targeting work in mice. It is now possible to make almost any desired change in the genome of the mouse (Robertson, 1991). ES cells are initially isolated from the inner cell mass of the blastocyst and grown in culture. In attempting to derive ES cells from cattle, problems arise because exactly analogous stages do not exist in embryos of mice and ungulates owing to differences in their embryonic development. The inner cell mass of the bovine embryo undergoes little development relative to the trophectoderm for several days in the elongating blastocyst,

which differs markedly from the way development of the inner cell mass occurs in the mouse. However, considerable progress has been made in recent years in establishing apparently stable ES-like stem cell lines from cattle embryos (Sims and First, 1993a). Perhaps, the most serious disadvantage of using ES cells for the transfer of foreign DNA in cattle is the fact that the calves born after the injection of ES cells will be chimeras. It takes 6 years before homozygote calves are born with transgenes in cattle (Wilmut *et al.*, 1992).

Gene transfer using primordial germ cells

Pluripotent primordial germ (PG) cells offers better opportunities than ES cells for making transgenic animals if they can be isolated and maintained in culture. The advantage of using such cells in place of ES cells is obvious; only a portion of ES cells could be differentiated to germ cells whereas in principle all PG cells will form gonads, leading to the generation of spermatogenic cells or oocytes. The first report on PG cells came from a mouse study by Resnick *et al.* (1992), opening up a new route for transgenic technology in cattle as well as in other livestock. Cherny *et al.* (1994) reported the establishment of pluripotent bovine PG cell-derived cell lines maintained in long-term culture. Lavoir *et al.* (1993, 1994) also reported the isolation and identification of female germ cells from bovine gonadal cell suspensions between days 35 and 130 of gestation. Whether these cells are truly able to differentiate to germ cells with integrated transgenes remains to be examined.

Gene transfer using nuclear transfer

Nuclear transfer technique could provide a powerful tool for producing transgenic animals in important farm livestock. Such possibility has now greatly expanded after the report of cloning lambs (Campbell *et al.*, 1996; Wilmut *et al.*, 1997) and calves (Sims and First, 1993b) by the nuclear transfer technique. Provided that the nuclear transfer allows high yield of cloned calves, the only remaining obstacle is how to select the nuclear-donor cells that carry transgenes of interest. Indeed, Krisher *et al.* (1995) claimed that bovine embryos that had been microinjected with DNA could be efficiently utilized as donor embryos in nuclear transfer. Germ cell nuclei could also be used as a nucleus donor for enucleated

oocytes since this manipulation was found to be possible in mice (Kato and Tsunoda, 1995). Moreover, Wilmut *et al.* (1997) reported the birth of live lambs from three cell populations established from the adult mammary gland, fetuses and embryos. These findings suggest that it is possible to obtain normal embryonic and fetal development from a wide variety of differentiated cells, and that the nuclear transfer technique allows to obtain genuine transgenic animals but not mosaic.

Sperm cell-mediated gene transfer

As a very simple and convenient method, and therefore of great interest, came from the technology of sperm-mediated transgenesis. The first indication goes back more than 20 years ago (Brackett *et al.*, 1971). Since then several reports implicate that this might be a possible way of introducing foreign genes into germ cells of animals (Gandolfi *et al.*, 1989; Lavitrano *et al.*, 1989; Castro *et al.*, 1990; Horan *et al.*, 1991). However, Brinster *et al.* (1989) have cast doubt about this because studies on sperm-mediated gene transfer in their own and several other laboratories failed to produce even a single transgenic mouse in more than 1300 births. So far, it has become gradually evident that the sperm of a variety of animal species could indeed bind foreign DNA (Lavitrano *et al.*, 1989; Peterson *et al.*, 1990; Gangé *et al.*, 1991; Horan *et al.*, 1991; Imai *et al.*, 1992). The question is whether or not the adsorbed DNA onto the surface of sperms can be efficiently delivered to pronucleus where the foreign DNA is integrated even though at a rare frequency. Thus, the most likely causes of discrepancy found in the previous reports may be the extremely low frequency at which sperm binds the DNA and helps integration processes. Unless innovative means is made to improve such low frequency of DNA binding and integration, the future of the sperm-mediated transgenesis technique remains elusive.

1.1.4. Screening Methods for Transgenesis at Pretransplantation Stages

One of the prohibitive costs in making transgenic cattle stems from the expense of maintaining recipient cattle bearing non-transgenic pregnancies. To avoid this, reliable and convenient embryo selection strategy should be devised. In principle, either DNA or protein

analysis could be utilized. So far, however, the DNA-based analysis has exclusively been studied. Although some modifications were made not to pick up false positive signals, DNA-based PCR analyses resulted to diagnose a dominant portion of non-transgenic embryos as being transgenic (Eyestone, 1994). The source of DNA causing these false positive signals is unknown. Some portion of this DNA may exist on the blastomere cell membranes or on the *zona pellucida*. The reported proportions of transgene positive bovine morulae or blastocysts by PCR analysis ranged from 21 to 85% (see Table 2), of which less than 10% would be truly transgenic. In contrast to these substantially high rates of detecting false positives, Seo *et al.* (1997) reported that the non-integrated injected DNA was almost completely eliminated by their modified PCR procedure. However, as the procedure is time-consuming and complicated, its use might be impractical.

With respect to the selection efficiency, simplicity and short time period required, protein-based analysis might be a more attractive alternative, although few attempts have been made hitherto. Takeda and Toyoda (1991) showed that the lacZ reporter gene expression could be detectable by X-gal staining in developing mouse embryos. Likewise in bovine embryos, Nakamura *et al.* (1995) demonstrated that the detection of the bacterial lacZ gene expression was possible after microinjecting the reporter gene driven by the SV40 promoter. However, the crucial disadvantage of the X-gal staining method was that developing embryos have to be fixed prior to histochemical staining for the detection of lacZ expression, and therefore, are no longer transplantable after the detection of the lacZ gene expression.

Thompson *et al.* (1995a) has solved the above shortcoming by using the live detection and screening method based on the bioluminescence generated from the firefly luciferase gene expression in mouse embryo. By using the same bioluminescence screening system, the firefly luciferase gene expression was found to be detected rapidly and easily in bovine preimplantation embryo (Muramatsu *et al.*, unpublished). Therefore, the use of firefly luciferase should permit live detection of a transgene before embryo transfer. The possible toxicity of luciferin, substrate for the bioluminescence reaction, to the embryonic development would be negligible since the detection can be completed within 10 min. Such

selection strategy may be equivalent to or more efficient than those reported by PCR-based selection method (Muramatsu *et al.*, unpublished, see Table 2). As might be suspected, however, this bioluminescence selection strategy may pick up many false positives due to the presence of the unintegrated, episomal reporter gene. The proportion of "germinal transgenic", i.e. truly transgenic embryos to "transfectgenic", i.e. DNA transferred embryos by the bioluminescence selection method remains unknown, but the results by PCR-based selection (Bowen *et al.*, 1994) suggest that bioluminescent embryos may still contain a dominant proportion of embryos with unintegrated luciferase gene. Nevertheless, this approach contributes to a substantial reduction in the number of recipient cattle, to approximately one-fifths, and hence is worth attempting.

Protein-based screening methods other than bioluminescence detection include the fluorescence analysis of bacterial β-galactosidase (Lin *et al.*, 1994b), and green fluorescent protein (Ikawa *et al.*, 1995; Peters *et al.*, 1995). However, in the fluorescence analyses by β-galactosidase and green fluorescent protein, irradiation at about 480 and 360 nm excitation wavelength respectively, might be deleterious to embryo development.

Admittedly, currently available DNA and protein-based selection methods do not allow to identify the integration status of transgenes in transplantable bovine embryos; in principle, they merely suggest the presence of transgenes. Although PCR analysis indicated 21% of the blastocysts carrying a transgene, only 7% of those PCR positive embryos were confirmed as truly transgenic (Bowen *et al.*, 1994). Thus, a further refinement in the currently available methods should be done or a totally different approach, not relying on PCR nor on protein analyses, should be developed to increase the accuracy of transgene screening in the live bovine embryos.

1.2. The Objectives of This Study

In order to establish the indirect screening method in bovine preimplantation embryos, the present study was conducted:

(1) To examine whether or not the product of transgene expression i.e. β-galactosidase, could be detected at early developmental stages of bovine embryos.

(2) To compare expression efficiency of four promoters in preimplantation bovine embryos by using lacZ reporter genes.

(3) To establish a new methodology for measuring bioluminescence activity easily in live developing bovine embryos by using firefly luciferase reporter genes.

(4) To investigate quantitatively the luciferase activity of viral and hybrid promoters in preimplantation bovine embryos by using firefly luciferase reporter genes.

Table 1. Expression of human proteins in milk of transgenic animal

Protein, species, and DNA[1]	Modification	Expression	
		Milk	Cell culutre[2]
		g/L	
α1-Antitrypsin, sheep:			
cDNA	N-Glycosylation	0.005 (Carver *et al.*, 1993)	0.044 (Paterson *et al.*, 1994)
gDNA	N-Glycosylation	>30 (Wright *et al.*, 1993)	0.044 (Paterson *et al.*, 1994)
Factor IX, sheep: cDNA	N-Glycosylation, γ-carboxylation, proteolytic cleavage	0.00002[3] (Clark *et al.*, 1989)	<0.005 (Anson *et al.*, 1985)
Protein C, pig: cDNA	N-Glycosylation, γ-carboxylation, proteolytic cleavage	≥1 (Velander *et al.*, 1992)	<0.01 (Foster *et al.*, 1990)
Tissue plasminogen activator, goat: cDNA	N-Glycosylation	3 (Ebert *et al.*, 1991)	0.0335 (Datar *et al.*, 1993)

[1]cDNA, complementary DNA; gDNA, genomic DNA.

[2]cDNA used exclusively; use of genomic sequences always gives lower yields in cell culture.

[3]Recent data (Yull *et al.*, 1995) indicate that a splicing abnormality leads to production of predominantly aberrant (nonsecretory?) protein. The errant gene has now been repaired.

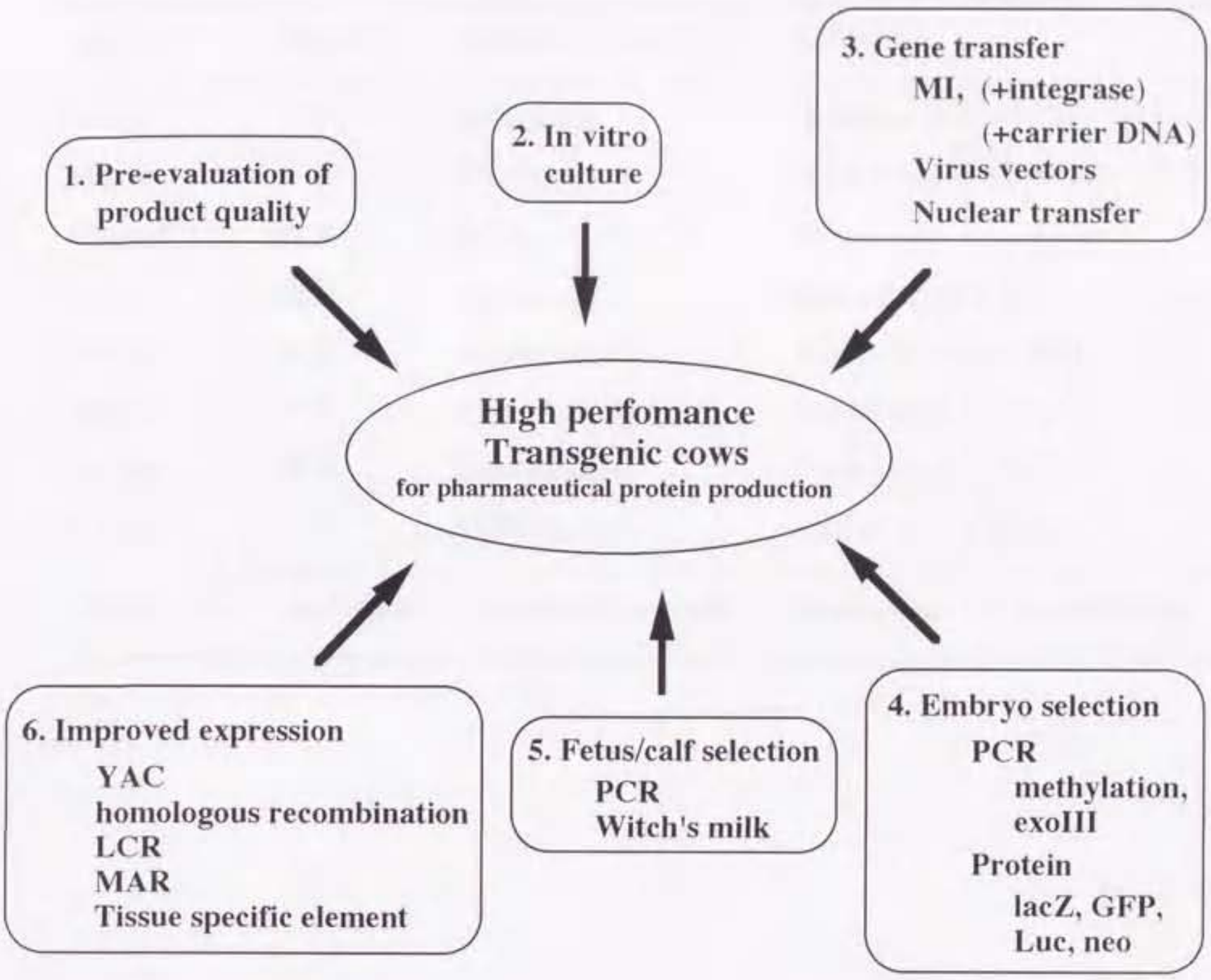


Figure 1. Various supporting technology for producing high performance transgenic cows that synthesize and secrete pharmaceutical proteins into the milk. Abbreviations used: MI, microinjection; PCR, polymerase chain reaction; exoIII, exonuclease III; GFP, green fluorescent protein; Luc, firefly luciferase; YAC, yeast artificial chromosome; LCR, locus control region; MAR, matrix attachment region.

Table 2. Proportions of transgenes detected by either PCR analysis or luciferase bioluminescence in preimplantation embryos

Species	Method	Transgene positive (%)	Reference
Mouse	PCR	36(morula)	Ninomiya *et al.* (1990)
Mouse	PCR	44(morula)	Burdon and Wall (1992)
Mouse	PCR	26(blastocyst)	〃
Mouse	PCR	40(morula)	Page *et al.* (1995b)
Bovine	PCR	54(blastocyst)	Behbbodi *et al.* (1993)
Bovine	PCR	60(blastocyst)	Horvat *et al.* (1993)
Bovine	PCR	21(blastocyst)	Bowen *et al.* (1994)
Bovine	PCR	85(blastocyst)	Krisher *et al.* (1994a)
Bovine	Luciferase	21(morula/blastocyst)	Muramatsu *et al.* (unpublished)

Chapter 2

EXPRESSION OF SV40-LACZ FUSION GENE INTRODUCED INTO BOVINE EMBRYOS AT EARLY STAGES OF DEVELOPMENT

2.1. Abstract

The experiment in this Chapter was conducted to assess gene expression of bacterial lacZ driven by the SV40 promoter at early developmental stages of bovine embryos. The lacZ gene was linearized with BamHI digestion, and introduced into the pronucleus by microinjection at 16-20 hrs after the commencement of *in vitro* fertilization. Intact bovine blastocysts were not stained with X-gal, suggesting that there is no endogenous β-galactosidase activity in these blastocysts. In contrast, the bovine blastocyst cells microinjected with the lacZ gene exerted a characteristic greenish-blue color originating from the bacterial β-galactosidase activity, albeit at a low rate, i.e. 2.1% of the total fertilized oocytes injected. It was concluded, therefore, that the lacZ gene driven by the SV40 promoter could be used for an indirect screening method in which the presence of transgene is evaluated from the product of transgene expression.

