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Zygotic Expression of *c-myc* Gene in Mouse Early Embryos: Functional Role of *c-myc* Promoter

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The *c-myc* proto-oncogene is involved in the control of normal cell proliferation and differentiation of many cell lineages. Although it has been suggested that *c-myc* may play an important role in the mammalian early development, it is unclear whether the embryonic *c-myc* mRNA is originated from zygotic gene expression or stored maternal message. Thus, we have constructed expression vectors, in which the 5′ flanking sequences including *c-myc* promoter region and a large non-coding exon I are fused with *E. coli lacZ* gene that encodes β-galactosidase as a reporter. As *c-myc* exon I contains a modulatory sequence, we designed two types of vectors (pcmyc-Gal1 and pcmyc-Gal2) to examine the role of exon I in *c-myc* expression. The former contains the complete exon I and the later has a deletion in 40 bp of modulator sequence located in the exon I of *c-myc*. These vectors were microinjected into fertilized one-cell embryos and β-galactosidase activity was examined by X-gal staining during early embryogenesis. β-galactosidase activity derived from *c-myc* promoter was decreased at two-cell stage. The expression level directed by pcmyc-Gal2 was similar to that of pcmyc-Gal1, indicating that the modulatory sequence in exon I may not be involved at least in the regulation of embryonic *c-myc* expression. In summary, the present study indicates that the *c-myc* promoter is functional at the early stage embryo, and the regulation of *c-myc* expression is under the control of "zygotic" clock of preimplantation mouse embryos.

KEY WORDS: *c-myc* Proto-Oncogene, Microinjection, Mouse, Embryos

The product of proto-oncogene myc family functions in the control of normal cell proliferation, differentiation and onset of neoplastic disease. Among the myc family, *c-myc* is the earliest discovered one and is known as one of the most prominent nuclear oncogenes (for reviews, see Cole, 1986; Lüscher and Eisenman, 1990). The c-Myc protein represents the paradigm for two board classes of transcription factor that contains either the helix-loop-helix (HLH) (Murre *et al.*, 1989) or the basic/leucine repeat structure (LH) (Landschulz *et al.*, 1988). The sequence-specific DNA binding activity of *c-myc* is executed by dimerization with another protein called Max (Blackwell *et al.*, 1990; Blackwood and Eisenman, 1991; Prendergast *et al.*, 1992). The expression of *c-myc* seems to confer the proliferation competence on cells and

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switch off during terminal differentiation in a variety of cell lines (Kelly *et al.*, 1983; Dani *et al.*, 1985). The biological role of *c-myc* on normal development is likely to induce the re-entry into cell cycle from a quiescent stage (Hann *et al.*, 1985; Thompson *et al.*, 1985). The microinjection of *c-myc* protein or transfection of *c-myc* gene into cultured cells induced cell division (Kingston *et al.*, 1984; Kaczmarek *et al.*, 1985). Recently, mRNA depletion of mouse two-cell embryos with antisense *c-myc* oligomers resulted in arrest of embryonic development at the eight-cell/morula stage (Paria *et al.*, 1992). Thus, it appears that *c-myc* may play a functional role in the cleavage and/or differentiation of mammalian early embryos.

In view of *c-myc* function involving the control of cell cycle, many investigators have examined the expression of *c-myc* gene in embryos during normal development by *in situ* hybridization (Schmid *et al.*, 1988; Yamada *et al.*, 1992), immunohistochemistry (Paria *et al.*, 1992), Northern blot hybridization (Zimmerman *et al.*, 1986), and reverse transcription coupled to polymerase chain reaction (RT-PCR) (Pal *et al.*, 1993). Recently, we reported that mRNA transcripts of *c-myc* were detectable at a whole stage of preimplantation mouse embryos by RT-PCR, and the promoter of *c-myc* was at least functional at blastocyst stage when the exogenous *c-myc* promoter fused with *E. coli lacZ* gene was microinjected into fertilized one-cell embryos. (Jeong *et al.*, 1995). But there still remains to elucidate the source of *c-myc* transcripts, which are found from two-cell to morular stage embryos. To resolve this question, we performed an experiment using a microinjection of *c-myc* promoter-*lacZ* fusion vectors into mouse early embryos. In our previous work, the discrepancy between endogenous expression of *c-myc* and exogenous expression of pcmyc-*lacZ* fusion gene may be due to the disintegration of pcmyc-*lacZ* fragment (Jeong *et al.*, 1995). To overcome this problem, we employed a transient expression system rather than a stable integration system in the present study.

