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Expression of LacZ Gene Controlled by Various Promoters in Mouse Preimplantation Embryos

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Abstract: *In this study, the activity of mouse phosphoglycerate kinase promoter (PGK) and murine embryonic stem cell virus promoter (MESV), enhanced by the connection of the R-segment and part of the U5 sequence (RU5) of the long terminal repeat of human T-cell leukemia virus type 1, was compared with that of simian virus 40 early promoter (SV40) and human cytomegalovirus early promoter (CMV). Escherichia coli β-galactosidase (LacZ) reporter gene connected to these promoters was microinjected into pronuclei of mouse zygotes, and their expression of developing embryos during the preimplantation period was evaluated histochemically with X-gal. No difference was observed in the proportion of embryos which developed into the morula stage among the promoter sequences at 96 h after hCG injection, but the expression of LacZ gene connected to MESV-RU5 (MESV-LacZ) was lower than that to PGK-RU5 (PGK-LacZ), CMV (CMV-LacZ) and SV40 (SV40-LacZ) in the morula stage embryos (P<0.05). In another experiment, more than 50% of embryos microinjected with PGK- and CMV-LacZ responded positively to X-gal staining at 48 h after hCG injection and the activity of these promoters continued at nearly the same rate from there onwards. However, the rate of expression of SV40- and MESV-LacZ was lower than that of PGK- and CMV-LacZ at 48 h after hCG injection (P<0.05). Although expression of MESV-LacZ was consistently low in proportion and weak in intensity, that of SV40-LacZ was high at 72 h after hCG injection and was equivalent to that of PGK- and CMV-LacZ at 96 h after hCG injection. Regardless of the promoters used, the expression of LacZ gene in the embryos showed various intensities of blue staining. The frequency of mosaic patterns and a weak intensity of gene expression in morphologically normal embryos had a tendency to be higher than in degenerated embryos or those whose development had been arrested.*

Key words: *Promoter, LacZ, Microinjection, Mouse, Embryo.*

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Transgenic animals are an increasingly popular experimental system because they offer the advantage of allowing the ability and impact of foreign gene expression within the entire organism to be studied [1]. Transgenic mice are most commonly used, while transgenic farm animals have also been developed for specific purposes, including agricultural, pharmaceutical and diagnostic applications [2–5]. However, the efficiency of producing transgenic animals by means of the microinjection technique, which is still the predominant method employed, is very low even in mice. There is little incentive to improve integration frequency in the mouse, because their costs are within an acceptable range [6], but increased efficiency of producing transgenic animals would have a significant impact in farm animals. One of the most widely discussed approaches is selection of transgenic embryos before they are transferred to recipients [7, 8].

Although it has been reported that expression of transgenes injected into the pronucleus can be found in over 30% of mouse embryos; the majority of these being mosaics [9–11], the behavior of such exogenous genes in developing embryos during the subsequent preimplantation period has not been fully clarified. The transgenic approach with a sensitive reporter gene, such as *Escherichia coli* β-galactosidase (LacZ), is an important method in the analysis of developmental regulation [12, 13]. To detect the expression of LacZ gene efficiently, it is important to select a strong promoter (containing both an enhancer element and a promoter) regulating preimplantation embryos. Of the promoter

sequences investigated, the simian virus 40 early promoter (SV40) and human cytomegalovirus immediate early promoter (CMV) are functional in an abundant tissue type, including preimplantation embryos [14, 15].

To investigate the pattern of expression of LacZ gene which allows efficiency in developing embryos during the subsequent preimplantation period, we have used two new constructs for microinjection of mouse zygotes. The mouse phosphoglycerate kinase (PGK) gene encodes the housekeeping enzyme, which is involved in glycolitic pathway, so that this promoter sequence could work on constitutively embryonic cells as well as on most somatic cells [16]. The mouse embryonic stem cell virus (MESV), derived from a mutant retrovirus, is exclusively expressed in ES cells [17], so that this promoter sequence could work in embryonic cells, too. Moreover, it was reported that the SV40 early promoter fused with the R-segment and part of the U5 sequence (RU5) of the long terminal repeat of human T-cell leukemia virus type 1 increased the expression level more than 1 order of magnitude over that of the SV40 promoter alone [18]. Taken together, we have used two promoters, PGK-RU5 and MESV-RU5, in which the SV40 has been replaced with PGK and MESV, respectively.