2.2. Introduction

Recent advancement of the nonsurgical techniques associated with *in vitro* fertilization (IVF) and culture of bovine embryos allows the supply of practically unlimited number of embryos available for embryo transfer (Goto *et al.*, 1988; Eyestone *et al.*, 1991). Based on this technology, transgenic cattle have been produced by microinjecting a foreign gene into the pronucleus of fertilized oocytes, followed by transferring the injected embryos to recipient cattle (Roschlau *et al.*, 1989; Krimpenfort *et al.*, 1991; Hyttinen *et al.*, 1994). Generally speaking, however, the production of transgenic farm animals is prohibitively expensive due to the long gestation period, small number of offspring per gestation and high maintenance costs including the need for a large number of recipient animals.

Besides these, a very low transgenesis rate is one of the major obstacles to be overcome. The reported efficiency of producing transgenic cattle was impracticably low. Krimpenfort *et al.* (1991) reported, for example, that the production rate of cattle expressing transgenes was only at 0.2% of microinjected embryos. This problem could be solved by developing a reliable screening method for identifying embryos that carry integrated transgenes prior to the embryo transfer. In this connection, several attempts have been made,

and reported in the literature.

One of the possible methods is to detect directly injected transgenes. The use of direct PCR with transgene-targeted primers for embryo selection has shown to yield a high number of false positives (Bowen *et al.*, 1993; Horvat *et al.*, 1993). A better selection method has been reported by using methylated gene constructs for microinjection with adequate restriction enzyme digestion prior to the PCR analysis (Jänne *et al.*, 1992). However, this method has been questioned by the observation that changes in DNA methylation patterns are independent of transgene integration in mouse embryos (Burdon and Wall, 1992).

Another possible approach might be the detection of marker proteins originated from a reporter gene microinjected together with transgenes of interest. This does not require a biopsy procedure which might decrease the survival rate of embryos transferable to recipient cattle. However, the indirect protein detection method may also confer false positive signals unless enough time is allowed to minimize transient gene expression originating from episomally existing microinjected genes.

In this Chapter, an attempt was made to establish basic conditions of the indirect screening method whether or not the product of transgene expression i.e. proteins, could be detected at early developmental stages of bovine embryos.

2.3. Materials and Methods

In Vitro Maturation and Fertilization of Ovarian Oocytes

In vitro maturation and IVF were done basically according to the method of Goto *et al.* (1994) with slight modifications. Cumulus-oocyte complexes were aspirated from small (1-7 mm in diameter) antral follicles in bovine ovaries obtained from a local slaughterhouse. They were washed twice with phosphate-buffered saline (PBS) supplemented with 0.3% (w/v) bovine serum albumin (Gibco, New York, USA), streptomycin (100 μg/ml) (Cosmobio, Tokyo, Japan), and penicillin (100 units/ml) (Cosmobio, Tokyo, Japan), followed by washing three times with maturation medium. The maturation medium was made up with Hepes-buffered TCM-199 (25 mM) (Gibco, New York, USA) supplemented with 10% (v/v) heat-treated fetal calf serum (Gibco, New York, USA), 10 mg/ml bovine

insulin (Wako, Osaka, Japan), streptomycin (100 μg/ml), and penicillin (100 units/ml). The oocytes with an intact, non-expanded cumulus mass and evenly granulated cytoplasm were then cultured in a humidified 5% CO_2 : 95% air atmosphere in an incubator at 39℃ for 24-25 hrs.

IVF was performed using frozen-thawed semen which had been diluted with BO medium (Brackett and Oliphant, 1975) without bovine serum albumin but with caffeine (10 mM) (Sigma, St. Louis, USA) and heparin (200 μg/ml) (Sigma, St. Louis, USA). The spermatozoa were washed with this medium, and centrifuged at 500 x g for 5 min. This washing procedure was repeated twice. The spermatozoa were then resuspended in the BO medium supplemented with 5 mM caffeine, 100 μg/ml heparin, and 10 mg/ml bovine serum albumin to the concentration of 12.5 x 10^6 cells/ml, and were preincubated for 10 min. IVF was conducted by co-incubating spermatozoa (25 x 10^6 cells/ml) with cumulus-oocyte complexes (10-15 per 50 μl drop) for 5 hrs.

In Vitro Culture of Fertilized Oocytes and Microinjection

After the 5-hr IVF, the cumulus-oocyte complexes were washed three times with the maturation medium, and cultured with the same maturation medium for development. At 12-13 hrs after the commencement of IVF, one-cell embryos were incubated in the TCM-199 medium without fetal calf serum but with 0.1% (w/v) hyaluronidase (Wako, Osaka, Japan) for 10 min, and were almost completely free from the residual cumulus cells by pipetting. The embryos were then washed three times with the maturation medium to remove the remaining hyaluronidase.

Denuded fertilized oocytes were centrifuged at 11,000 x g for 10 min to visualize pronuclei, and subjected to microinjection of a transgene as described by Hogan *et al.* (1986). The transgene used was the bacterial lacZ reporter gene fused downstream to the simian virus 40 (SV40) early promoter and enhancer (pSV-β-Galactosidase Control; pSVElacZ) (Promega, Madison, USA) (Figure 2). The gene was linearized by BamHI digestion, gel-purified with SUPREC-01 (TaKaRa, Kyoto, Japan), diluted with the TE buffer (10 mM Tris-HCl / 0.1 mM EDTA, pH 7.5) to give the final concentration of 3.6 μg/ml, and filtered

through a 0.20 μm filter. Embryos were microinjected with 1 to 3 pl of the DNA solution (500 to 1500 copies of the transgene) at approximately 16-20 hrs after the commencement of IVF.

After microinjection, the embryos were co-cultured for 7 days with the cumulus cells that had been prepared prior to *in vitro* maturation at 39℃ in a 5% CO_2 : 95% air atmosphere. The medium for embryo development was the same as that for *in vitro* maturation.

Detection of Transgene Expression

Expression of the lacZ gene was detected by histochemical X-gal staining for bacterial β-galactosidase at 7 days after microinjection. The X-gal staining was done after removing *zona pellucida* and the cumulus cells by digesting with 0.2% (w/v) pronase (Wako, Osaka, Japan) at 39℃ for 5 min. The bovine embryos were first washed twice in PBS and were fixed in 1% glutaraldehyde in PBS. After 10 min they were washed twice in PBS and stained in a solution of 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Wako, Osaka, Japan), 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS at 39℃ overnight.

Statistical Analyses

The data were treated statistically by the χ^2 test to assess the effect of centrifugation.

2.4. Results

The number of bovine embryos reaching different developmental stages (from the 2-cell to blastocyst) with or without the centrifugation treatment is given in Table 3. The results indicated that the embryos developed to blastocysts at about 10 to 11% of the total irrespective of the centrifugation treatment. The development of bovine embryos was not significantly affected by the centrifugation treatment according to the χ^2 test.

Intact bovine blastocysts were not stained with X-gal, except for a few cumulus cells (Figure 3), suggesting that these blastocysts did not have endogenous β-galactosidase

activity. The bovine oocytes expressing bacterial β-galactosidase by X-gal staining are presented in Figure 4. Out of 48 oocytes microinjected with pSVElacZ, 11 zygotes (22.9%) showed cleavage (> 2 cells) and two (4.2%) developed to the blastocyst stage. A characteristic blue color due to β-galactosidase activity was seen in some embryos microinjected. The number of embryos expressed β-galactosidase activity at each developmental stage is given in Table 4. Out of 48 fertilized oocytes, six 1-cell stage oocytes, one 2-cell stage oocyte and one blastocyst were stained blue with X-gal, showing the expressing of the transgene, pSVElacZ. The overall efficiency for obtaining the β-galactosidase-positive blastocysts was at 2.1% (1/48). In one 2-cell stage oocyte stained blue, all blastomeres showed bacterial β-galactosidase activity. A β-galactosidase-positive blastocyst showed a sign of mosaicism.

2.5. Discussion

The development of a good method for screening the presence of the transgene and its integration into the embryonic genome is of the most importance to reduce the production cost of transgenic farm animals. In the literature, there have been several reported methods mainly based on the amplification of transgenes by means of PCR with some modification. So far, however, all of these have suffered from the shortcoming that they tend to confer a large number of false positives (Burdon and Wall, 1992; Jänne *et al.*, 1992, Bowen *et al.*, 1993; Horvat *et al.*, 1993). Alternative quick and reliable methodology is definitely needed.

In this Chapter, an attempt was made to establish basic conditions of the indirect screening method whether or not the product of transgene expression, i.e. marker proteins, could be detected at early developmental stages of bovine oocytes. Bacterial β-galactosidase was chosen in the present study as a marker protein to be detected. Because no endogenous β-galactosidase activity was detected in non-injected control bovine embryos, it was considered that the lacZ gene would serve as a good reporter gene.

As was expected, some embryos exhibited the blue color characteristic to bacterial β-galactosidase by X-gal staining. The expression efficiency of bovine embryos microinjected with the transgene at 16.6% (8/48) of 1-cell to blastocyst embryos was comparable to that

reported in the literature (Kubisch *et al.*, 1994). Particularly notable was the fact that, albeit at a low gene expression rate, one blastocyst exhibited the blue color characteristic to bacterial β-galactosidase by X-gal staining. Thus, these results indicate that the lacZ gene fused to the SV40 promoter could be used for identifying the presence of the product of reporter gene expression, hence the reporter gene itself, in bovine embryos at the morula or blastocyst stage.

In most bovine embryos expressing bacterial β-galactosidase, blue staining was observed in a part of embryos. Similarly in most studies with transgenic cattle, mosaicism was demonstrated presumably due to integration of the gene at a stage later than the one-cell stage after microinjection. Strictly speaking, however, the results obtained by X-gal staining in the present study may not be comparable with the mosaicism reported by others because of the transient gene expression due to episomally existing microinjected genes. Therefore, in the indirect protein-detection method, it is of crucial importance to identify when, in the course of embryo development, transient gene expression becomes negligible.

Although microinjection of DNA into mouse egg pronuclei has become relatively common, the application of this technique to domestic animals was impeded by the opacity of their eggs. In cattle, to overcome this impediment, centrifugation of cow ova may be used, which stratifies the cytoplasm and leaves the pronuclei in a clear equatorial layer of the fertilized egg. It was found previously that the centrifugation treatment had no detectable adverse influence on survival rates of the cattle embryos (Wall and Hawk, 1988), which is in good agreement with the present result.

Bovine insulin was added to the maturation and development medium used in the present experiment to elevate the overall efficiency of bovine embryo development, because the hormone is known to stimulate the proliferation of cultured cumulus cells (Hoshi *et al.*, 1991). However, the efficiency of embryos successfully differentiated to blastocysts in this medium was not significantly elevated compared with that in the medium without bovine insulin (data not shown), and remained low compared with those reported in the literature (Eyestone *et al.*, 1991; Hamano and Kuwayama, 1992). Therefore, the method for culturing IVF embryos should be improved further. In addition to the culture conditions, the time of

microinjection may also be an important factor affecting the embryo development efficiency. Krisher *et al.* (1994a) demonstrated that microinjection at 11 hrs after the commencement of IVF resulted in higher survival rates of bovine embryos than did the microinjection at 15-19 hrs, which were close to the present condition.

The present protein detection method dose not require a biopsy procedure which might decrease the survival rate of embryos transferable to recipient cattle. Obviously, however, the indirect protein detection method may also confer false positive signals if a significant portion of the gene expression is transient, resulting from episomally existing microinjected genes. It was implied from the PCR study that cattle may not degrade DNA as readily as mice (Krisher *et al.*, 1994a). If this were true, a double check would have to be made to eliminate the false positive signals as much as possible by using the combination of direct PCR and indirect protein detection methods, for example.

Because the detection of β-galactosidase expression by X-gal staining requires fixation of cells, the microinjected bovine embryo is no longer usable for embryo transfer to recipient cattle even if the presence and expression of the reporter gene has been confirmed. Therefore, the development of a live detection method of transgenic embryos is necessary. Lin *et al.* (1994b) have demonstrated that this is possible in embryos of transgenic zebrafish expressing the bacterial lacZ gene by using chemiluminescence. However, this methodology should be improved further, because irradiation at about 480 nm excitation wavelength might be harmful to embryo viability.

2.6. Summary

1. The centrifugation treatment had no detectable adverse influence on survival rates of the bovine embryos.

2. Intact bovine blastocysts did not exhibit endogenous β-galactosidase activity.

3. Because expression of the lacZ gene was observed in microinjected bovine embryos from the 1-cell through blastocyst stage, the SV40 early promoter and enhancer were able to drive the reporter gene expression in bovine embryos at early stages of development.

4. At 7 days after DNA microinjection, the overall efficiency for obtaining the β-

galactosidase-positive blastocysts was at 2.1%.

pSVElacZ (pSV-β-Galactosidase Control)

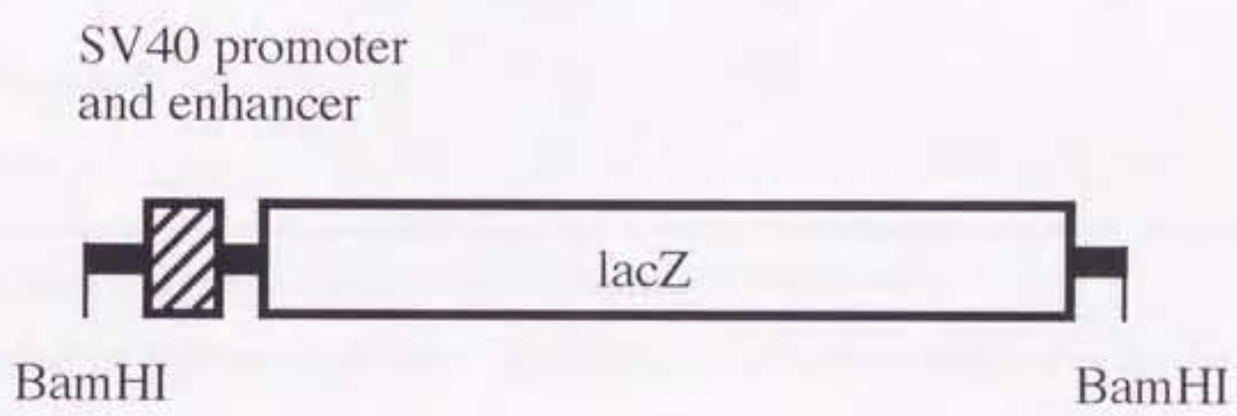


Figure 2. SV40-lacZ fusion gene (pSVElacZ). The plasmid DNA was linearized with BamHI, a restriction endonuclease, and dissolved in TE buffer (10 mM Tris-HCl / 0.1 mM EDTA, pH 7.5) to give the concentration at 3.6 μg/ml. The SV40 early promoter and enhancer drive transcription of the bacterial lacZ gene which is translated into the β-galactosidase enzyme.

