Activation of Mouse Metallothionein-I Promoter in Mouse Preimplantation Embryos After Pronuclear Microinjection

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Abstract. To analyze expression of developmental genes and their regulatory mechanisms in mouse preimplantation embryos, pronuclei of *in vitro*-fertilized eggs were microinjected 8–10 h after insemination (hpi) with a fusion gene composed of the mouse metallothionein-I promoter (MT-I) and the *Escherichia coli* β -galactosidase gene (*lacZ*). The eggs were cultured up to the blastocyst stage in Whitten's medium supplemented with 100 μ M EDTA. Expression of the *lacZ* gene was examined by staining the embryos with X-gal as a substrate. Expression was first detected at the 2-cell stage, 27 hpi; the proportion of embryos expressing the *lacZ* gene reached a maximum of 80% at 33–48 hpi. The proportion declined from the 4-cell stage onward, but some embryos still showed positive staining at the blastocyst stage. α -Amanitin blocked the expression of *lacZ* gene only when added before 12 hpi, suggesting that transcription had started during the pronuclear stage rather than in cleavage stages. The presence of EDTA in the culture medium reduced the expression markedly from the morula stage on, but 25 μ M ZnCl₂ enhanced expression after the 4-cell stage in the presence of EDTA. These results suggest that transcription is active at the pronuclear stage and that the MT-I promoter becomes inducible by ZnCl₂ after activation of the embryonic genome.

Key words: Mouse preimplantation embryo, Embryonic genome activation, Metallothionein-I promoter, X-gal staining.

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Transcription of genes is active during oogenesis in the mouse [1]; then poly(A)⁺ RNA decreases at the oocyte maturation from germinal vesicle breakdown until ovulation [2]. After that, poly(A)⁺ RNA [3], α -actin mRNA [4] and histone mRNA [5] increase during the course of early embryonic development. It has been deduced that activation of the embryonic mouse genome first occurs at the 2-

cell stage [6]; which appears to be independent of DNA synthesis [7], cell cycle [8] and nuclear/cytoplasm ratio [9]. Recent studies suggest that activation of genes in the zygote may occur at the G2 phase of the 1-cell stage [10]. However, the mechanism has not been clarified.

Microinjection of an easily detectable marker gene into the pronuclear egg is an especially useful technique for investigating transcriptional activity in preimplantation embryos. In a previous report [11] we showed that the SV40-*lacZ* gene

injected into the pronuclei was expressed from the 4-cell to the blastocyst stages in preimplantation embryos cultured *in vitro*.

In the work reported here we examined *lacZ* expression driven by the promoter of an inducible gene, mouse metallothionein-I (MT-I), in order to analyze an epigenetic effect on gene expression during preimplantation development. Our results suggest that this construct (ML) was transcribed during the pronuclear stage; β -galactosidase activity became detectable at the late 2-cell stage, and this activity was enhanced by the addition of ZnCl_2 to the culture medium after embryonic genome activation.

Materials and Methods

In vitro fertilization and culture of embryos

In vitro fertilization was performed as described by Toyoda *et al.* [12]. Briefly, female F1 (C57BL/6N $\times$ DBA2) mice, 2–5 months old, were superovulated by intraperitoneal injections of equine chorionic gonadotropin and human chorionic gonadotropin, given 48 h apart. Spermatozoa obtained from the *cauda epididymis* of mature (3–5 month-old) male F1 (C57BL/6N $\times$ DBA2) mice were preincubated for 1 h in fertilization medium (TYH). The time course was described as hours post insemination (hpi), 0 hpi being the time when the preincubated sperm were introduced into the fertilization medium containing eggs. Fertilized eggs were cultured in Whitten's medium [13]. Ethylenediamine-tetraacetic acid (EDTA, disodium salt) [14, 15], zinc chloride (ZnCl_2) and α -amanitin were added to the culture medium according to the experimental design; to clarify when the exogenous ML gene began to be transcribed, α -amanitin (10 $\mu\text{g}/\text{ml}$) was added to the Whitten's medium supplemented with 100 μM EDTA at different times between 12 and 24 hpi, and β -galactosidase activity was examined at 36 hpi. To clarify the effects of supplementation with ZnCl_2 on expression of ML, the embryos were transferred to the culture media supplemented with 25 μM ZnCl_2 at 12, 24, 48 and 72 hpi, then x-gal staining was carried out at 24, 48, 72 and 96 hpi, respectively.