The objective of this study was to assess the effects of activity of PGK-RU5, MESV-RU5, SV40 and CMV on developing mouse embryos during the preimplantation period. To detect gene expression, LacZ reporter gene was connected to these promoters and microinjected into pronuclei of mouse zygotes. Developmental stages of these embryos were scored at various times and the product of the LacZ gene in embryos was detected by X-gal staining.

Materials and Methods

Preparation of DNA constructs for microinjection

DNA constructs used in this experiment are shown in Fig. 1. The 4.3 kb fragment containing SV40 early promoter and enhancer connected to the LacZ coding region (SV40-LacZ) was obtained after AseI/PstI digestion of the plasmid pSV-β GAL (Promega Co., Madison, WI, USA). The 4.5 kb fragment containing CMV early promoter and enhancer connected to the LacZ coding region (CMV-LacZ) was also obtained after EcoRI/HindIII digestion of the plasmid pCMV β (Promega Co., Madison, WI, USA). Plasmid vector, pSY3 and pIN2 were kindly provided by Dr. T. Yokota (Institute of Medical Science, University of Tokyo). The LacZ coding region was introduced into the multi cloning site of pSY3 (pSY3-β GAL). The 5.0 kb fragment containing PGK-RU5

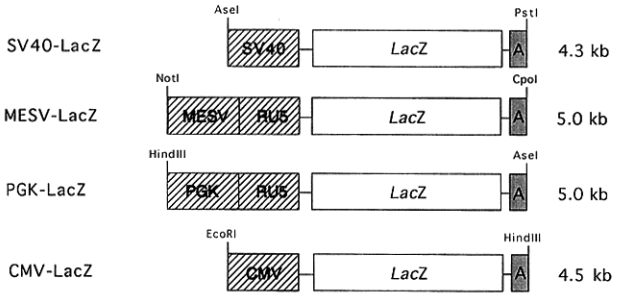

Fig. 1. Structure of DNA constructs used for microinjection into pronucler stage embryos. The bacterial LacZ coding region is shown as open boxes. Black boxes indicate SV40 polyadenylation signals. Shaded boxes indicate promoters. The restriction enzyme sites used to obtain the injected DNA are indicated above each diagram.

promoter connected to the LacZ coding region (PGK-LacZ) was obtained after HindIII/AseI digestion of pSY3-β GAL. Similarly, the LacZ coding region was introduced into the multi cloning site of pIN2 (pIN2-β GAL) and the 5.0 kb fragment containing MESV-RU5 promoter fused with the LacZ coding region (MESV-LacZ) was obtained after NotI/CpoI digestion of pIN2-β GAL.

Each DNA fragment was separated on agarose gel, purified with a gel extraction kit (Qiagen, Germany) and dissolved in 10 mM Tris-HCl and 0.1 mM EDTA solution (pH 7.4) at a concentration of 5.0 μg/ml.

Microinjection and in vitro culture

Female F1 (C57BL/6J X C3H) mice were superovulated with 5 i.u. eCG followed by 5 i.u. hCG 48 h later. After being left with F1 (C57BL/6J X C3H) males overnight, the females were killed, and fertilized pronuclear eggs were collected from the oviducts at 20 h after hCG injection. The manipulation and microinjection into pronuclei of eggs were carried out as described by Hogan *et al.* [19] The microinjected eggs were cultured in Whitten's medium supplemented with 100 μM EDTA [20] covered with paraffin oil at 37°C in 5% CO_2-air atmosphere. At 1 h after microinjection, the surviving eggs continued to be cultured and their subsequent development was scored morphologically at intervals of 24 h. The embryos that reached more than the 2-cell, 8-cell, morula and blastocyst stages at 48, 72, 96 and 120 h after hCG injection, respectively, were judged to have normal development.