Table 3. Effect of centrifugation of the development of bovine embryos

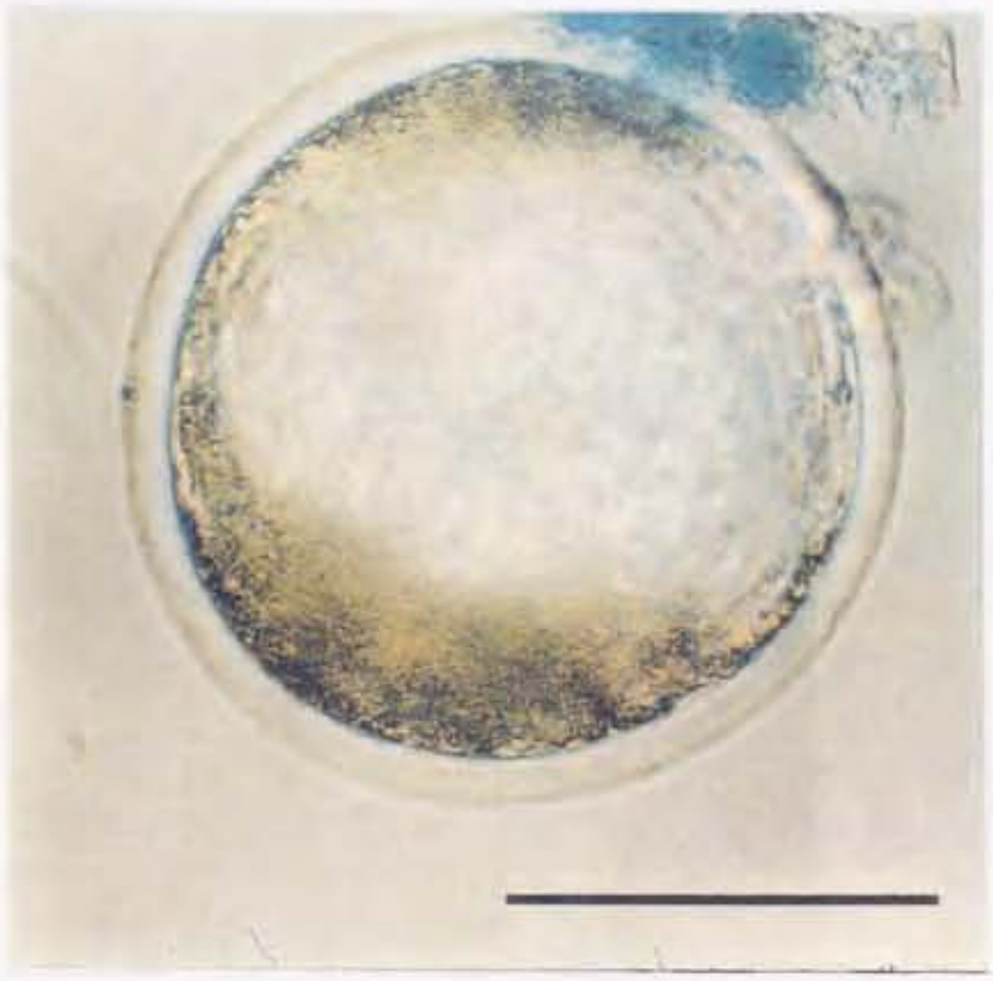
Developmental stage	Centrifugation treatment[1]			
	—		+	
	No.	%[2]	No.	%[2]
2-Cell	262	—	38	—
4-Cell	203	77.5	28	73.7
8-Cell	169	64.5	28	73.7
16-Cell	138	52.7	24	63.2
Compact morula	105	40.1	18	47.4
Blastocyst	30	11.5	4	10.5

[1]Centrifuged at 11,000 x g at room temperature for 10 min.

[2]Proportion of embryos that have successfully completed each stage to the initial number of fertilized oocytes.

There was no significant effect of the centrifugation treatment on the proportion of embryos according to the χ^2 test ($\chi^2 = 1.10$, df = 5, $P > 0.1$).

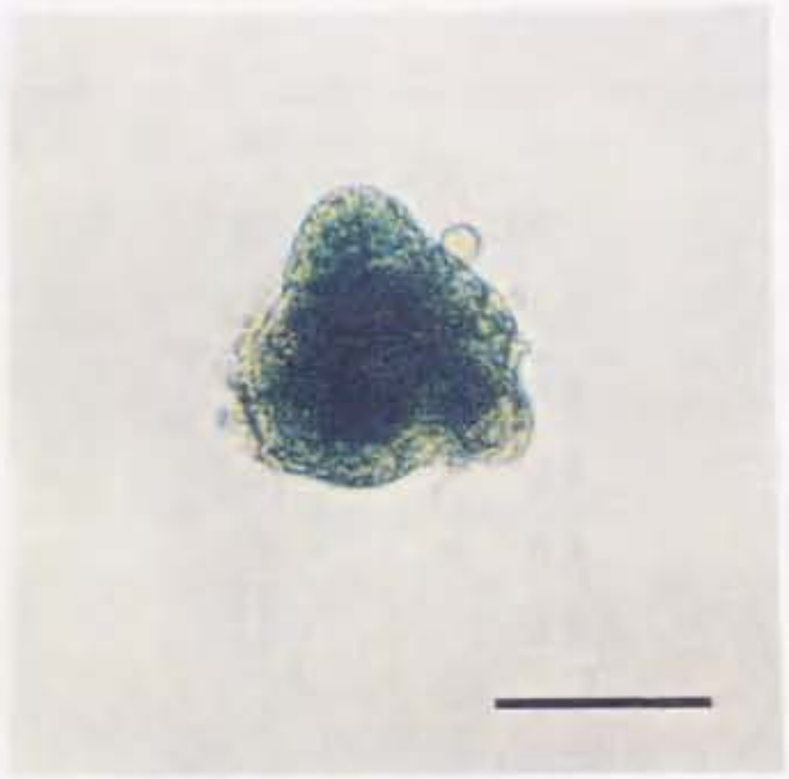
(A)



(B)

Figure 3. X-gal staining of intact bovin blastocysts with (A) or without (B) cumulus cells and *zona pellucida* which was removed by digesting with 0.2% pronase (x 400). No greenish-blue color characteristic to bacterial β-galactosidase was detected in the control blastocysts except for the cumulus cells. Scale bar represents 100 μm.

(A)



(B)

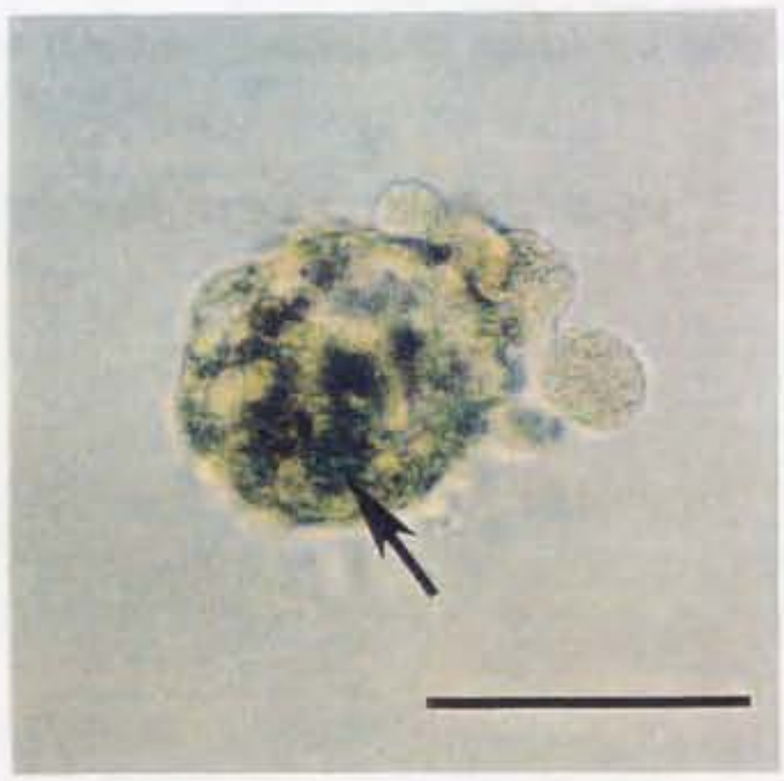


Figure 4. X-gal staining of bovine embryos at 2-cell (A, x 200) and blastocyst (B, x 300) stages after microinjecting pSVElacZ into the pronucleus of *in vitro* fertilized bovine oocytes at the 1-cell stage. The cells located in the middle of the blastocyst exhibit a blue color (indicated as an arrow) originating from bacterial β-galactosidase activity in (B). *Zona pellucida* and the cumulus cells were removed by digesting with 0.2% (w/v) pronase at 39℃ for 5 min, and thereafter the X-gal staining was conducted. Scale bars represent 100 μm.

Table 4. Expression of SV40-lacZ fusion gene during the development of bovine fertilized oocytes for up to 7 days after microinjection

Developmental stage	Total No.[1]	%[2]	No. of expression[3]
Injected embryos	48	—	—
1-Cell	48	100	6
2-Cell	11	22.9	1
4-Cell	10	20.8	0
8-Cell	5	10.4	0
16-Cell	3	6.3	0
Compact morula	2	4.2	0
Blastocyst	2	4.2	1

[1]The number of embryos that have successfully developed to each stage.

[2]The proportion of embryos that have successfully completed each stage to the initial number of fertilized oocytes.

[3]The number of bovine fertilized oocytes stained blue with X-gal.

Chapter 3

LACZ GENE EXPRESSION DRIVEN BY FOUR PROMOTERS IN BOVINE PREIMPLANTATION EMBRYOS

3.1. Abstract

The experiment in this Chapter was conducted to compare the expression efficiency of four lacZ reporter genes in preimplantation bovine embryos. Four bacterial lacZ (TKlacZ, 6WTKlacZ, SVElacZ and MiwlacZ) genes were introduced by microinjecting into the pronucleus of fertilized bovine oocytes, and the injected embryos were cultured to the morula or blastocyst stage. Detection of the transgene expression was done by X-gal staining. The development was not significantly affected by the difference in the four transgenes injected. Among the transgenes tested the MiwlacZ gene carrying the Rous sarcoma virus (RSV) long terminal repeat (LTR) and chicken β-actin promoter showed higher expression efficiency (62.5%, n=24) and stronger expression of the lacZ gene than any other transgenes at the morula and blastocyst stages. Most putative transgenic embryos injected with four transgenes showed a mosaic pattern of lacZ gene expression, but in the group injected with the MiwlacZ gene the proportion of X-gal positive blastomeres seemed to be larger than those injected with any other lacZ genes in preimplantation bovine embryos. These results suggest that a reporter gene driven by the Miw promoter (RSV LTR and chicken β-actin promoter) may be expressed efficiently and strongly in bovine preimplantation embryos.

3.2 Introduction

A quick and reliable detection system for transgenes introduced into bovine embryos may permit the reduction in the number of recipient cows needed. So far, most extensively used methodology to detect an exogenous DNA for embryo selection has been based on polymerase chain reaction (PCR) (Behboodi *et al.*, 1993; Horvat *et al.*, 1993; Bowen *et al.*, 1994; Krisher *et al.*, 1994a). However, this system requires microsurgical biopsy which may decrease the survival rate of embryos transferable to recipient cattle. The results in Chapter 2 suggested that marker proteins originated from a reporter gene microinjected together with a transgene of interest could be useful for an indirect screening method in which the presence of transgene is evaluated from the product of reporter gene expression. Furthermore, it was found that the selection strategy based on bioluminescence detection generated by firefly

luciferase gene expression may be equivalent to or more efficient than those reported by PCR-based selection methods (Muramatsu and Nakamura, 1997).

In the last few years, several studies about promoter and enhancer activities in bovine embryos have been made by using the bacterial lacZ reporter gene. The results in Chapter 2 found, for example, that the SV40 early promoter and enhancer could drive the lacZ gene expression throughout the developmental stages from the one-cell to blastocyst. Kubisch *et al.* (1995) have shown that the activity of human cytoplasmic β-actin promoter is the strongest at the morula stage. The activity of chicken cytoplasmic β-actin promoter, on the other hand, was stronger before the 16-cell stage than the morula stage (Gangé *et al.*, 1993). Lemme *et al.* (1994) have found that the 6W enhancer and thymidine kinase promoter were able to drive the lacZ gene expression from the 1-cell through 16-cell stages of bovine embryos. However, there are few reports comparing promoter activities at the preimplantation stages, i.e. morula or blastocyst, of bovine embryogenesis.

The experiment in this Chapter was conducted to compare expression efficiency of four lacZ reporter genes in preimplantation bovine embryos, in the hope to construct a good reporter gene usable for the indirect protein detection method.

3.3. Materials and Methods

Transgene Constructs for Microinjection

The structure of the transgenes introduced into fertilized bovine oocytes is shown in Figure 5. The p6WTKlacZ (a gift from Dr. P. Gruss, Max Planck Institute of Biophysical Chemistry, Germany) consisted of the lacZ gene with the 6W (a hexamer of 1W) enhancer and a minimal thymidine kinase (TK) promoter whose intrinsic octamer motif had been removed. The 1W fragment was the oligonucleotides containing the μE4 and Oct binding site (Schöler *et al.*, 1989). The plasmid pTKlacZ was created by excising a 330bp BamHI fragment of the 6W enhancer from p6WTKlacZ. The pSV-β-Galactosidase Control plasmid (pSVElacZ) was obtained commercially. The pMiwlacZ consisted of RSV LTR, chicken β-actin promoter, β-actin first exon/intron, splice acceptor sites of β-actin second exon and chicken δ-crystallin third exon, bacterial β-galactosidase coding sequences, and two polyA

addition signals originating from herpes simplex virus thymidine kinase and SV40 early genes (Suemori *et al.*, 1990). The transgenes were linearized by unique restriction endonuclease digestion, and gel-purified with SUPREC-01. The linearized gene constructs of p6WTKlacZ, pTKlacZ, pSVElacZ and pMiwlacZ were resuspended in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA to an equimolar concentration to have 500 copies of the transgene in 1 pl, i.e. 3.7 μg/ml, 3.5 μg/ml, 3.6 μg/ml and 4.7 μg/ml respectively, and filtered through a 0.20 μm filter.

Egg Manipulation, Microinjection, and Detection of Transgene Expression

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 in Chapter 2. At 6-8 days after microinjection, expression of the lacZ gene was detected by histochemical X-gal staining for bacterial β-galactosidase as described in Chapter 2.

Statistical Analyses

Data were analysed statistically by the χ^2 test.

3.4. Results

The developmental rate of bovine embryos injected with the four lacZ genes is shown in Table 5. The development of bovine oocytes was not significantly ($P>0.05$) affected by the four transgenes injected: zygotes survived after microinjection with four different lacZ genes had cleavage rates of 71.2% (94 of 132 zygotes survived), 66.1% (72 of 109), 77.5% (79 of 102) and 70.8% (75 of 106) in the TKlacZ, 6WTKlacZ, SVElacZ and MiwlacZ gene groups, respectively. The proportions of oocytes developed to the blastocyst stage to the total oocytes survived were similar among the four genes, ranging from 9.2 to 12.7%.