Materials and Methods

Preparation of one-cell embryos

Fertilized one-cell embryos were obtained from mature CBA × C57BL/6 F1 mice (Laboratory Animal Breeding Center, Seoul National University, Seoul) after superovulation and mating with the same strain of males (Hogan *et al.*, 1986). Fertilized one-cell embryos were then collected from the oviducts of plugged mice and treated with 100 unit/ml of hyaluronidase (Sigma) to remove adherant cumulus cells. For microinjection, embryos were centrifuged at 10,000×g for 3 min to visualize the pronucleus more easily and incubated in M16 medium containing 0.1 mM EDTA until microinjection.

Construction of *c-myc* promoter-*lacZ* fusion vectors

All cloning of *c-myc* promoter-*lacZ* fusion vectors were carried out by standard method (Sambrook *et al.*, 1989). The mouse *c-myc* genomic DNA including 5′ flanking sequence, exon I, and a part of intron I (Yang *et al.*, 1986, a kindly gift from Dr. K.S. Rhee, University of Texas, Galveston, USA), which is originated from Balb/c mouse strain, was cloned into *XbaI* site of pUC19 vector. From this vector, pcMYC2, about 1.8 kb fragment containing the 5′ flanking sequence with a first exon was isolated by cutting with *BgeII* and inserted into *BamHI* site of pTK2.5Gal, which consists of *tk* promoter, *E. coli lacZ* and SV 40 poly (A) additional sequence. In pTK2.5Gal vector, *tk* promoter was removed by a partial digestion with *BamHI*. The resulted vector was named pcmyc-GalI (Fig. 1). For construction of pcmyc-Gal2, pcMYC2 was digested with *XhoI* and *SalI* to isolated a 2.0 kb fragment, which contains a 5′ flanking region (2.0 kb in length) and a part of the first exon of *c-myc*. The fragment was then ligated to a *SalI* fragment of pTK2.5Gal and maned pcmyc-Gal2

For microinjection, plasmids were purified by a CsCl gradient ultracentrifugation. DNA was dissolved at a concentration of 2 mg/ml in injection buffer (10 mM Tris-HCl, pH 7.4, 0.25 mM EDTA) (Brinster *et al.*, 1985) and diluted at appropriate concentrations before microinjection.

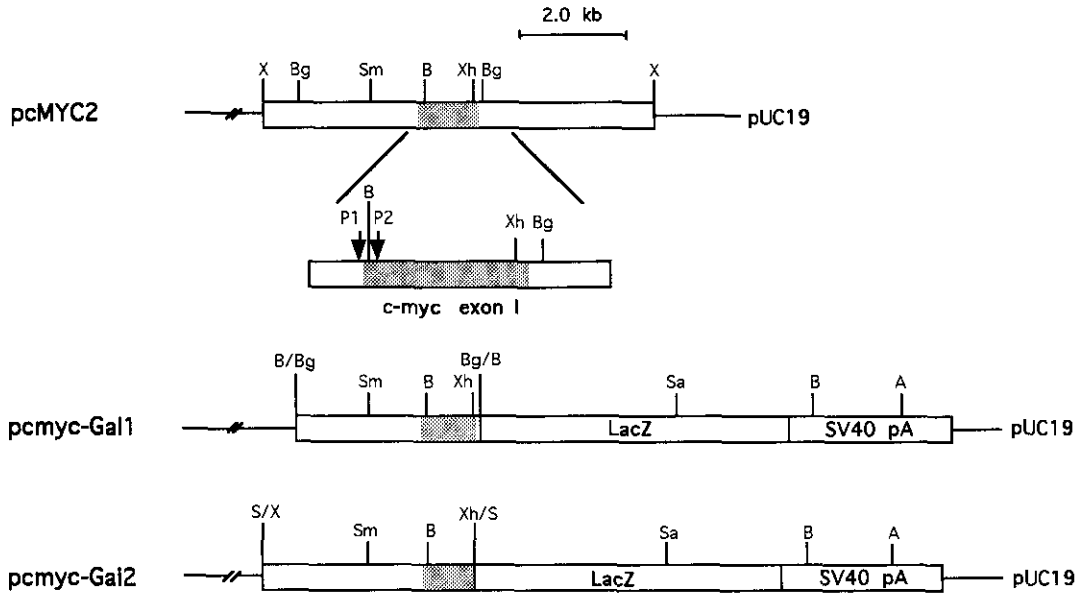

Fig. 1. Map of pcmyc-Gal1 and pcmyc-Gal2. Details for construction see Materials and Methods. Dotted box indicates the first exon of *c-myc*. P1 and P2 mean the position of two promoters of *c-myc* gene. A: ApaI, B: BamHI, Bg: BglII, S: SalI, Sa: SacI, Sm: SmaI, X: XbaI, Xh: XhoI.