Construction of pML

Plasmid pML was constructed according to the general scheme outlined in Fig. 1. Plasmid pCH110

[16] was digested with *Hind*III and *Bam*HI; then a 3.8-kb fragment containing an *E. coli lacZ* gene was treated with T4 DNA polymerase and ligated to *Bam*HI linker (Takara Co. Ltd.). After digestion with *Bam*HI, the *lacZ* fragment was inserted into the *Bgl*II site of the plasmid pMK [17].

DNA preparation for microinjection

Plasmid pML was digested with *Pst*I, and the 5.7-kb fragment (designated ML) containing the *lacZ* gene fused to mouse MT-I promoter was purified in a silica matrix (GeneClean, Bio 101 Inc.) after electrophoresis in a 0.8% agarose gel. ML was dissolved in 10 mM Tris-0.1 mM EDTA solu-

Fig. 1. Construction of plasmid pML. Arrow indicates an ampicillin-resistance gene. E, *Eco* RI; P, *Pst* I; B, *Bam* HI; G, *Bgl* II; H, *Hind* III; TATA, TATA box; ATG, translation initiation; SV40, SV40 promoter; MT-I, MT-I promoter; HSV-TK, herpes simplex virus thymidine kinase gene.

tion (pH 7.4). At 8–10 hpi, the DNA solution at the concentrations of 0.05–50 ng/ μ l was microinjected into a pronucleus of each fertilized mouse egg.

Detection of lacZ expression

X-gal staining for detection of β -galactosidase activity was performed as described previously [11]. Briefly, embryos were rinsed with PBS (pH 7.2) and then fixed for 10 min at 4 C with 0.25% glutaraldehyde in PBS. Embryos were washed in PBS and then incubated for 12 h at 37 C in air, in a staining solution consisting of 0.04% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), 1 mM MgCl₂, 10 mM potassium ferricyanide, and 10 mM potassium ferrocyanide in PBS.

Results

Development of injected embryos

To evaluate the survival and subsequent development of the mouse embryos after DNA injection,

0.05–50 ng/ μ l (approximately 10–10000 copies) of ML was microinjected into the pronuclei; subsequently, 66–95% of zygotes cleaved into 2-cell embryos within 24 hpi (Table 1). Development of these embryos beyond the 2-cell stage in media containing EDTA is summarized in Table 2, which shows that there was no significant difference between 0.05 and 5 ng/ μ l, although development was markedly inhibited after injection of 50 ng/ μ l of ML.

Frequency of embryos expressing lacZ in each developmental stage

When examined at 24 h intervals, β -galactosidase activity was initially detected at 48 hpi in 4-cell embryos derived from eggs injected with ML at concentrations of 0.05–5 ng/ml (Fig. 2A). The frequency of embryos showing X-gal staining increased in a dose-dependent manner, reaching the highest level (72%) in the embryos that had been injected with 5 ng/ μ l of ML. Injection of 50 ng/ μ l resulted in an entirely different pattern of expression; staining was observed in 73.9% of 2-

Table 1. Development to 2-cell stage of mouse embryos after the microinjection of ML gene

Concentration of DNA (ng/ μ l)	Number of embryos	
	Survived ^a	Development to 2-cell stage (%)
0	86	74 (98.6) ^a
0.05	255	242 (94.9) ^a
0.5	304	281 (92.4) ^a
5	304	199 (65.5) ^b
50	111	81 (73.0) ^b

^aSurvival of the eggs was judged by morphology at 1 h after microinjection. Values with different superscript represent significant difference in the same column ($p < 0.05$).