In a parallel study, a group of one-cell embryos were microinjected only with the same buffer without transgenes to evaluate whether DNA injection influenced the developmental

rate of the embryos to the blastocyst stage. Of the 40 embryos survived after pronuclear injection, 15.0% (6 of 40) developed to the blastocyst stage. The developmental rate of the microinjected oocytes to the blastocyst stage was not significantly ($P>0.05$) influenced regardless of the presence of DNA (data not shown).

The efficiency for obtaining the β-galactosidase-positive oocytes (one-cell to blastocyst stages) after injecting with each lacZ gene is presented in Table 6. The expression efficiencies of SVElacZ (28.4%) and MiwlacZ (36.8%) were significantly ($P<0.05$) higher than those of TKlacZ (9.8%) and 6WTKlacZ (12.8%) throughout the developmental stages. In morulae and blastocysts, the expression efficiency of the MiwlacZ (62.5%) construct was significantly ($P<0.05$) higher than those of TKlacZ (25.0%), 6WTKlacZ (21.1%) and SVElacZ (22.7%). The proportion of lacZ positive morulae and blastocysts did not seem to be increased by the presence of the 6W enhancer. Uninjected intact embryos and buffer-injected embryos showed no endogenous β-galactosidase activity throughout the preimplantation development.

The embryos injected with four different lacZ gene were stained blue with various degrees of intensity. Table 7 presents the intensity of bacterial β-galactosidase expression in the X-gal positive oocytes. Over 70% of the positive embryos expressing the MiwlacZ construct were stained dark blue. In bovine embryos injected with three other lacZ genes, β-galactosidase activity was generally low, and the degree of staining was weak; over 50% of the embryos had cytoplasmic spotty staining or light blue staining. Thus, among the transgenes tested the MiwlacZ gene driven by the RSV LTR and chicken β-actin promoter showed stronger staining of X-gal than three other transgenes over the entire developmental stages. Table 7 also represents X-gal staining intensity at morula and blastocyst stages. Approximately one half of the morulae and blastocysts injected with the MiwlacZ construct were stained very dark blue, indicating that the Miw promoter activity did not decrease as development proceeded. The number of 6WTKlacZ-positive morulae and blastocysts were too small to assess whether the 6W enhancer was active in the inner cell mass at these developmental stages.

The pattern of staining in the injected embryos is given in Table 8 according to the

area stained, i.e. strong staining in the whole embryo, and mosaic staining above 50% or less. Typical examples of these staining pattern are also shown in Figure 6. In bovine embryos injected with the TKlacZ, 6WTKlacZ and SVElacZ gene constructs, almost all stained zygotes were proved to be expression mosaics. In addition, all positive embryos beyond the four-cell stage demonstrated the mosaicism. In contrast, about 16% of X-gal positives in MiwlacZ-injected zygotes were stained strong blue in the whole embryo without the sign of mosaicism.

3.5. Discussion

In this Chapter, an attempt was made to compare the promoter activity in the hope to construct a good reporter gene usable for the indirect protein detection method. We have already found that bioluminescence imaging of the firefly gene expression might serve as a rapid and convenient screening system of foreign genes in bovine embryos (Muramatsu and Nakamura, 1997). The Miw promoter, whose activity was the strongest at the morula and blastocyst stages in the present experiment may be useful in the above diagnosis purpose.

It was reported that approximately 21% of bovine blastocysts harboured the transgenes microinjected (Bowen *et al.*, 1994). However, because of episomally existing transgenes, not all embryos expressing the reporter gene were transgenic (Muramatsu and Nakamura, 1997). If the production of transgenic cattle is performed without the embryo selection for transgenes, a vast number of recipient cows for embryo transfer would be needed. As a new and convenient protein detection system, Takada *et al.* (1997) suggested that green fluorescent protein (GFP) was a good reporter, and found that of total GFP positive murine embryos microinjected with a GFP reporter gene, more than 90% embryos possessed transgenes in their genome. Moreover, Methot *et al.* (1995) demonstrated that there was a correlation between the intensity of a marker protein and degree of expression of the co-injected transgene of interest. Therefore, selecting bovine zygotes expressing a reporter gene would increase the rate of transgenic cattle production.

The pMiwlacZ was designed to attain strong expression of the lacZ gene in mouse embryos at a variety of developmental stages (Suemori *et al.*, 1990). The chicken β-actin

promoter activity was known to be exceptionally strong and the activity was not decreased after stable chromosomal integration in transfection experiments (Fregien and Davidson, 1986). Sands *et al.* (1993) have reported that the expression rate of transgenic mouse embryos harboring the chicken cytoplasmic β-actin-lacZ fusion gene was higher than that of embryos having the chicken skeletal α-actin-lacZ fusion gene. The RSV LTR is not only strong but also confers a wide expression range after transfection into a variety of eukaryotic cells (Gorman *et al.*, 1982; Ueno *et al.*, 1987). Because in the present experiment, the majority of MiwlacZ-injected morulae or blastocysts exhibited the β-galactosidase activity in over the 50% region or the whole embryo as shown in Table 8, the Miw promoter activity might be resistant to transcriptional repression as development proceeded. It was considered, therefore, that a reporter gene driven by the Miw promoter might be expressed efficiently and strongly in bovine preimplantation embryos.

The SV40 early promoter has been widely used in transfection experiments involving eukaryotic cells and in transgenic mice experiments. Takeda and Toyoda (1991) reported that the lacZ gene driven only by the SV40 early promoter was expressed in murine morulae at the highest frequency. In bovine oocytes, however, the percentage of embryos expressing the lacZ gene governed by this promoter declined significantly from 8-cell stage to the morula and blastocyst stages (Kubisch *et al.*, 1995). Such low expression efficiency at the morula or blastocyst stages of bovine embryos was also observed in the present experiment.

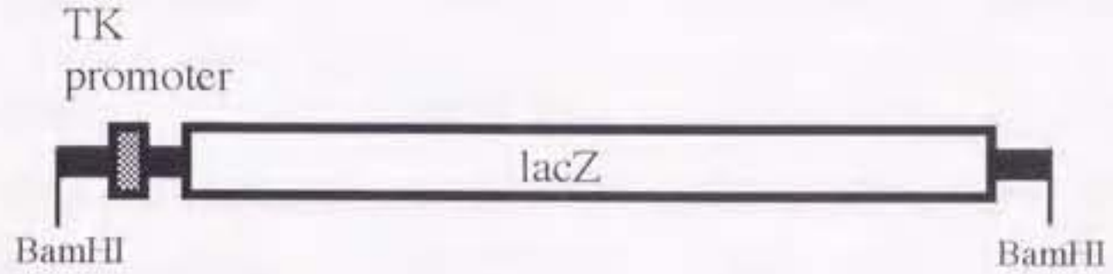
Schöler *et al.* (1989) have found that the 6W enhancer containing six octamer motifs, each of which consists of a binding site of a transcription factor, Oct3, is active in the inner cell mass but not in the trophectoderm of transgenic mouse blastocysts. It was expected, therefore, that the number of embryos expressing the p6WTKlacZ would increase specifically at the morula or blastocyst stage when the selection of the bovine embryos harboring transgenes is performed. However, the expression rate of oocytes microinjected with p6WTKlacZ at these developmental stages was not increased by the presence of the 6W enhancer in the present experiment. The numbers of positive morulae and blastocysts were probably too small to detect the activation effect of the 6W enhancer in the inner cell mass of these developmental stages.

The rate of embryos successfully differentiated to blastocysts *in vitro* was approximately 12% as described in Chapter 2. This efficiency was low compared with those reported in the literature (Eyestone *et al.*, 1991; Goto *et al.*, 1994). The true reason for this was unknown, but it might be related to the culture medium used or more likely to oocyte quality obtained from a local slaughterhouse. However, the rates of bovine blastocysts developed from the embryos survived after DNA microinjection into the pronucleus ranged from 10 to 15%, which were comparable to that reported in the literature (Behboodi *et al.*, 1993). The present results indicated that bovine embryos survived after injection into the pronucleus can develop to blastocysts at the same rate as non-injected embryos as observed by Behboodi *et al.* (1993). In mouse embryos (Brinster *et al.*, 1985) and bovine embryos (Gangé *et al.*, 1993), it has been shown that high concentration of plasmid DNA (≧10 μg/ml) interferes with the development of embryos. The concentration (about 4 μg/ml) in the present experiment did not appear to affect the developmental rate of bovine embryos up to blastocyst stage.

3.6. Summary

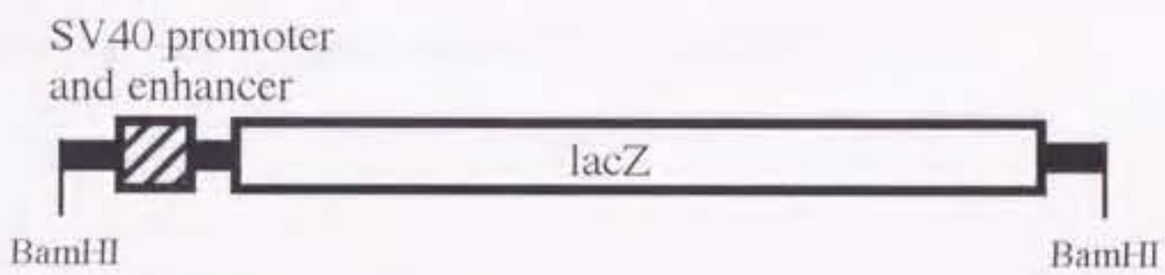
1. The development was not significantly affected by the difference in the four transgenes injected.
2. Among the transgenes tested the MiwlacZ gene carrying the RSV LTR and chicken β-actin promoter showed higher expression efficiency (62.5%, n=24) and stronger expression of the lacZ gene than any other transgenes at the morula and blastocyst stages.
3. Most putative transgenic embryos injected with four transgenes showed a mosaic pattern of lacZ gene expression, but in the group injected with the MiwlacZ gene the proportion of X-gal positive blastomeres seemed to be larger than those injected with any other lacZ genes in preimplantation bovine embryos.

(A) pTKlacZ



(B) p6WTKlacZ

(C) pSVElacZ



(D) pMiwlacZ

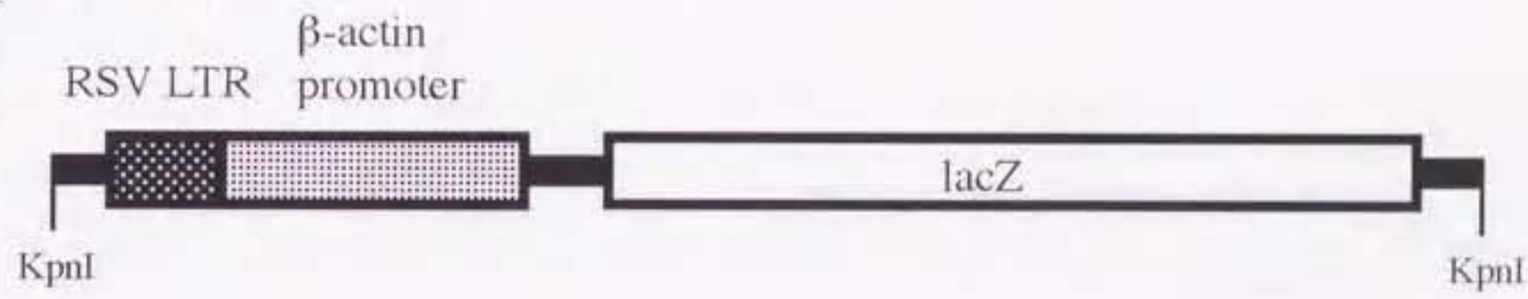


Figure 5. Structures of microinjected transgenes: (A) pTKlacZ, (B) p6WTKlacZ, (C) pSVElacZ, and (D) pMiwlacZ. These plasmid DNAs were linearized with unique restriction endonucleases and dissolved in a buffer (10 mM Tris-HCl (pH 7.5) / 0.1 mM EDTA) to give an equimolar concentration at 3.5 μg/ml (pTKlacZ), 3.7 μg/ml (p6WTKlacZ), 3.6 μg/ml (pSVElacZ) and 4.7 μg/ml (pMiwlacZ). LacZ: bacterial lacZ gene encoding β-galactosidase; 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.

Table 5. Development of bovine embryos cultured for up to 8 days after microinjection with four different lacZ genes

	Transgene			
Developmental stage	TKlacZ	6WTKlacZ	SVElacZ	MiwlacZ
1-Cell (%)[1]	38 (28.8%)	37 (33.9%)	23 (22.5%)	31 (29.2%)
2-Cell and 4-cell (%)[1]	36 (27.3%)	30 (27.5%)	33 (32.4%)	22 (20.8%)
8-Cell and 16-cell (%)[1]	30 (22.7%)	23 (21.1%)	24 (23.5%)	29 (27.4%)
Morula and blastocyst (%)[1]	28 (21.2%)	19 (17.4%)	22 (21.6%)	24 (22.6%)
Total	132	109	102	106

[1]Proportion of oocytes that have developed to each stage to the number of total oocytes survived after microinjection.

There was no significant difference between the genes microinjected in the developmental rate of bovine embryos according to the χ^2 test ($P>0.05$).

Table 6. Expression of four different lacZ genes in developing bovine embryos

Transgene	Expression efficiency (%)	
	Entire developmental stages[1] (1-cell to blastocyst)	Morula to blastocyst[2]
TKlacZ	13/132 (9.8%)[a]	7/28 (25.0%)[a]
6WTKlacZ	14/109 (12.8%)[a]	4/19 (21.1%)[a]
SVElacZ	29/102 (28.4%)[b]	5/22 (22.7%)[a]
MiwlacZ	39/106 (36.8%)[b]	15/24 (62.5%)[b]

[1]No. of embryos expressing lacZ gene / No. of embryos survived after microinjection.

[2]No. of morulae and blastocysts expressing lacZ gene / No. of morulae and blastocysts developing after microinjection.

[a, b]Values with different superscripts are significantly different as assessed by the χ^2 test ($P<0.05$).

Table 7. Comparison of X-gal staining intensity in developing bovine embryos after microinjection with four different lacZ genes

Intensity of X-gal staining[1]	Transgene			
	TKlacZ	6WTKlacZ	SVElacZ	MiwlacZ
Entire developmental stages (1-cell to blastocyst)				
+	4	2	12	6
++	6	7	5	4
+++	0	2	6	7
++++	3	3	6	22
Total	13	14	29	39
Morula / blastocyst				
+	1	1	2	3
++	5	1	1	3
+++	0	0	1	2
++++	1	2	1	7
Total	7	4	5	15

[1]The intensity of the staining in embryos expressing each transgene was classified into four grades according to the area stained under the light microscope. The intensity was classified as: +, weak (spotty staining); ++, moderate (light blue staining); +++, strong (dark blue staining); ++++, very strong (very dark blue staining).