Microinjection

Microinjection of *c-myc-lacZ* fusion gene constructs into fertilized one-cell embryos was carried out as described previously (Hogan *et al.*, 1986). Briefly, 15-20 embryos were transferred to a depression slide in M2 medium overlaid with mineral oil, and held firmly in place with holding pipet. The injection pipet filled with DNA solution was inserted into the male pronucleus by using a micromanipulator (Leitz) equipped with a microscope. About 1-2 pl of DNA solution was injected into the male pronucleus of each embryo. The injected embryos were cultured in M16 medium containing 0.1 M EDTA under oil at 37° C in a humidified atmosphere of 5% CO_2 in air.

β-Galactosidase activity in pcmyc-Gal injected-embryos

Expression of *lacZ* gene in embryos was determined by histochemical staining with 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal). The microinjected one-cell embryos were cultured *in vitro* and collected at appropriate stages. The detection of β-galactosidase activity was performed as described previously (Lee *et al.*, 1994). The embryos were thorologhly rinsed with phosphate buffered-saline (PBS), fixed with 0.25% glutaraldehyde in PBS for 10 min and incubated in X-gal staining buffer (0.4 mg/ml X-gal, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6.3H_2O$, 4 mM $MgCl_2$ in PBS) for overnight at 37°C.

Results and Discussion

Mammalian *c-myc* gene contains three exons, the first of which is a large non-coding sequence (Stanton *et al.*, 1983). The *c-myc* mRNA synthesis is normally initiated from two promoters (P1 and P2) (Battey *et al.*, 1983), which are positively regulated by the 5′ flanking sequences and the modulator sequences located in the first exon of *c-myc*. The major promoter, P2 requires a 3′ terminal 60 bp of the first exon of *c-myc* for efficient transcription. But P1, a 5′ *c-myc* promoter is not activated by the first exon. A 450 bp segment of the first exon of *c-myc* has, however, an enhancer activity similar to that of SV 40 (Yang *et al.*, 1986; Moberg *et al.*, 1992). Because differential enhancer activity is directed

either by the 5′ flanking sequence or by the first exon of *c-myc*, we have constructed two types of vectors to examine zygotic expression of *c-myc* gene. As shown in Fig. 1, the pcmyc-Gal1 vector contains a DNA fragment including the 5′ flanking sequence (1.8 kb in length), complete exon I and a part of the first intron of *c-myc*, which contains a positive modulator sequence in the exon I. The pcmyc-Gal2 vector has a 2.0 kb of the 5′ flanking sequence and a segment of exon I, which is deleted a 40 bp of modulator sequence located in the exon I of *c-myc*.

To examine the functional role of *c-myc* promoter, we microinjected the pcmyc-Gal1 into the male pronuclei of fertilized one-cell embryos at relatively high concentration (1-2 pl injection of 50-200 μg/ml concentration of DNA). The microinjected one-cell embryos were cultured *in vitro* for about 30 hr and then stained with X-gal to detect β-galactosidase activity. The result is summarized in Fig. 2. More than half of injected one-cell embryos were developed to two-cell embryos, although the rate of development is rather decreased. The β-galactosidase activity was detectable when a high concentration of DNA (100 μg/ml) was injected. About 30% of one-cell embryos, but not two-cell embryos showed β-galactosidase activity. As shown in Fig. 2, the total number of β-galactosidase positive embryos was increased as microinjected DNA concentration increased. When DNA was injected at a concentration of 200 μg/ml, about 48% of one-cell embryos (12/26) and 9% of two-cell embryos (3/31) were positive to X-gal staining. Neither one-cell nor two-cell embryos were stained to X-gal in the control group (only buffer without DNA). The representative staining pattern of these embryos is shown in Fig. 5. These results clearly indicate that exogenous *c-myc* promoter is functional in mouse one- and two-cell embryos.