Table 2. Development of mouse embryos *in vitro* after the microinjection of ML gene

Concentration of DNA (ng/ μ l)	Percentage of embryos developed to		
	4-cell (48 hpi)	Morula (72 hpi)	Blastocyst (96 hpi)
0	97.3 ^a (73)	94.6 ^a (72)	67.6 ^a (50)
0.05	89.3 ^b (56)	66.7 ^b (60)	50.0 ^b (54)
0.5	89.6 ^b (67)	59.4 ^b (64)	48.4 ^b (62)
5	85.7 ^b (35)	68.6 ^b (35)	56.7 ^b (30)
50	5.6 ^c (36)	8.3 ^c (36)	5.6 ^c (34)

Values with different superscript represent significant difference in the same column ($p < 0.05$). Number of embryos examined is shown in parentheses.

Fig. 2. (A) Staining frequency of embryos microinjected at the pronuclear stage with 0.5–50 ng/μl ML. Two-cell, 4-cell, morula and blastocyst embryos were examined at 24, 48, 72 and 96 hpi, respectively. Embryos were cultured in Whitten's medium supplemented with EDTA. The number of embryos examined is shown in parentheses. (B) Staining frequency of 2-cell stage embryos 24–36 hpi after microinjection with ML (5 ng/μl) at the pronuclear stage (8–10 hpi). Embryos were cultured in Whitten's medium supplemented with EDTA. Number of embryos examined is shown in parentheses.

cell stage embryos as early as 24 hpi, although the intensity of the staining was weak. Staining could not be examined at later stages because after 24 hpi development of those embryos was strongly suppressed.

Timing of ML expression

To clarify when the exogenous β -galactosidase began to express, X-gal staining was carried out at 3 h intervals from 24 hpi to 36 hpi, using embryos that had been injected with 5 ng/μl of ML. All of the embryos examined were at the 2-cell stage during this period, and β -galactosidase activity was detected initially at 27 hpi in 33% of the embryos (Fig. 2B). The frequency increased as the culture period lengthened.

To examine whether the β -galactosidase activity detected at 36 hpi had originated from newly synthesized mRNA, its expression was examined in embryos cultured in the presence of α -amanitin, an inhibitor of RNA polymerase II. 0.1–500 μg/ml of α -amanitin was added to the culture medium containing EDTA at 12 hpi, and X-gal staining was carried out at 36 hpi. The addition of α -amanitin suppressed the expression of the *lacZ* gene in a

dose-dependent manner, a significant inhibition being observed at 10 μg/ml or higher concentrations of α -amanitin (Fig. 3A). At these concentrations, α -amanitin also prevented the development of uninjected embryos to the 4-cell stage (Table 3). To further clarify the beginning of transcription, 10 μg/ml of α -amanitin was added at different times between 12 and 24 hpi, and β -galactosidase activity was examined at 36 hpi by X-gal staining. The results presented in Fig. 3B show that the activity was detected in 35% of embryos that had been treated with α -amanitin at 15 hpi, when all of the embryos were still at the 1-cell stage. The inhibitory effect of α -amanitin on β -galactosidase activity was less under the treatment after the embryos had already developed to the 2-cell stage.

Effects of EDTA and ZnCl₂ on induction of ML expression

After pronuclear microinjection of ML, embryos were incubated in Whitten's medium with or without 100 μM EDTA. Media were supplemented with 25 μM ZnCl₂ at 24 h (12 h as for examination of 2-cell stage embryos) before X-gal staining. The

Fig. 3. (A) Inhibition of ML expression by α -amanitin. α -Amanitin (0.1–100 $\mu\text{g/ml}$) was added at 12 hpi to Whitten's medium containing EDTA, and X-gal staining was carried out at 36 hpi. Number of embryos examined is shown in parentheses. (B) Inhibition of ML gene expression with 10 $\mu\text{g/ml}$ of α -amanitin. Open bars indicate percentages of embryos stained. Number of embryos examined is shown in parentheses. Thick line indicates percentages of embryos developed to 2-cell stage when α -amanitin was added to the culture medium. Dotted line tracks percentages of 2-cell embryos at the time of X-gal staining (36 hpi).