Table 8. Distribution of X-gal staining pattern in developing bovine embryos microinjected with four different lacZ reporter genes

Pattern of X-gal staining	Transgene			
	TKlacZ	6WTKlacZ	SVElacZ	MiwlacZ
No. of positive embryos (≧2-cell stage)[1]	12	14	23	37
Whole embryo	0	1	0	6
Mosaic ≧50% area stained	3	4	9	21
<50% area stained	9	9	14	10

[1]Number of X-gal-positive embryos at 2-cell to blastocyst stage.

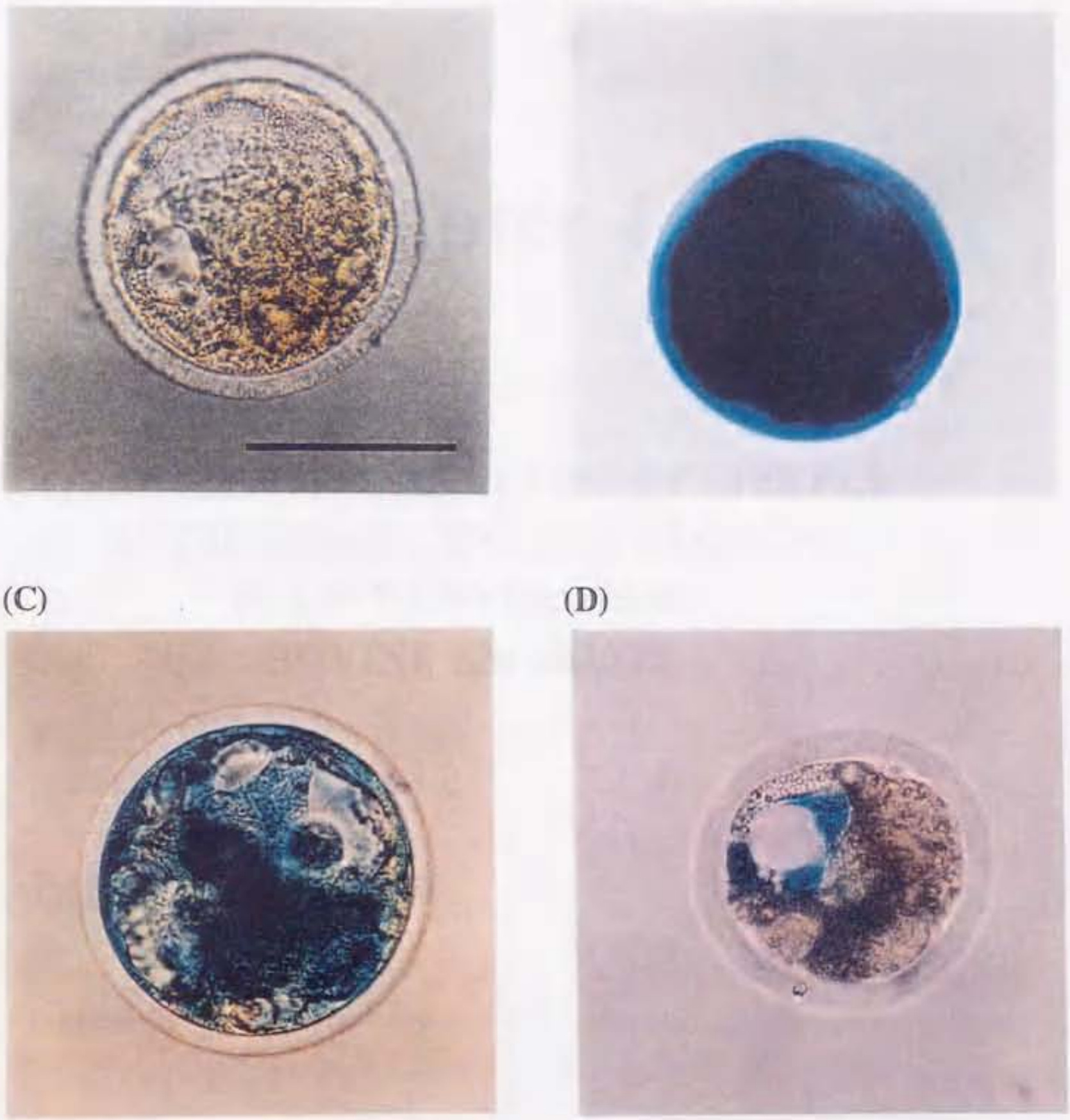


Figure 6. Bovine blastocysts stained with X-gal. (A) Non-injected blastocyst (under the light microscope, ×300). (B, C, D) Typical X-gal positive bovine blastocysts after microinjecting with lacZ reporter genes (under the light microscope, ×300): (B) MiwlacZ-injected blastocyst showing β-galactosidase activity in the whole embryo; (C) MiwlacZ-injected blastocyst showing β-galactosidase activity in more than 50% area; (D) SVElacZ-injected blastocyst showing β-galactosidase activity in less than 50% area. Scale bar represents 100 μm.

Chapter 4

QUANTITATIVE ANALYSIS OF FIREFLY LUCIFERASE GENE EXPRESSION IN LIVE DEVELOPING BOVINE EMBRYOS

4.1. Abstract

The two experiments in this Chapter were designed to develop the new methodology to measure the bioluminescence activity easily in live developing bovine embryos by photoncounting. In experiment 1, equimolar pSVElacZ 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, and lacZ gene expression in the same embryos was assayed by X-gal staining. All the luciferase-positive embryos showed some bacterial β-galactosidase activity irrespective of the intensity. In experiment 2, to examine the stability of the luciferase, bioluminescence intensity of bovine embryos injected with SVEluc was measured with a luminometer at 2 days after microinjection. The results indicated that the bioluminescence could be analysed at any time within 30 min because the luciferase activity was constant during the measurement period from 5 to 30 min. The present results demonstrated that the bioluminescence activity in live developing bovine embryos could be measured quickly by photoncounting.

4.2. Introduction

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 two experiments in this Chapter were conducted to establish a new methodology for measuring bioluminescence activity easily in live developing bovine embryos.

4.3. Materials and Methods

Transgene constructs for microinjection

The transgenes used are given in Figure 7. The pSVlacZ (pSV-β-Galactosidase Control) and pSVEluc (pGL2-Control) containing the SV40 early promoter and enhancer were obtained commercially (Promega, Madison, USA). The transgenes were linearized by unique restriction endonuclease digestion, and gel-purified with SUPREC-01. The linearized gene constructs of pSVlacZ and pSVEluc 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 in Chapter 2.

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, pSVEluc was microinjected at an concentration of 500 copies of the transgene in 1 pl, i.e. 3.2 μg/ml. The microinjected zygotes were cultured for 2 days, and assayed 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 embryo cultured for 2 days was transferred into 50 μl of the fresh culture medium held in a plastic tube, 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. In experiment 1, luciferase activity was measured at 5 min after the addition of D-luciferin and sucrose. In experiment 2, luciferase activity was measured at similarly 5, 15, 20, 25, and 30 min.

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 described in Chapter 2.

Statistical analyses

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).

4.4. Results

Gene expression of bovine oocytes injected with both pSVElacZ and pSVEluc in experiment 1 is presented in Table 9. 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.

In experiment 2, luciferase activity in 10 responders that were microinjected with pSVEluc and developed normally to the 4 to 16-cell stage was measured. Figure 8 displays the time course of luciferase activity in the responders. The luciferase activity was almost constant throughout the measurement period from 5 to 30 min, and there was no significant difference in the luciferase level detected at each time ($P>0.05$).

4.5. Discussion

In this Chapter, an attempt was made to develop a new method for measuring firefly luciferase activity in living bovine embryos by photoncounting with a luminometer. So far, it has been reported that by using a luminometer the firefly luciferase gene expression has been analysed quantitatively 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 this Chapter a noninvasive monitoring system of firefly luciferase gene expression in live bovine embryos.

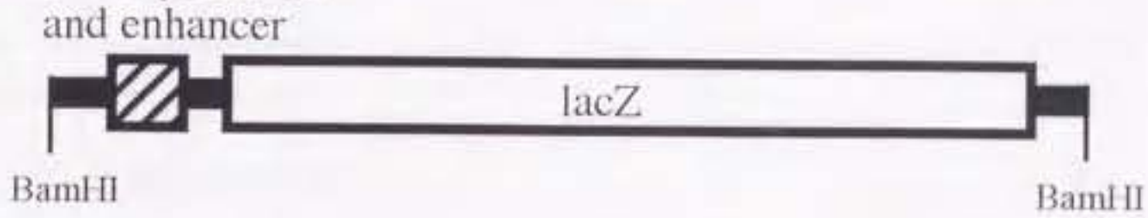
In the present experiments, ATP was not added to the luciferin solution, although ATP is essential for the bioluminescence reaction which firefly luciferase catalyzes (Alam and Cook, 1990). The reason for removing ATP was that it might help to evaluate whether or not the embryo was alive. If the embryo is alive, endogenously generated ATP could be used for the bioluminescence reaction as a sole ATP source. On the other hand, if the embryo is already dead, hence endogenous ATP cannot be produced and is depleted, then no bioluminescence would be exerted irrespective of the remaining firefly luciferase activity. Moreover, we have confirmed that the development of bovine embryos to the blastocyst stage was normal when luciferin (500 μM) was present in the medium for up to 3 hrs, whereas the 6-hr exposure to luciferin significantly reduced the percentage of embryos that successfully developed to blastocysts (Muramatsu *et al.*, unpublished results). So long as the bioluminescence measurement is done within 30 min, the value should be stable as demonstrated in Figure 7 and no detrimental effect would be detected.

As shown in Table 9, 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 firefly 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.

4.6. Summary

1. In experiment 1, all the luciferase-positive embryos showed some bacterial β-galactosidase activity irrespective of the intensity.
2. In experiment 2, the luciferase activity in luciferase expressing embryos was constant during the measurement period from 5 to 30 min.

(A) pSVElacZ



(B) pSVEluc (pGL2-Control)

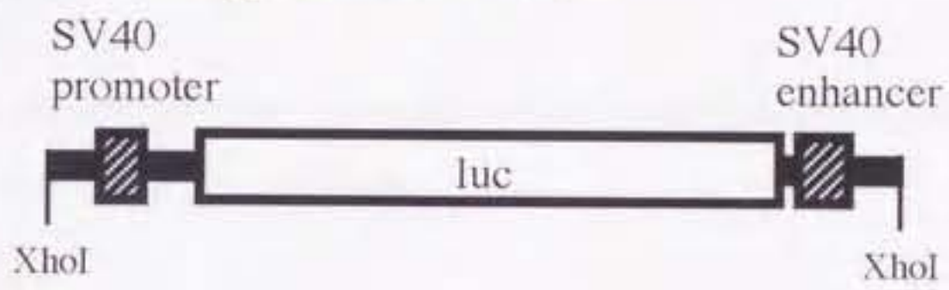


Figure 7. Structures of microinjected transgenes: (A) pSVElacZ and (B) pSVEluc (pGL2-Control). These plasmid DNAs were linearized with unique restriction endonucleases and dissolved in TE buffer (10 mM Tris-HCl (pH 7.5) / 0.1 mM EDTA) to give an equimolar concentration at 3.6 μg/ml (pSVElacZ) and 3.2 μg/ml (pSVEluc). Abbreviation: SV40, simian virus 40; lacZ, bacterial lacZ gene encoding β-galactosidase; luc, firefly luciferase cDNA.

Table 9. Gene expression at four- to 16-cell stages of bovine embryos microinjected with both the lacZ and firefly luciferase genes

	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 pSVElacZ (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.

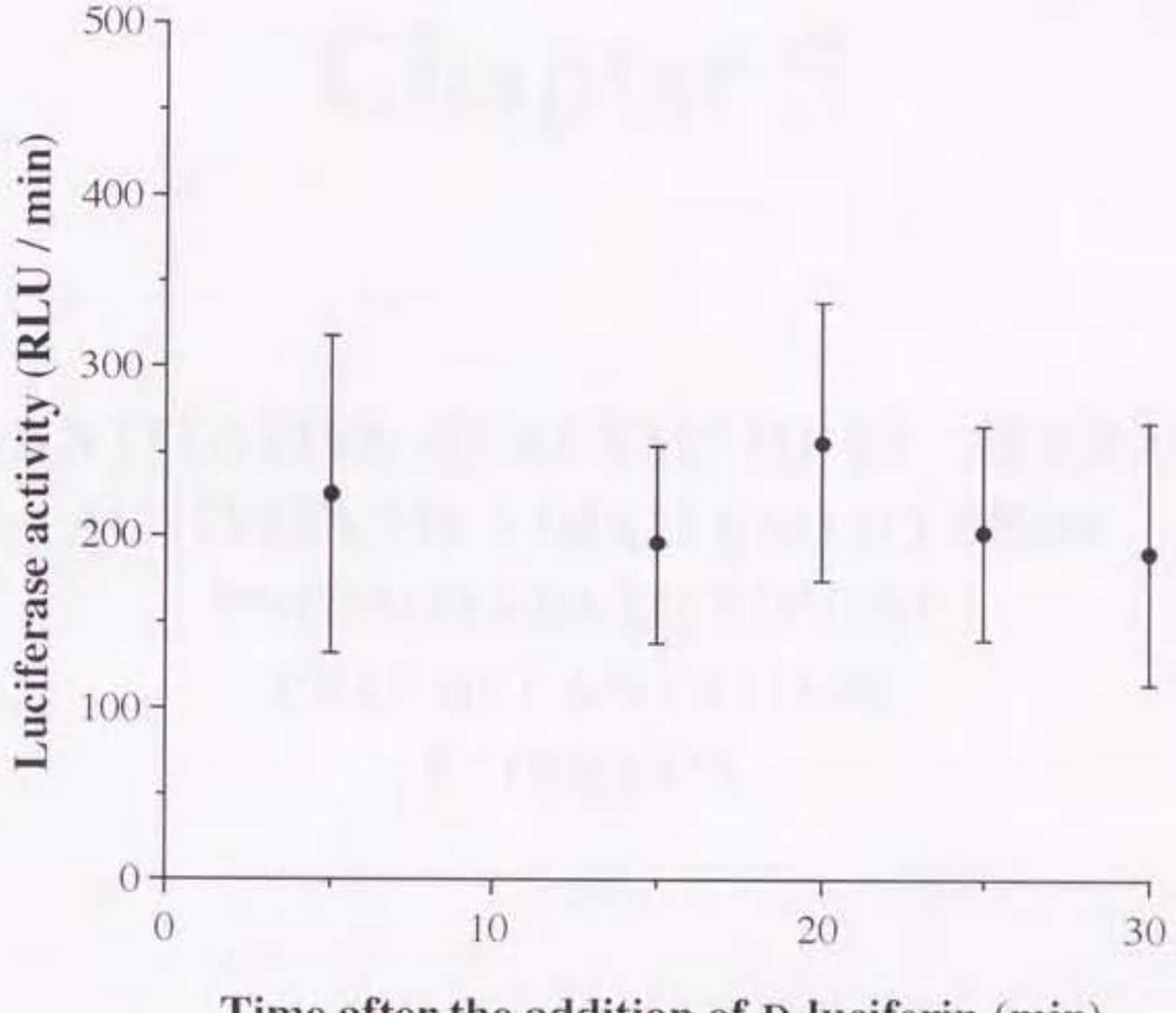


Figure 8. Time course of luciferase activity in bovine embryos microinjected with pSVEluc. The live embryos that cleaved normally and developed to 4 to 16-cell stage were analysed at 2 days after microinjection. Each data point represents means ± SEM of 9 or 10 replicates. There was no significant difference in the luciferase activity detected between measurement points ($P>0.05$).