On the basis of data in Fig. 2, the concentration of injected DNA is fixed to 200 μg/ml, and then the effect of culture time on β-galctosidase activity was examined. After injection of pcmyc-Gal1, embryos were cultured for 22, 24, and 30 hr, respectively (these times correspond to 40, 44, and 48 hr post hCG injection). The number of embryos showing β-galactosidase activity increased as a function of culture time (Fig. 3). At any time points, the overall rate of X-gal staing in one-cell stage embryos was higher than that in two-cell stage embryos, indicating that exogenous *c-myc* promoter appears to be functional in one-cell stage embryos and its activity turns off during the

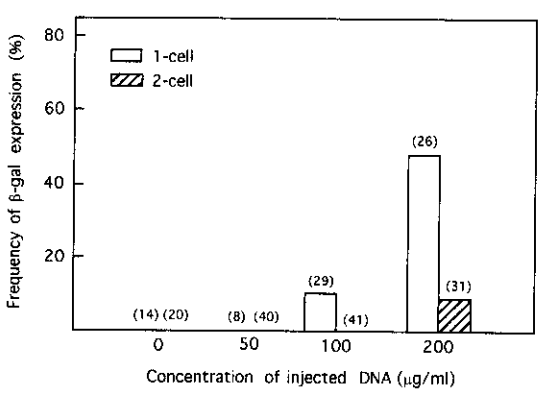

Fig. 2. The frequency of β-galactosidase activity of embryos microinjected with different concentrations of pcmyc-Gal1 vector. After microinjection into the fertilized one-cell embryos at 18 hr post hCG injection, embryos were cultured for 30 hr and then stained with X-gal to detect β-galactosidase. Open and hatched box indicate one-cell and two-cell embryos, respectively. Total number of embryos examined is in parenthesis.

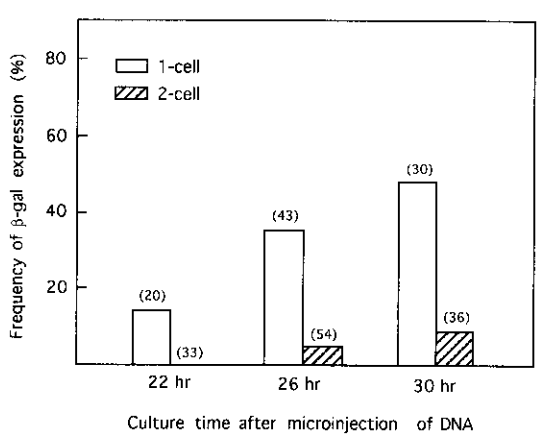

Fig. 3. X-gal staining frequency of embryos microinjected with pcmyc-Gal1 vector at various culture time. After microinjection with 200 μg/ml of DNA, embryos were cultured and stained with X-gal to detect β-galactosidase at 22, 26 and 30 hr, respectively. Open and hatched box indicate one-cell and two-cell embryos, respectively. Total number of embryos examined is in parenthesis.

second cleavage when maternal message may be degraded and/or the embryonic transcription turns on. However, further cultivation of embryos up to 80 hr failed to detect a significant X-gal staining in four-cell and morula stage embryos. Considering that the stability of microinjected DNA as a circular form into a nucleus is stable for up to 3 days (Wirak *et al.*, 1985; Martinez-Salas *et al.*, 1988), the failure in detection of *c-myc* promoter activity in four-cell and morula stage embryos indicates that there is no functional re-activation of *c-myc* promoter at these stages of embryo development. Microinjection of another *c-myc-lacZ* fusion vector, pcmyc-Gal2 into fertilized one-cell embryos resulted in a similar pattern of X-gal staining (Fig. 4). It appears that the regulation of *c-myc* gene expression in mouse embryos may be unaffected by modulator sequences present in exon I. It seems likely then that *c-myc* transcription is under the control of P1 promoter during early embryo development as previously suggested (Yang *et al.*, 1986).

From the above microinjection experiments using two expression vectors, pcmyc-Gal1 and pcmyc-Gal2, it is clear that mouse one-cell embryos have a transcription machinery enough for activation for *c-myc* gene expression. Recently, Worrad *et al.* (1994) observed that the pronucleus concentration of two transcription factors, Sp1 and TATA box-binding protein (TBP) are increased after fertilization of mouse eggs. It suggests that mouse one-cell embryos are transcriptionally active. Although, we failed to detect β-galactosidase activity directed by *c-myc* promoter in mouse early embryonic stages except blastocyst in our previous work (Jeong *et al.*, 1995), *c-myc* transcripts present in all stages of early mouse embryos as judged by RT-PCR (Jeong *et al.*, 1995) and by *in situ* hybridization (Pal *et al.*, 1993). The discrepancy may be due to the disintegration of a low concentration of linearized DNA injected into pronuclei of one-cell embryos, which may not be sufficient for enough promoter activity. However, the microinjection of high concentration (200 μg/ml) of circular DNA is successful in the induction of β-galactosidase expression directed by *c-myc* promoter in early embryonic stages. In fact, Wiekowski *et al.* (1991) successfully detected a luciferase activity directed by thymidine kinase promoter in one-cell embryos after microinjection of circular DNA at a concentration of 200 μg/ml into a male pronucleus. The possibility of artificial activation due to a high concentration of injected DNA can be excluded in our experiments, since only *lacZ* structual gene without *c-myc* promoter (200 μg/ml) failed to induce β-galactosidase activity (data not shown).