Table 3. Effect of α -amanitin on preimplantation development

α -amanitin ($\mu\text{g/ml}$)	No. of eggs examined	Percentage of embryos developed to			
		2-cell (24 h)	4-cell (48 h)	Morula (72 h)	Blastocyst (96 h)
0	32	100.0 ^a	84.4 ^a	93.8 ^a	87.5 ^a
0.1	26	100.0 ^a	76.9 ^a	92.3 ^a	76.9 ^a
1	27	100.0 ^a	40.7 ^b	18.5 ^b	0.0 ^b
10	29	100.0 ^a	0.0 ^c	0.0 ^c	0.0 ^b
100	27	100.0 ^a	0.0 ^c	0.0 ^c	0.0 ^b

Values with different superscript represent significant difference in the same column ($p < 0.05$). α -Amanitin was added to the medium at 12 hpi.

results of the assays for β -galactosidase activity are shown in Fig. 4. In the presence of EDTA, much higher activity was observed at the morula and blastocyst stage when ZnCl_2 was added to the culture medium (Fig. 4A). In the absence of EDTA, however, the activity was comparable in the embryos from 4-cell through blastocyst stage whether or not ZnCl_2 was in the culture medium (Fig. 4B).

Discussion

Most of the embryos microinjected with 0.05–5 ng/ μl of ML and cleaved to the 2-cell stage developed to the blastocyst stage by 96 hpi in Whitten's

medium supplemented with 100 μM of EDTA. On the other hand, the development to the 4-cell stage was markedly suppressed when the embryos were injected with a higher concentration (50 ng/ μl) of ML. This result indicates that injection of too much DNA inhibits subsequent development of embryos. We therefore chose an ML concentration of 5 ng/ml to examine transcriptional activity of the injected gene in preimplantation embryos. We consider this concentration, which is definitely lower than that used by other investigators [18–20], to approximate the actual physiological context of zygotic gene activation.

None of the 2-cell embryos expressed the exogenous gene product before 24 hpi except for those

Fig. 4. Effects of supplementation with 25 μ M ZnCl₂ on expression of ML in preimplantation mouse embryos. Embryos were microinjected with ML (5 ng/ μ l) at the pronuclear stage and cultured in Whitten's medium with (A) or without (B) EDTA. Results from embryos cultured in medium with and without ZnCl₂ are depicted as slanted and open bars, respectively.

injected with the higher concentrations of ML mentioned above. Initially, *lacZ* gene product was detected at 27 hpi by X-gal staining, and the frequency of positively stained embryos increased after that. After the 4-cell stage the frequencies decreased, but about one-fifth of the blastocysts still showed *lacZ* staining. Stevens *et al.* [21] examined expression of mouse MT-I-*E. coli* β -galactosidase fusion gene (plasmid pMT β gal and its insert) in mouse preimplantation embryos after pronuclear microinjection at a concentration of 1.67 ng/ μ l. In their experiments *lacZ* expression was detected in few or none of the embryos in the absence of heavy metal. In the present study, more than half of the embryos showed positive staining without ZnCl₂ supplementation even in the presence of EDTA. To clarify the causes of this discrepancy, we tried using the same DNA solvent (10 mM Tris-1mM EDTA) used by them, but 66.7% of microinjected embryos at the 4-cell stage (48 hpi) still showed positive staining (data not shown). Therefore the difference between our results and those of Stevens *et al.* is probably not due to the composition of the DNA solvents employed. In their report, the length of the MT-I promoter is not clear, and their constructs can be expected to produce a fusion protein consisting of *tufB*, *E. coli* peptide elongation factor, and the *lacZ* gene; our ML construct contains a 1.8-kb *EcoRI*-*PstI* fragment and is designed to express a fusion protein consisting of tryptophanyl tRNA synthetase

and *lacZ* [16]. Differences in the efficiency of transcription and/or translation of each construct, together with subtle changes in the structure of the fusion proteins produced, may provoke the discrepancy in these results. Differences in mouse strains and/or culture media could be also ascribed to the differences in the expression.