Detection of LacZ gene expression

The β-galactosidase activity of the embryos was ex-

amined by a histochemical staining procedure with slight modifications [21]. Embryos were washed twice with PBS (pH 7.4) containing 3 mg/ml polyvinylpyrolidone and then fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 10 min at 4°C. After washing twice in PBS, the embryos were placed in PBS (pH 7.2) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal: Gibco BRL, Grand Island, NY, USA), 2 mM $MgCl_2$, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide and incubated for 16 h at 37°C in air. The expression of LacZ gene in embryos was scored by staining with blue deposits in the cytoplasm according to the pattern (whole *versus* mosaic) and intensity (weak or spot; + *versus* strong; ++).

Statistical analysis

Data on development and expression were analyzed by a chi-square procedure. Differences with $P<0.05$ were considered statistically significant.

Results

Effects of various promoter sequences on LacZ expression in the embryos which developed to the morula stage at 96 h after hCG injection

The percentages of microinjected embryos that survived at 1 h after microinjection, and of surviving embryos that continued to develop to the morula stage, did not differ among the promoter sequences (Table 1). Nevertheless, when the morula stage embryos that appeared to be morphologically normal were subjected to X-gal staining, the expression of MESV-LacZ (20.6%) was lower than that of PGK- (36.9%), CMV- (45.8%) and SV40-LacZ (36.4%, $P<0.05$, Table 2).

Frequency, pattern and intensity of embryos expressed LacZ gene fused with various promoters at different stages of preimplantation development

As the following experiments were conducted to detect the expression of LacZ gene with various promoters at different stages of preimplantation development, β-galactosidase activity was examined in the microinjected embryos at 48, 72, 96 and 120 h after hCG injection.

More than 50% of embryos microinjected with PGK- and CMV-LacZ responded positively to X-gal staining at 48 h (51.2% and 59.6%) after hCG injection and the activity of these promoters continued at nearly the same rate thereafter (58.5–69.0% and 60.4–65.3%, Fig. 2). But the rate of expression of SV40- and MESV-LacZ was lower than that of PGK- and CMV-LacZ at 48 h (3.5% and 2.7%) after hCG injection ($P<0.05$). Although expression of MESV-LacZ was consistently low in proportion (19.4–28.6%) and weak in intensity, that of SV40-LacZ was high at 72 h (40.0%) after hCG injec-

Table 1. Development of mouse embryos after the microinjection of LacZ gene connected with various promoter sequences

DNA construct	No. of embryos		No. (%*) of embryos developed to:		
	Injected	Survived	2-cell ≦ (48 h**)	8-cell ≦ (72 h**)	Compact morula ≦ (96 h**)
SV40-LacZ	191	149 (78.0)	133 (89.3)	104 (69.8)	110 (73.8)
MESV-LacZ	146	108 (74.0)	96 (88.9)	66 (61.1)	68 (63.0)
PGK-LacZ	138	115 (83.3)	98 (85.2)	79 (68.7)	84 (73.0)
CMV-LacZ	112	93 (83.0)	75 (80.6)	59 (63.4)	59 (63.4)

*: Number of embryos developed/Number of embryos survived. **: Time of observation (h after hCG injection).

Table 2. LacZ gene expression of embryos developed to the compact morula stage at 96 h after hCG injection

DNA construct	No. of embryos examined	No. (%) of embryos expressing LacZ gene
SV40-LacZ	110	40 (36.4)[a]
MESV-LacZ	68	14 (20.6)[b]
PGK-LacZ	84	31 (36.9)[a]
CMV-LacZ	59	27 (45.8)[a]

All of compact morula represented in Table 1 were stained with X-gal. [a,b]: Values with superscripts are significantly different ($P<0.05$).