Chapter 5

QUANTITATIVE ANALYSIS OF LUCIFERASE ACTIVITY OF VIRAL AND HYBRID PROMOTERS IN BOVINE PREIMPLANTATION EMBRYOS

5.1. Abstract

The two experiments in this Chapter were conducted to investigate quantitatively the luciferase activity of gene constructs with viral and hybrid enhancers and promoters in bovine preimplantation embryos by using firefly luciferase reporter genes. In experiment 1, the luciferase expression of fertilized oocytes injected with four gene constructs (TKEluc, TK6WEluc, SVEluc and Miwluc) was analysed by using a photon imaging system at 2 or 6 days following microinjection. The results from experiment 1 indicated that the reporter gene governed by the Miw promoter (RSV LTR and chicken β-actin promoter) was expressed more intensively in bovine morulae and blastocysts than three other gene constructs. In experiment 2, the effect of SV40 enhancer was investigated when fused downstream to the luciferase cDNA of the Miwluc vector. The results showed that SV40 enhancer further activated the luciferase activity of the Miw promoter in bovine preimplantation embryos. It was concluded, therefore, that the Miw promoter together with the SV40 enhancer would confer the strongest expression of the firefly luciferase reporter gene among the gene constructs tested in preimplantation bovine embryos.

5.2. Introduction

In the last few years, it has been suggested that the use of *Vargula* or firefly luciferase should permit rapid and live detection of a transgene before embryo transfer (Thompson *et al.*, 1995a; Menck *et al.*, 1997; Muramatsu and Nakamura, 1997). Live detection of luciferase activity by the bioluminescence imaging keeps embryos away from the biological stress such as biopsy for diagnosis of by PCR (Behboodi *et al.*, 1993; Horvat *et al.*, 1993; Bowen *et al.*, 1994; Krisher *et al.*, 1994a) and the irradiation for detection of either bacterial β-galactosidase (Lin *et al.*, 1994b) or green fluorescent protein (GFP) (Ikawa *et al.*, 1995). Matsumoto *et al.* (1994) have reported that the short exposure (<3 hrs) of D-luciferin, the substrate of the luciferase bioluminescence reaction, has no effect on the embryonic development. Thus, the use of firefly luciferase reporter gene allows continuous and noninvasive monitoring of gene expression in preimplantation embryos.

With this luciferase detection method, the reporter gene driven by a strong promoter is

necessary as a good diagnosis tool for the detection of transgenes in preimplantation bovine embryos. In the literature, some viral and hybrid promoters are known to be functional in developing embryos. For example, the Miw promoter that harbours both the chicken β-actin promoter and the RSV LTR showed the strong activity in mouse embryos (Suemori *et al.*, 1990). Schöler *et al.* (1989) have found that the 6W enhancer elevated the promoter activity in the trophectoderm of mouse blastocysts. The SV40 early promoter has been widely used in transfection experiments involving mammalian cells and in transgenic mice experiments, and its activity is generally known to be strong. In bovine embryos, however, the percentage of embryos expressing the lacZ gene governed by this promoter declined significantly from the eight-cell stage to the morula and blastocyst stages (Kubisch *et al.*, 1995).

The two experiments in this Chapter were conducted to investigate quantitatively the luciferase activity of viral and hybrid promoters in preimplantation bovine embryos by using firefly luciferase reporter genes, in the hope to construct a good reporter gene usable for the indirect protein detection method.

5.3. Materials and Methods

Transgene Constructs for Microinjection

The transgene constructs used are given in Figure 9. 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 derived from pSVEluc by replacing the SV40 promoter region for the fragment of 6W (a hexamer of 1W) enhancer and the TK promoter derived from p6WTKlacZ. The 1W fragment was the oligonucleotides containing the μE4 and Oct binding sites (Schöler *et al.*, 1989). The pGL2-Control plasmid containing the SV40 early promoter and enhancer (pSVEluc) was obtained commercially. The pMiwluc was constructed by fusing the regulatory region of pMiwZ (Suemori *et al.*, 1990) upstream to the firefly luciferase cDNA and the SV40 polyadenylation signal sequences. The pMiwEluc plasmid was created by fusing the regulatory sequence of pMiwlacZ upstream to a combined sequences of firefly luciferase cDNA, SV40 polyadenylation signal, intron and SV40 enhancer derived from

pSVEluc.

The gene constructs were linearized by unique restriction endonuclease digestion, and gel-purified with SUPREC-01. The linearized transgenes of pTKEluc, pTK6WEluc, pSVEluc, pMiwluc and pMiwEluc were dissolved in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA to an equimolar concentration of 3.2 μg/ml, 3.4 μg/ml, 3.2 μg/ml, 3.8 μg/ml and 4.2 μg/ml respectively, and cleaned up with a 0.20 μm filter.

Egg Manipulation and Microinjection

Bovine ovaries were obtained at a local slaughterhouse and were transported to the laboratory in 0.85% (w/v) physiological saline at 37℃. Cumulus-oocyte complexes collected from ovarian follicles were matured, fertilized, microinjected with transgenes, and cultured as described in Chapter 2.

In experiment 1, microinjection of four gene constructs (TKEluc, TK6WEluc, SVEluc and Miwluc) was performed. The microinjected embryos were similarly cultured for 2 or 6 days, and analysed for bioluminescence intensity. Experiment 2 was carried out by microinjecting with pMiwluc or pMiwEluc. The microinjected early embryos were cultured for 2 days and assayed for bioluminescence intensity.

In both experiments, non-injected control oocytes were removed from cumulus cells, centrifuged, and inspected for pronuclear visibility to examine whether or not fertilization was performed.

Detection of Firefly Luciferase Expression

In experiments 1 and 2, bioluminescence intensity was determined by using the photon imaging system (ARGUS-50/VIM, Hamamatsu Photonics Co., Ltd., Hamamatsu, Japan), because the measurement of multiple embryos could be done at once. Bovine embryos cultured for 2 or 6 days were transferred into a 10 μl drop of the culture medium covered with oil on a microscopic chamber. To the drop, 10 μl of PBS containing 500 μM D-luciferin was added, and the embryos were stored at 37℃ for 5 min. Subsequently, bioluminescence imaging was done in a dark room for 5 min.

Statistical Analyses

For testing the significance of differences in the developmental rate of bovine embryos injected with the four gene constructs (experiment 1), the χ^2 test was used. The quantitative data of luciferase activity were treated statistically by analysis of variance, and significance of differences between means were assessed by a Duncan's multiple range test by using General Linear Model Procedures (SAS Institute, 1985).

5.4. Results

Developmental rates of bovine embryos microinjected with the four gene constructs in experiment 1 are shown in Figure 10. The TKEluc, 6WTKEluc, SVEluc and Miwluc vectors were microinjected into 100, 96, 92 and 89 fertilized oocytes, respectively. As a control group, 54 uninjected zygotes were also cultured. No significant difference was found in the proportions of embryos developed to the morula or blastocyst stage to the total embryos, ranging from 16 to 22%, among the treatment groups irrespective of DNA injection, and kind of DNA injected. Values for bioluminescence intensity analysed by the photon imaging system in Experiment 1 are shown in Figure 11. The TKEluc, 6WTKEluc, SVEluc and Miwluc vectors were microinjected into 90, 105, 93 and 135 fertilized oocytes, respectively. The embryos that cleaved normally and developed to the 4 to 16-cell stage were assayed at 2 days after microinjection. At 2 days after microinjection, the SVEluc-injected zygotes tended to have a higher expression level of the firefly luciferase than those of three other groups, and significant difference was detected between the SVEluc and the two TK promoters ($P<0.05$). Similarly, the TKEluc, 6WTKEluc, SVEluc and Miwluc vectors were introduced into 100, 96, 92 and 89 fertilized oocytes, respectively. The embryos that cleaved normally and developed to the morula or blastocyst stage were assayed at 6 days following microinjection. At 6 days after gene transfer, the luciferase activity of the zygotes injected with the Miwluc gene tended to be higher than that of zygotes injected with the three other gene constructs with significant difference between the Miwluc and TK6WEluc genes ($P<0.05$).

Figure 12 presents the effect on gene expression level of SV40 enhancer in bovine

embryos at 2 days after microinjection when fused downstream to the luciferase cDNA of the Miwluc vector. The Miwluc and MiwEluc vectors were microinjected into 90 and 100 fertilized oocytes, respectively. The embryos that cleaved normally and developed to the 4 to 16-cell stage were assayed at 2 days after microinjection. The bioluminescence values were significantly enhanced by the presence of SV40 enhancer ($P<0.05$).

5.5. Discussion

In this Chapter, an attempt was made to compare quantitatively the transcriptional activity of gene constructs with viral and hybrid promoters and enhancers. The Miw promoter is known to be strong in mouse embryos (Suemori *et al.*, 1990). The Miw promoter consists of the chicken β-actin promoter and the RSV LTR. The chicken β-actin promoter, one half of the Miw promoter, is a strong constitutive promoter (Fregien and Davidson, 1986), while the RSV LTR, the other half, is not only strong but also exhibits minimum variation of transcriptional activity among various cell types (Gorman *et al.*, 1982). As was expected, the activity of the luciferase gene driven by the Miw promoter tended to be higher than that of three other gene constructs at 6 days after gene transfer (see Figure 11). The fact that the SV40 enhancer further increased the transcriptional activity of the Miw promoter suggests that the Miw promoter in combination with the SV40 enhancer may be useful for the detection of reporter gene expression. Taken together, the pMiwEluc plasmid may serve as a good diagnosis tool for the presence of transgenes in preimplantation bovine embryos.

To the substrate solution for the photon imaging system, 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. In addition, the photon imaging system was used in the present experiments to save the measurement time and to minimize the possible disturbance of embryonic development by the exposure to D-luciferin, the substrate of bioluminescence

reaction.

The proportion of eggs that developed to the morula and blastocyst stages ranged from 16 to 22% irrespective of the DNA treatment. The present results implied that bovine embryos surviving after pronucleus microinjection can develop to the morula and blastocyst stages at the same rate as intact embryos as observed by Behboodi *et al.* (1993). Additionally, the development of microinjected zygotes did not depend on the difference of the promoter fused to the firefly luciferase gene. Therefore, the firefly luciferase may not be deleterious to bovine embryos development before implantation.

In this Chapter, more responders were obtained than expected. Previously, it was observed that the proportion of morulae/blastocysts expressing the luciferase gene was 21%, when luciferase-positive embryos by eyes were counted (Muramatsu *et al.*, unpublished results; Muramatsu and Nakamura, 1997). In this Chapter, however, luciferase activity was analysed by computer-assisted image processing, which increased the overall sensitivity of the luciferase analysis. This might explain why the higher proportion of luciferase-positive embryos was obtained.

Schöler *et al.* (1989) have suggested that the 6W enhancer containing six octamer motifs, each of which consists of a binding site of Oct3, is active in the inner cell mass but not in the trophectoderm of transgenic mouse blastocysts. Accordingly, it was expected that the luciferase activity in embryos injected with pTK6WEluc would be enhanced specifically at the morula and blastocyst stages. However, the expression level of pTK6WEluc at these developmental stages was not significantly increased by the presence of the 6W enhancer in this Chapter. No activation by the 6W enhancer was also observed with lacZ gene constructs as described in Chapter 3. The true reason for this was unknown, but the affinity between the octamer motifs and bovine Oct3 might not be so strong as that found in mouse blastocysts (Schöler *et al.*, 1989).

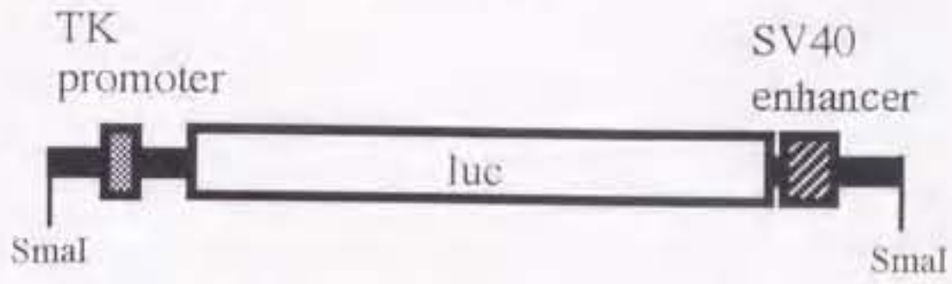
The SV40 early promoter has been widely used in gene regulation experiments of embryos. Kubisch *et al.* (1995) have found that the expression efficiency of the lacZ gene governed by the SV40 early promoter decreased significantly from eight-cell to the morula and blastocyst stages of bovine embryos. In this Chapter, it was also observed that the SV40

early promoter activity declined rapidly as the development proceeded from 2 to 6 days after microinjection (Figure 11). Thus, the SV40 driven luciferase gene may not be suitable for the diagnosis of the presence of transgenes.

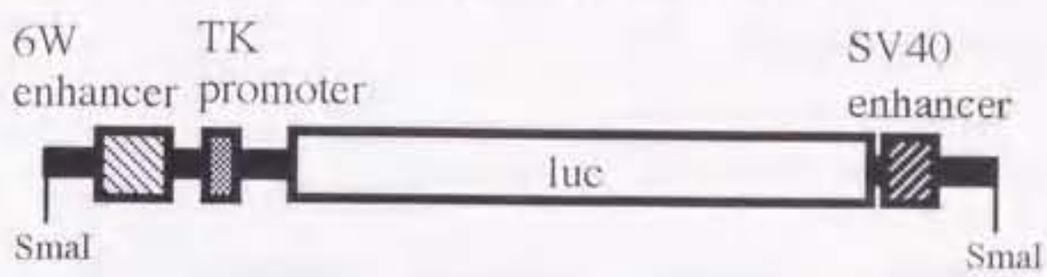
5.6. Summary

1. In experiment 1, no significant difference was found in the proportions of embryos developed to the morula or blastocyst stage to the total embryos, ranging from 16 to 22%, among the treatment groups irrespective of DNA injection, and kind of DNA injected.
2. In experiment 1, the firefly luciferase gene governed by the Miw promoter (RSV LTR and chicken β-actin promoter) was expressed more intensively in bovine morulae and blastocysts than three other gene constructs.
3. In experiment 2, SV40 enhancer significantly activated the luciferase activity of the Miw promoter in bovine embryos at 2 days after microinjection.

