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Expression of *c-myc* Proto-oncogene in Preimplantation Mouse Embryos

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The *c-myc* proto-oncogene, one of the immediately early genes, is expressed in various mammalian cell types and heavily involved in the regulation of cell proliferation and differentiation. To determine endogeneous expression pattern of *c-myc* gene in preimplantation mouse embryos, we employed a reverse transcription coupled to polymerase chain reaction (RT-PCR). Transcript of *c-myc* was detected at fertilized embryos as a maternal transcript. At the early two-cell stage, transcript of *c-myc* gene was hardly detected, but appeared at late two-cell embryos as a zygotic transcript. The level of *c-myc* expresion was increased at later stages and peaked at blastocyst stage. To examine the functional role of promoter region for *c-myc* gene transcription, we fused the 5' upstream region (1.8 kb) including exon I of *c-myc* genomic DNA with *E. coli lacZ* gene (named as pcMYC-lacZ). pcMYC-lacZ was microinjected into the pronucleus of mouse one-cell embryoys, and β-galactosidase activity was determined by histochemical staining with X-gal at different stages. β-galactosidase activity was detected only at blastocyst, but not at the earlier stage embryos. This result indicates that *c-myc* gene is transcriptionally active during mouse preimplantation development.

KEY WORDS: *c-myc*, Gene Expression, Microinjection, RT-PCR, Mouse Embryo

Molecular control of mammalian preimplantation embryogenesis remains largely unexplored, mainly due to the difficulty of obtaining sufficient quantities of timed embryos for experimentation. Nonetheless, knowledge about the changes in gene expression during this period is essential to understanding the mammalian development. Several lines of evidence clearly point to the early activation of the embryonic genome (for review, see Telford, 1990). The inhibition of transcription at one-cell stage blocks subsequent development after the first cleavage, implicating that turn-on the zygotic gene occurs at the two-cell stage. In an effort to identify genes expressing at the early mammalian developmental stage, several investigators have applied the polymerase chain reaction (PCR) technique, *in situ* hybridization and immunohistochemistry. Thus, by combining these tools, it is likely to circumvent the problem of obtaining sufficient embryonic materials for study (Rappolee *et al.*, 1988). And microinjection technique introducing the exogenous DNA into fertilized one-cell embryo is also a powerful tool for studying the molecular mechanism of gene expression in developing mammalian embryos (Palmiter and Brinster, 1985).

The *c-myc* gene is one of the cellular homolog of viral v-*myc* oncogene and is a member of a gene family which includes L-*myc* and N-*myc*. *c-*

myc is a nuclear DNA-binding phosphoprotein that is highly conserved among diverse chordate throughout evolution (for review, see Cole, 1986). The *c-myc* protein binds to DNA and functions as a transcription factor. 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* positively correlates with DNA synthesis and cell proliferation (Adams *et al.*, 1985) and is rapidly induced in various cell types after stimulation with mitogen (Kelly *et al.*, 1983). The biological function of *c-myc* is likely to induce the cell to reenter into cell cycle from a quiescent stage. The microinjection of *c-myc* protein or the transfection of *c-myc* gene into the cultured cells induced cell division (Kingston *et al.*, 1984; Kaczmarek *et al.*, 1985).

As mammalian development is a consequence of proliferation and differentiation, it has been hypothesized that *c-myc* is involved in the regulation of normal development. Indeed, many investigators have examined the expression pattern of *c-myc* gene at various stages of embryos 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 (RT) coupled to PCR (Pal *et al.*, 1993). Recently, it has been shown that application of antisense *c-myc* oligonucleotides on two-cell embryos arrested the development at the eight/morula stage (Paria *et al.*, 1992), indicating a key role of *c-myc* in the regulation of preimplantation mouse embryo. However, it still remains to be resolved whether the promoter of *c-myc* functions in preimplantation mouse embryo. In this paper, we therefore examined whether *c-myc* gene is transcriptionally active during preimplantation mouse embryonic development. To do this, we have used RT-PCR protocol to detect *c-myc* transcript and microinjected pcMYC-lacZ consisting of the 5′ region of *c-myc* gene fused with *lacZ* gene into fertilized one-cell embryo.

Materials and Methods

Preparation of embryos

Fertilized one-cell, two-cell, morula, and blastocyst embryos were collected from mature CBAxC57BL/6 F1 mice (Laboratory Animal Research Center, Seoul National University, Seoul) after superovulation and mating to the same strain of males (Hogan *et al.*, 1986). Fertilized one-cell embryos were collected from the oviduct of plugged mice and treated with 100 unit/ml of hyaluronidase (Sigma) to remove cumulus cells. For microinjection, eggs 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. For isolation of total RNA, the embryos were treated with 0.5% pronase (Calbiochem), thoroughly washed and stored at liquid N_2 tank.

Total RNA isolation from embryos

The stored embryos were thawed in a 400 μl of denaturing solution consisting of 4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5 % sarcosyl and 0.1 M 2-mercaptoethanol, and then vortexed for 3 min followed by addition of 500 ng yeast tRNA, 0.1 vol of 2 M Na-acetate (pH 4.0), 1 vol of phenol and 0.2 vol of chloroform. After centrifugation at 12,000 ×g for 20 min, aqueous phase was collected and RNA was precipitated with 2.5 vol of cold ethanol. The pellet was washed with 70% ethanol, dried in air and dissolved in 10 μl of DEPC-treated water as described previously (Pal *et al.*, 1993).