However, it still remains to be resolved why β-galactosidase activity is not detected in eight-cell and morular stage embryos in the present

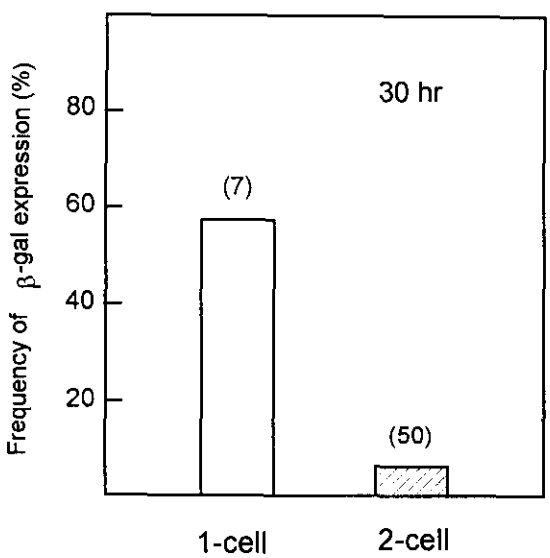

Fig. 4. X-gal staining frequency of embryos microinjected with pcmyc-Gal2 vector (200 μg/ml of DNA) at 30 hr after microinjection. Open and hatched box indicate one-cell and two-cell embryos, respectively. Total number of embryos examined is in parenthesis.

Fig. 5. Photographs of embryos stained with X-gal. The activity of β-galactosidase inexpressed as blue color. A: One-cell embryos B: Four-cell embryos.

experiment. It may be due to an extremely low activity of *c-myc* promoter in early mouse embryos. Indeed, the level of *c-myc* mRNA is decreased from two-cell to morular stage, and is barely detectable at the morula stage as determined by RT-PCR (Pal *et al.*, 1993; Jeong *et al.*, 1995). Since mRNA depletion by treatment with *c-myc* antisense oligomers to two-cell stage embryos arrested normal development at morular stage (Paria *et al.*, 1992), *c-myc* expression is involved in normal development of early mouse embryos and its mRNA may be synthesized as early as morula stage. However, it cannot be ruled out that mRNAs synthesized in one-cell embryos, as shown in the present study, may be used in the later stages of development without complete degradation during re-organization of gene expression at the two-cell stage.

In conclusion, *c-myc* gene is transcriptionally active in early mouse embryos and its expression is under the control of "zygotic clock" during early embryo development. To elucidate the functional role of c-Myc protein in the preimplantation embryogenesis, further studies are necessary to examine another transcription factor, Max, which forms a heterodimer with c-Myc to serve in DNA binding (Blackwell *et al.*, 1990; Blackwook and Eisenman, 1991; Prendergast *et al.*, 1992). This issue is currently under investigation.

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생쥐 초기배아에서 *c-myc* Proto-Oncogene Promoter의 기능적 활성화

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c-myc proto-onocgene은 여러 세포들의 분화와 형질전화에 뿐만 아니라 정상세포의 분열조절에도 관여한다고 알려져왔다. 특히 생쥐의 초기배아에서 *c-myc* mRNA가 발현되고 antisense *c-myc* oligomer의 미세주입에 의해 배발생이 억제된다는 연구결과는 *c-myc*이 초기배아의 발생 및 분열에 관여하는 것을 시사한다. 그러나 최근까지 초기배아에 존재하는 *c-myc* promoter의 기능적 활성화에 관한 연구는 미진하였다. 이를 위하여, *c-myc* promoter와 대장균의 *lacZ* 유전자를 결합시킨 두 종류의 vector(pcmyc-Gal1, pcmyc-Gal2)를 만들어 수정란의 전핵에 미세주입한 후, 배 발생에 따른 *c-myc* promoter의 활성화를 *lacZ* 유전자의 산물인 β-galactosidase에 의한 X-gal 염색으로 조사하였다. 미세주입된 초기 배아는 2세포기 배아를 포함하는 여러 발생단계에서 β-galactosidase의 활성을 보였다. 이는 *c-myc* 유전자가 배아의 게놈유전자로부터 발현되며, 또한 궁극적으로 초기 배아의 발생과정에 중요한 역할을 하고 있음을 시사하고 있다.