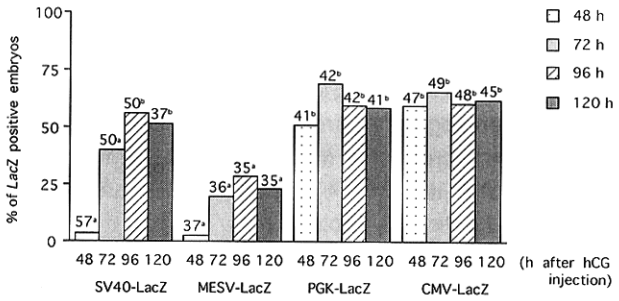

Fig. 2. Expression of LacZ gene fused with various promoter sequences. Embryos injected with different promoters were fixed at various times after hCG injection and stained with X-gal as a substrate at pH 7.2. The total number of embryos examined for LacZ gene expression is indicated above each column. [a, b]Values with different superscripts were significantly different at the same number of hours after hCG injection.

tion and was equivalent to PGK- and CMV-LacZ at 96 h (56.0%) after hCG injection.

Regardless of the promoters used, the embryos expressing the LacZ gene showed various degrees of patterns and intensities of blue staining (Table 3); i) the cytoplasm of some blastomeres was stained with blue spots, whereas the whole cytoplasm was blue in others, ii) all of the embryos that showed mosaic staining were composed of unstained and stained blastomeres, or weakly and strongly stained blastomeres, iii) the embryos that degenerated or those whose development had been arrested also showed a mosaic staining pattern similar in intensity to that observed in normal developing embryos, iv) but the frequency of mosaic patterns and the weak gene expression in morphologically normal embryos had a tendency to be higher than in degenerated embryos or in those whose development had been arrested, and v) all of the blastocysts developed at 120 h after hCG were classified as having spotty or weak intensity of gene expression.

Discussion

Some of the factors that reduce the efficiency of producing transgenic animals are the low embryo survival rate and low incidence of transgene integration [6]. With regard to the survival rate, it was reported that DNA concentrations greater than 10 μg/ml have detrimental effects on development [22]. Since a "near miss" of the pronucleus was expected to result in an effort to insert adequate quantities of DNA, concentrations employed in this study (5.0 μg/ml) were about 2- to 4-fold higher than in many published protocols [19, 23]. Nevertheless, 63–74% of microinjected embryos continued to develop to the morula stage, regardless of the different promoters used.

Nevertheless, the pattern of LacZ gene expression of microinjected embryos during the preimplantation stage depended on the activities of promoter sequences. The regulatory elements, such as promoter and enhancer sequences, have their own specific transcriptional activity that will determine the embryos in which the gene is to be expressed and the time and magnitude of expression.

In spite of the enhancement by RU5 sequences, activity of MESV-RU5 was consistently lower in proportion and weaker in intensity than that of other promoters. And blue staining was not restricted to the inner cell

Table 3. The proportion of pattern and intensity of LacZ positive embryos

DNA construct	Intensity / Pattern	48 h*				72 h*				96 h*				120 h*			
		1-cell		2-cell≦		<8-cell		8-cell≦		<Morula		Morula≦		<Blastocyst		Blastocyst≦	
		+	++	+	++	+	++	+	++	+	++	+	++	+	++	+	++
SV40-LacZ	Whole	3.5	0.0	0.0	0.0	4.0	14.0	2.0	0.0	2.0	8.0	2.0	0.0	10.8	13.5	0.0	0.0
	Mosaic	0.0	0.0	0.0	0.0	6.0	2.0	6.0	6.0	6.0	8.0	28.0	2.0	8.1	5.4	13.5	0.0
MESV-LacZ	Whole	0.0	0.0	2.7	0.0	2.8	0.0	0.0	0.0	8.6	0.0	0.0	0.0	11.4	0.0	0.0	0.0
	Mosaic	0.0	0.0	0.0	0.0	8.3	0.0	8.3	0.0	8.6	0.0	11.4	0.0	8.6	0.0	2.9	0.0
PGK-LacZ	Whole	4.9	9.8	4.9	12.2	2.4	23.8	4.8	2.4	0.0	16.7	4.8	0.0	2.4	17.1	2.4	0.0
	Mosaic	0.0	0.0	14.6	4.9	9.5	9.5	11.9	4.8	4.8	4.8	23.8	4.8	4.9	4.9	26.8	0.0
CMV-LacZ	Whole	2.1	21.3	14.9	6.4	6.1	36.7	4.1	0.0	2.1	20.8	6.3	2.1	2.2	33.3	2.2	0.0
	Mosaic	0.0	0.0	4.3	10.6	2.0	0.0	10.2	6.1	2.1	2.1	22.9	2.1	4.4	2.2	17.8	0.0