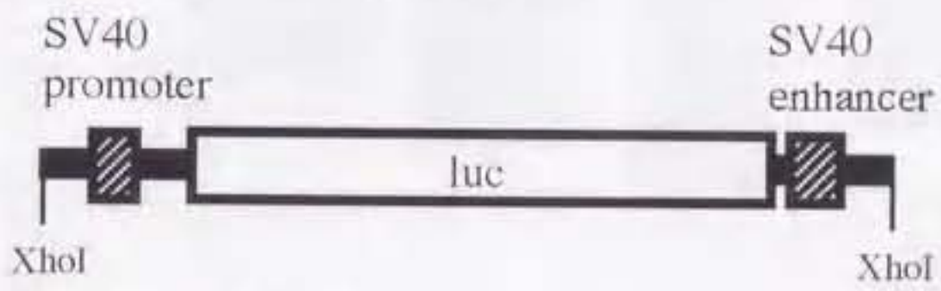
(A) pTKEluc [TK(P)+SV(E)]



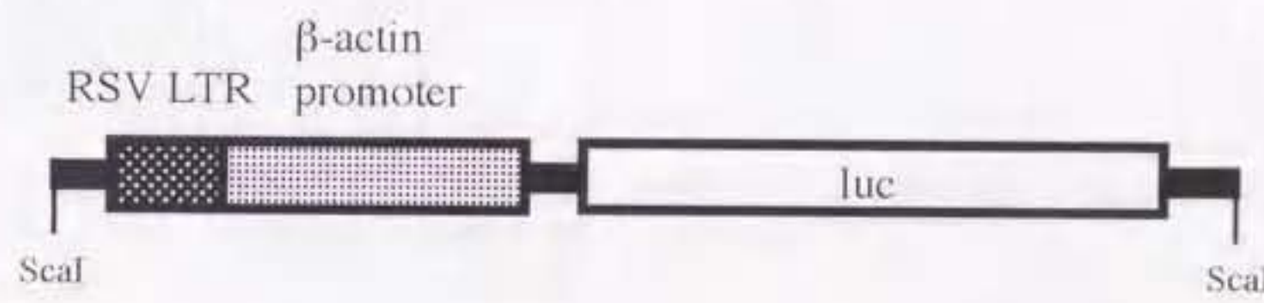
(B) pTK6WEluc [TK(P)+6W(E)+SV(E)]



(C) pSVEluc [SV(P)+SV(E)]



(D) pMiwluc [Miw(P)]



(E) pMiwEluc [Miw(P)+SV(E)]

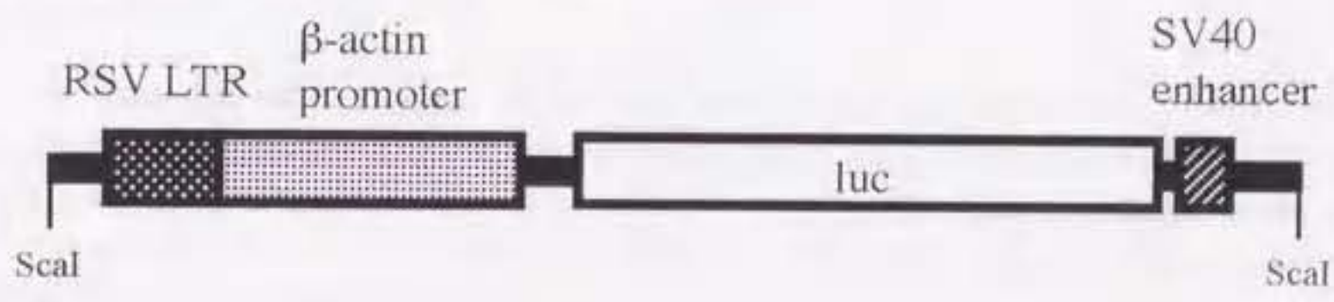


Figure 9. Constructs of microinjected transgenes: (A) pTKEluc; (B) pTK6WEluc; (C) pSVEluc; (D) pMiwluc; and (E) pMiwEluc. These plasmid DNAs were linearized with unique restriction endonucleases and dissolved in TE buffer (10 mM Tris-HCl (pH 7.5) / 0.1 mM EDTA) to give an equimolar concentration at 3.2 μg/ml (pTKEluc), 3.4 μg/ml (pTK6WEluc), 3.2 μg/ml (pSVEluc), 3.8 μg/ml (pMiwluc) and 4.2 μg/ml (pMiwEluc). Abbreviation used: luc, firefly luciferase cDNA; TK, thymidine kinase; SV40, simian virus 40; 6W, hexamer of the 1W fragment containing an octamer motif; RSV, Rous sarcoma virus; LTR, long terminal repeat; β-actin, chicken β-actin.

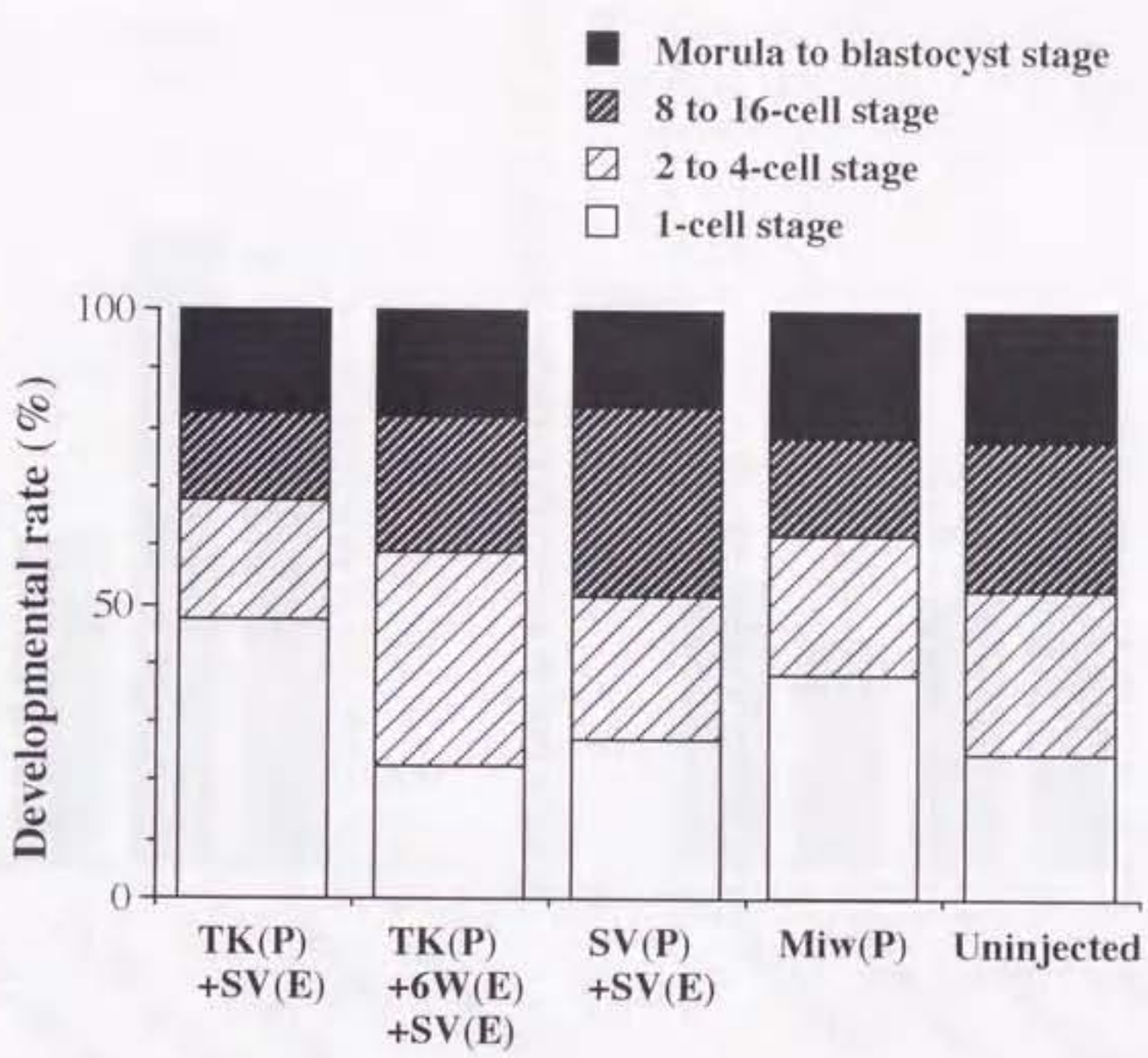


Figure 10. Developmental rates of bovine embryos microinjected with the four different gene constructs. They were cultured for 6 days after microinjection and developed to morula and blastocyst stages. There was no significant difference in the developmental rate of bovine embryos between the treatment groups according to the χ^2 test ($P>0.05$).

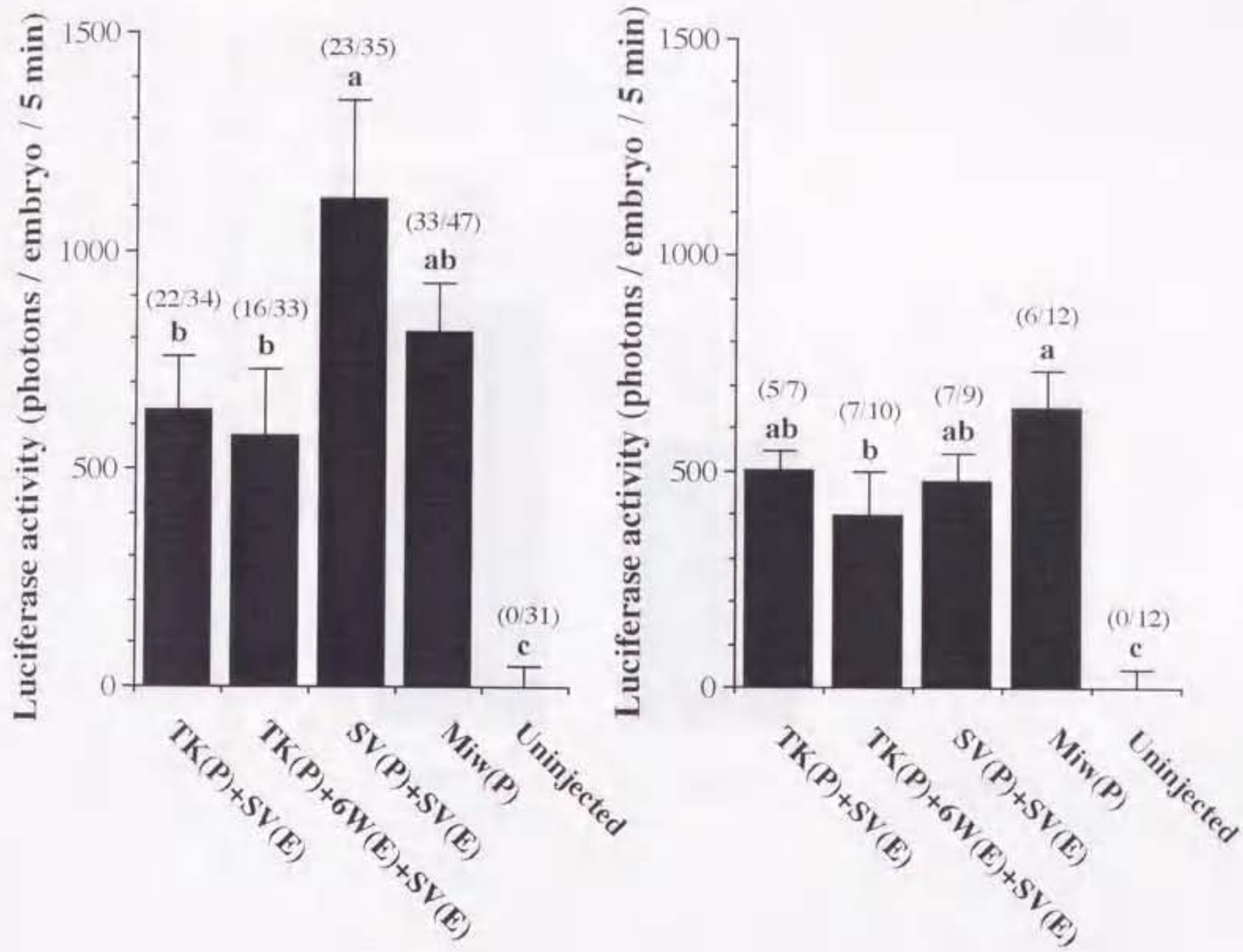


Figure 11. Luciferase gene expression analysed by using a photon imaging system. (A): Comparison of luciferase activity directed by four different gene constructs at 2 days after microinjection. The zygotes that developed normally to the 4 to 16-cell stage were assayed. The data are presented as means ± SEM. Numbers of embryos assessed (responders / analyzed embryos) are indicated in parentheses for each treatment. [a-c]Means not having the same letter are significantly different at $P<0.05$. (B): Comparison of luciferase activity directed by four different gene costructs at 6 days after microinjection. The zygotes that developed normally to the morula or blastocyst stage were assayed. The data are presented as means ± SEM. Numbers of embryos assessed (responders / analyzed embryos) are indicated in parentheses for each treatment. [a-c]Means not having the same letter are significantly different at $P<0.05$.

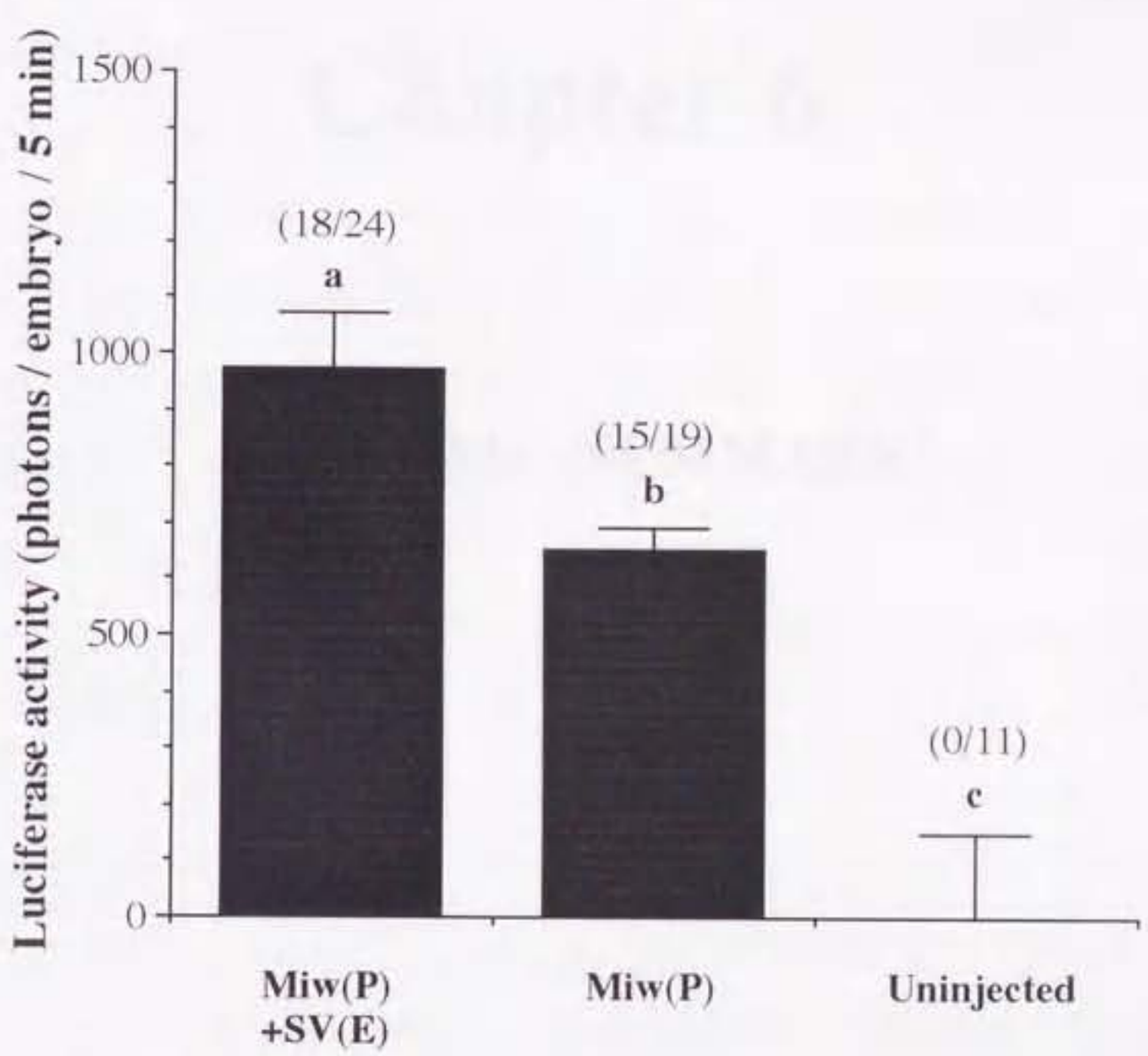


Figure 12. The effect of the SV40 enhancer on the firefly luciferase gene expression in bovine preimplantation embryos. The embryos that cleaved normally and developed to the 4 to 16-cell stage were assayed at 2 days after microinjection. The data are presented as means ± SEM. Numbers of embryos assessed (responders / analyzed embryos) are indicated in parentheses for each treatment. [a-c]Means not having the same letter are significantly different at $P<0.05$.