β -Galactosidase activity was detected initially at 27 hpi in 33% of 2-cell embryos, and the frequency of detection increased thereafter. The addition of α -amanitin at 12 hpi suppressed the expression of the injected gene in a dose-dependent manner, but complete inhibition did not appear if the embryos were treated with the drug only after 15 hpi. Flach *et al.* [22] reported that activation of the embryonic genome occurs both 18–21 hpi and 26–29 hpi, on the basis of the effect of α -amanitin on patterns of protein synthesis. Recent studies suggest that transcription may be already initiated prior to the 2-cell stage in the mouse embryo. Ram and Schultz *et al.* [20] showed that SV40 early promoter-driven luciferase gene was expressed in G2 phase of 1-cell mouse embryos, and Wiekowski *et al.* [23] reported that the male pronucleus may foster a higher level of transcription than the female pronucleus. Bouniol *et al.* [24] reported endogenous transcription by RNA polymerase II takes place at late 1-cell stage (27 h after hCG, estimated at 15 hpi) using a fluorescent method allowing the detection of *in vivo* RNA synthesis. Our results suggest that ML was transcribed at same time mentioned above, and the

transcription of ML may reflect that activation of embryonic genome occurs during the pronuclear stage.

The activity of β -galactosidase, however, became apparent in our experiments only at 27 hpi. The interval of 12 h between the estimated time of transcriptional initiation (~15 hpi) and the detection of gene product (~27 hpi) may suggest that the ML transcript was under some control of translation, or it may reflect the time needed for accumulation of translated product in an amount sufficient for detection by the X-gal staining method. Vernet *et al.* [25] observed that *lacZ* RNA, but not *lacZ* DNA, microinjected during the first cleavage was expressed at 18–19 hpi and suggested that there may be a negative control for translation before 18–19 hpi. We detected the product of ML in nearly the same period as the endogenous genes expressed in early stage embryos reported by Andrews *et al.* [26].

EDTA exerted a slight but significant inhibition on expression of the ML gene. Inhibition was not obvious at the 4-cell stage, but became apparent at the morula stage, 72 hpi. This results implies that the expression of ML in late 2-cell and 4-cell stage embryos is independent of environmental factors, while the expression at later stages is not. Contaminating Zn^{2+} in our culture medium was estimated to be ~0.6 μM by atomic-absorption analysis (data not shown); addition of 25 μM of $ZnCl_2$, a concentration sufficient to induce the expression of genes from MT-I promoter [27, 28], did not suppress development of the embryos used in this

study (data not shown). As EDTA did not reduce the expression in the presence of 25 μM $ZnCl_2$, it is likely that EDTA suppressed the MT-I promoter by chelating the minute amount of contaminating Zn^{2+} or unidentified heavy metals.

Addition of $ZnCl_2$ did not induce the expression of ML in 2-cell embryos before 24 hpi, but enhancement of expression was observed in 4-cell and later stages. Brinster *et al.* [27] showed that cadmium enhanced expression of HSV-tk driven by mouse MT-I promoter in late 2-cell embryos. Ao *et al.* [28] showed that expression of pMT-HPRT was induced by 50 mM cadmium in late 2-cell embryos. Muller *et al.* [29] reported that expression of Hsp70 was induced by heat treatment in blastocysts but not in 1-cell embryos, and Bevilacqua *et al.* [30] showed that hsp68-*lacZ* gene became heart-inducible in late morulae. Andrews *et al.* [26] observed that expression of endogenous MT-I was induced by heavy metals (Zn and Cd) in 4-cell embryos, but not in the 2-cell stage. From these features, together with our present results, it is suggested that preimplantation mouse embryos develop the capacity to respond to environmental conditions after activation of the embryonic genome.

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