LacZ positive embryos represented in Fig. 2 were classified according to their staining pattern and intensity. The figure indicates percentages of embryos examined. *: Time of observation (h after hCG injection).

mass in blastocyst. MESV is a mutant derived from Moloney murine leukemia virus (MLV) and is active in ES cells [17], but the replication of MLV was not permissive in the preimplantation mouse embryos [24]. And vectors derived from MLV did not allow retroviral expression in stem cells, because of *de novo* methylation, lack of enhancer function, the presence of negative transacting factors and the involvement of intragenic sequences [17]. Although MESV-RU5 promoter was not fully functional during preimplantation development under our experimental conditions, further experiments will be needed to determine whether it allows expression in embryos at more advanced developmental stages.

Several groups have reported that the expression of LacZ gene fused with SV40 promoter is not detected or is at a very low level in the 2-cell stage embryos [11, 25, 26]. In this respect, our results were consistent with these reports. Takeda and Toyoda [11] observed that expression of SV40-LacZ was initially detectable at the 4-cell stage, maximized at the morula and decreased at the blastocyst stage. In contrast, PGK-RU5 and CMV allowed the expression of LacZ gene even at the 2-cell stage. As for transcriptional characteristics of microinjected DNA, transcription factor SP1-sensitive promoters were active in 2-cell mouse embryos in which the embryonic genome had yet to be activated [25]. Although SV40, as well as PGK and CMV, is sensitive to SP1 [16, 25, 27], Gorman *et al.* [28] suggested that a factor repressing SV40 promoter might exist in the undifferentiated cells. As expression of microinjected DNA is thought to be under the control of the mechanism responsible for zygotic gene regulation, SV40 may be activated only at a very low level at the 2-cell stage. On the other hand, more than 50% of embryos microinjected with PGK- and CMV-LacZ responded positively to X-gal staining at the 2-cell stage and the activity of these promoters continued at nearly the same rate from then onwards, but the activity of SV40 was increased at the 8-cell stage and was equivalent to that of PGK and CMV at the morula stage.

The staining frequency of embryos in this study was extremely high, as compared with the frequency of producing transgenic animals when measured as a proportion of newborns in which the gene is integrated [29]. The embryos expressing the LacZ gene showed various intensities of blue staining and a majority of these embryos were expression mosaics. Also, the proportion of positive blastomeres tended to decrease as development proceeded, and morula and blastocyst seldom showed uniform LacZ activity. It has been postulated that such mosaicism could have resulted either from the selective silencing of reporter genes as development progressed or from development-depend "diluting-out" of reporter gene; nonintegrated DNA was maintained through successive cell divisions and became unequally partitioned between blastomeres, so that a decreasing population of cells maintained the episomal DNA as development progressed [30].

In summary, the present results showed that more than 50% of embryos microinjected with PGK- and CMV-LacZ responded positively to X-gal staining from the 2-cell stage on, although the proportion of positive blastomeres in these embryos decreased as development proceeded. Further studies will have to be conducted to ascertain whether this parameter accurately reflects the fate of the injected DNA during embryo development.

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