Chapter 6

GENERAL SUMMARY

The present study was conducted, (1) to examine whether or not the product of transgene expression i.e. β-galactosidase, could be detected at early developmental stages of bovine embryos, (2) to compare expression efficiency of four promoters in preimplantation bovine embryos by using lacZ reporter genes, (3) to establish a new methodology for measuring bioluminescence activity easily in live developing bovine embryos by using firefly luciferase reporter genes, and (4) to investigate quantitatively the luciferase activity of viral and hybrid promoters in preimplantation bovine embryos by using firefly luciferase reporter genes. Both the bacterial β-galactosidase and the firefly luciferase activities generated by transgene expression could be detected in bovine preimplantation embryos (Chapters 2 and 4). It was suggested, therefore, that the lacZ and firefly luciferase genes could be used as a reporter gene for the screening method.

E.coli β-galactosidase, a tetramer with subunit size of 1023 amino acids, catalyzed the hydrolysis of various β-galactosides, including lactose (Fowler and Zabin, 1983). Bacterial β-galactosidase activity can be monitored histochemically by cleavage of the substrate X-gal. Practically, β-galactosidase activity in bovine embryos that were introduced with the lacZ gene by microinjection could be assayed histochemically as shown in Chapters 2 and 3. However, the crucial disadvantage of the X-gal staining method is that developing embryos have to be fixed prior to histochemical staining for the detection of lacZ expression, and therefore, are no longer transplantable after the detection of lacZ gene expression. As an alternative approach, β-galactosidase activity can be monitored by the fluorescence from the cleavage of fluorescein-di-β-D-galactopyranoside (FDG) as a substrate. However, in this fluorescence analyses, irradiation at about 480 nm excitation wavelength might be deleterious to embryo development.

Firefly luciferase catalyzes the bioluminescence reaction (Alam and Cook, 1990). So far, it has been reported that by using a luminometer the firefly luciferase gene expression has been analysed quantitatively in the cell extract of mouse preimplantation embryos (Majumder *et al.*, 1993; Mélin *et al.*, 1993; Thompson *et al.*, 1994, 1995b). However, the embryos subjected to sonication can no longer be used for examining the luciferase activity at later developmental stages nor for transplantation to recipient cattle. To overcome this

shortcoming, the present study demonstrated that two noninvasive systems were available for monitoring firefly luciferase gene expression in live bovine embryos. In Chapter 4, by using a luminometer, luciferase gene expression could be assayed quantitatively in live bovine embryos, while in Chapter 5 and in the previous study, by using a photon imaging system (Muramatsu and Nakamura, 1997), gene expression could be directly visualized *in situ* and assayed quantitatively in living embryos from early stages of development. In addition, it was confirmed that so long as the bioluminescence measurement was done in a short time, no harmful effect would be detected. The development of bovine embryos to the blastocyst stage was normal when luciferin (500 μM) was present in the medium for up to 3 hrs, whereas the 6-hr exposure to luciferin significantly reduced the percentage of embryos that successfully developed to blastocysts (Muramatsu *et al.*, unpublished results). Therefore, the bioluminescence screening method established in the present study would be of great use for the detection of transgenes in preimplantation bovine embryos.

In the present luciferase screening method, there are at least three advantages over other methods. First, this method does not confer embryos the biological stress such as biopsy for diagnosis of by PCR (Behboodi *et al.*, 1993; Horvat *et al.*, 1993; Bowen *et al.*, 1994; Krisher *et al.*, 1994a) and the irradiation for detection of either bacterial β-galactosidase (Lin *et al.*, 1994b) or green fluorescent protein (GFP) (Ikawa *et al.*, 1995). Secondly, its manipulation is easy and quick. Thirdly, from offspring derived from mosaic animals with transgenes, transgenic embryos could be detected easily and credibly. However, all indirect screening methods may confer false positive signals like PCR methods if a significant portion of the gene expression results from episomally existing microinjected genes. Thus, to increase reliability of screening for integrated transgene is of crucial importance to apply the present indirect screening method in practice.

Among the some promoters tested in the present study, the Miw promoter containing the RSV LTR and chicken β-actin promoter showed higher expression efficiency and stronger expression than other promoters as demonstrated in Chapters 3 and 5. Suemori *et al.* (1990) have reported that the Miw promoter showed strong activity in mouse embryos. Therefore, this promoter could drive structural genes efficiently in mouse or bovine embryos

at early stages of development. Additionally, the SV40 enhancer was found to significantly increase the transcriptional activity of the Miw promoter as shown in Chapter 5. These results suggest that the Miw promoter in combination with the SV40 enhancer may be useful for the detection of reporter gene expression.

As described in Chapter 1, nuclear transfer technique could provide a powerful tool for producing transgenic animals in important farm livestock. Wilmut *et al.* (1997) reported the birth of live lambs from fetal and adult differentiated cells. This report indicated that a wide variety of cultured and differentiated cells could be used as donor cells. If transgenes were stably transferred into donor cells, the offspring derived from the transfected donor cells would become genuine transgenic, and be free from mosaicism. So far, most transgenic domestic animals are generated by the direct microinjection of DNA fragments into the pronuclei of fertilized eggs. Whitelaw *et al.* (1993) reported that most G_0 transgenic mice generated by microinjection were mosaic. Such mosaicism has also been detected in transgenic pigs (Pursel *et al.*, 1989) and sheep (Simons *et al.*, 1988; Rexroad *et al.*, 1991). In the near future, by utilizing the present screening system for selecting donor cells for nuclear transfer, the production efficiency of transgenic cattle would be more improved.

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APPENDICES

List of Publications Related to This Thesis

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

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

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Nakamura, A., Okumura, J. and Muramatsu, T. (1998) Quick detection of firefly luciferase gene expression in live developing bovine embryos by photoncounting. Asian-Australasian J. Anim. Sci. (in press).

Other Publications

Muramatsu, T., Nakamura, A. and Okumura, J. (1994) Expression of a foreign gene introduced into bovine embryos at early stages of development. *In:* Animal Cell Technology: Basic & Applied Aspects vol. 6. Edited by Kobayashi, T., Kitagawa, Y. and Okumura, K. pp. 255-258. Kluwer Academic Publishers, Dordrecht, Netherlands.

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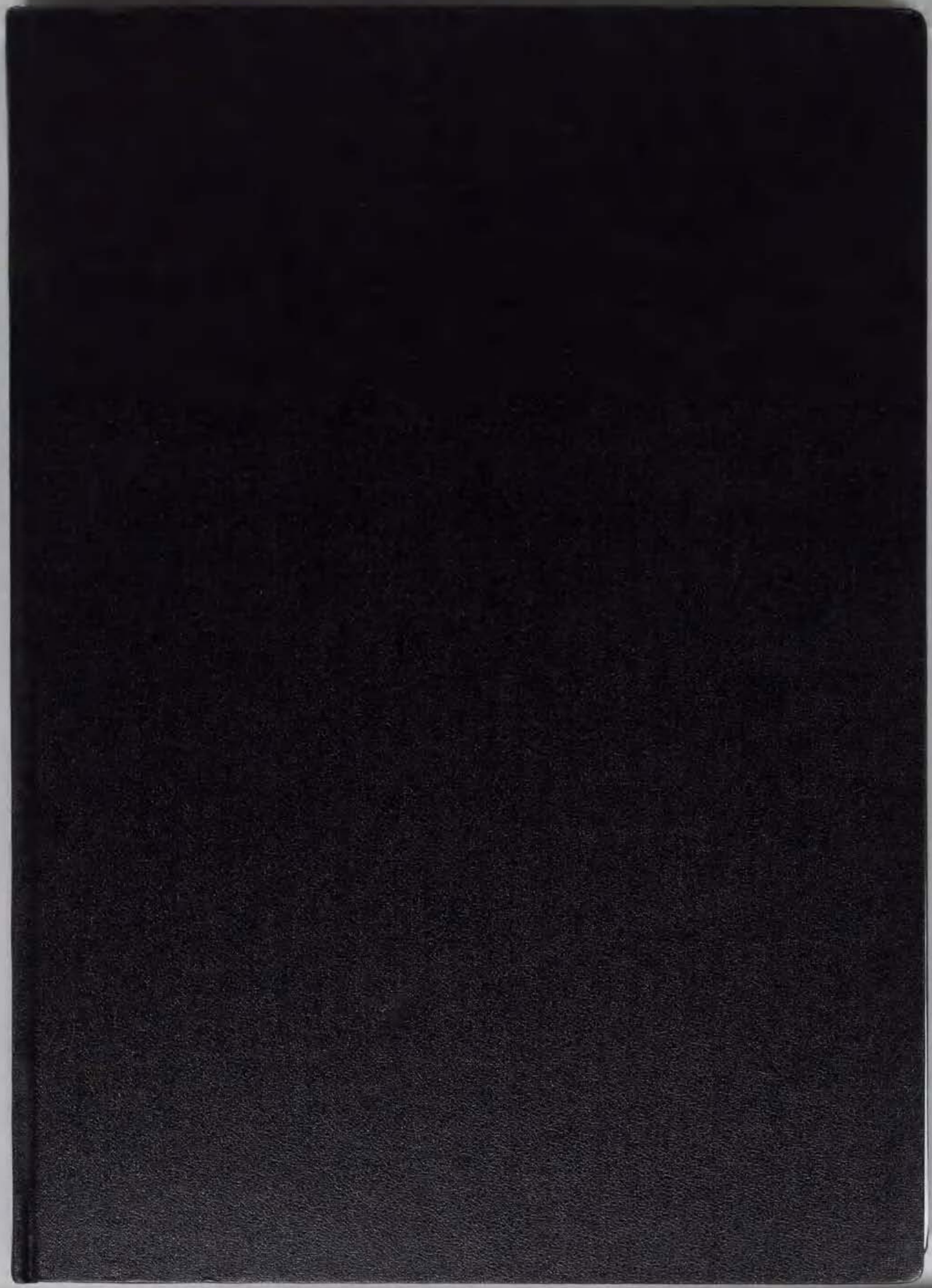

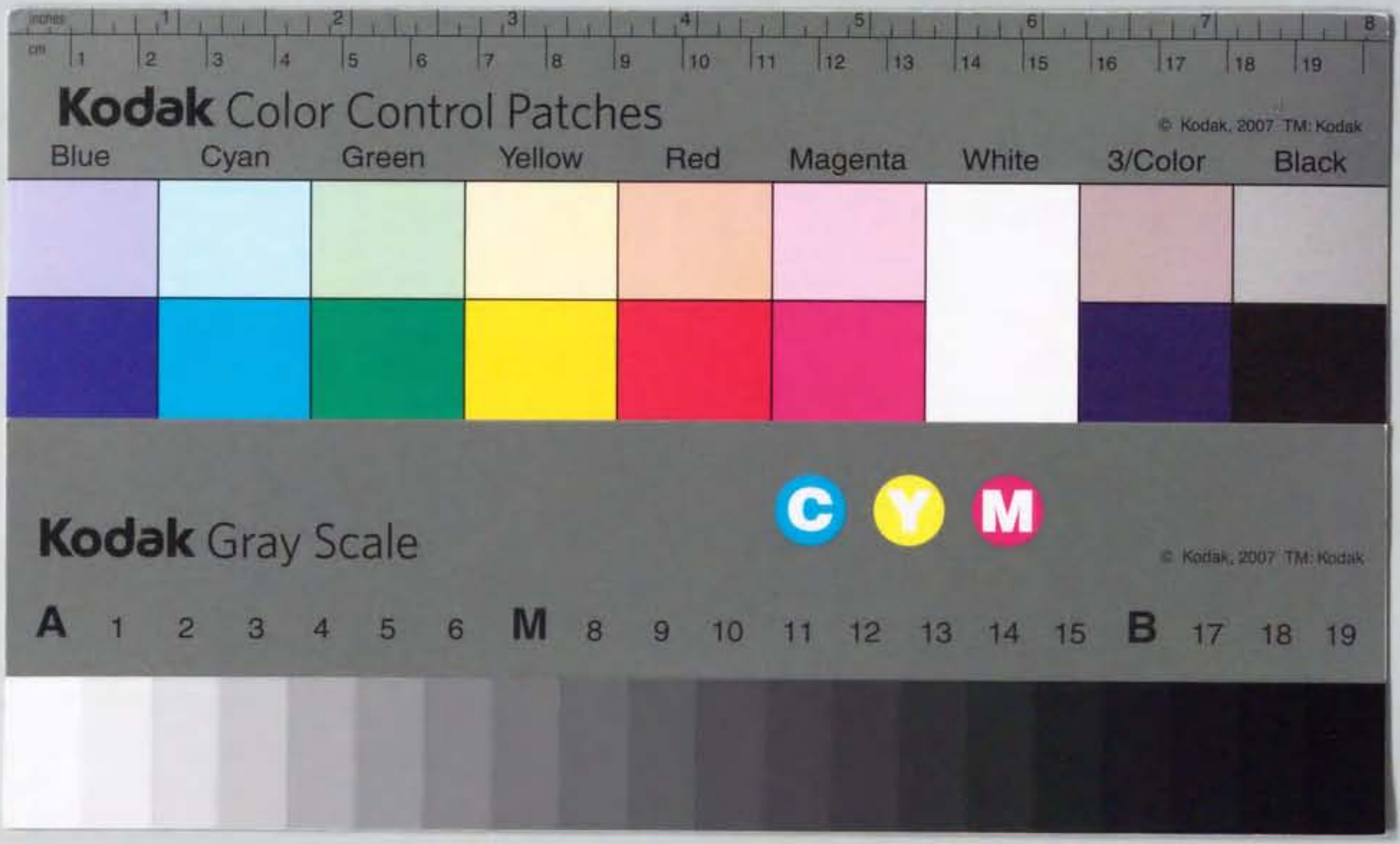
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