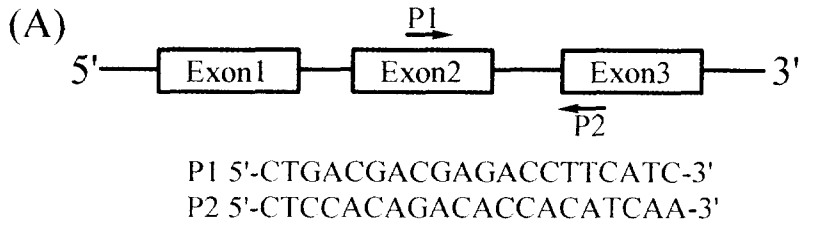
Reverse transcription and polymerase chain reaction (RT-PCR)

RT-PCR was performed as described previously (Saiki *et al.*, 1988; Rappolee *et al.*, 1988). Briefly, total RNA was incubated with 100 pmole of hexamer and reverse transcribed by adding 20 μl of reaction buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 mM dNTP, 0.5 μl of RNase-inhibitor (1 unit/μl, Boehringer Mannheim Biochemical), and 1 μl of reverse transcriptase (10 units/μl, Promega) for 1 hr at 37°C. After synthesis of the first strand of cDNA, 4 μl of cDNA sample was

used for RCR reaction. Primer set is CTG ACG ACG AGA CCT TCA TC for 5′ primer (P1) and CTC CAC AGA CAC CAC ATC AA for 3′ primer (P2), which correspond to the 965-984 and 1391-1410 nucleotide sequence of murine *c-myc* cDNA respectively (Stanton *et al.*, 1983, Fig. 1a). The PCR reaction was performed for 35 cycles on Hybaid heating block, where each cycle consists of denaturing at 95°C for 5 min, primer annealing at 56°C for 5 min and primer extension at 72°C for 2 min, respectively. Yeast tRNA was used for a negative control of RT-PCR procedure. After PCR amplification, PCR products were subjected to 1.4% agarose gel electrophoresis and a relative value of PCR products were estimated by densitometric scanning. For southern blot hybridization, electrophoresed PCR products were transferred to nitrocellulose membrane by capillary transfer. The blot was hybridized with *c-myc* probe as a standard protocol (Sambrook *et al.*, 1989).

Construction of pcMYC-lacZ

Cloning of the pcMYC-lacZ was carried out by standard method (Sambrook *et al.*, 1989). The mouse *c-myc* genomic DNA originated from Balb/c strain, a kindly gift from Dr. K.S. Rhee (Texas University, Galveston, Texas, USA) includes the 5′ flanking sequence, exon I, and a part of intron I (Yang *et al.*, 1986). From this sequence about 1.8 kb fragment contained the 5′ flanking sequence and the first exon was isolated by cutting with *BglII* and inserted into *BamHI* site of pTK 2.5Gal, which consists of *tk* promoter, *E. coli* lacZ and SV poly(A) additional sequence. From pTK2.



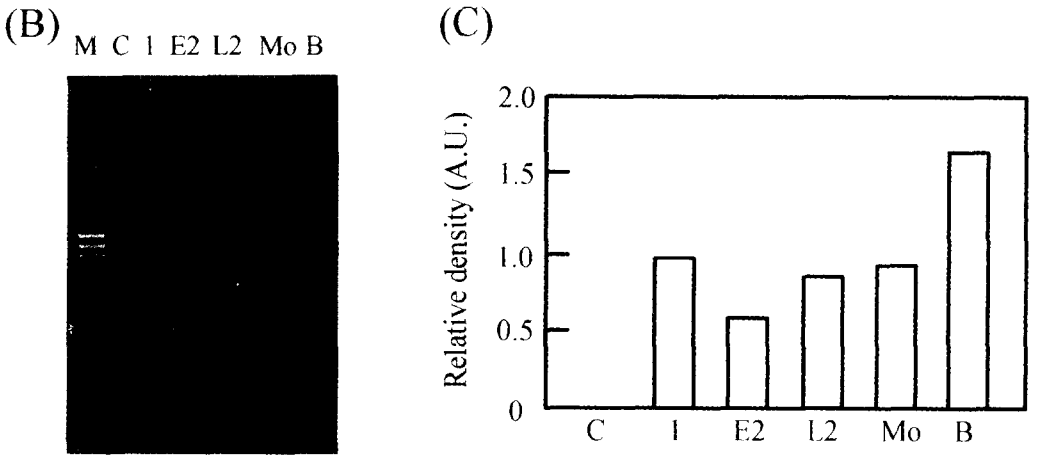


Fig. 1. Amplification of *c-myc* transcript by RT-PCR in preimplantation mouse embryos. Total RNAs isolated from twenty of embryos at appropriate stages were reverse transcribed and then resulting cDNAs were amplified with *c-myc* primers (P1 and P2) for 35 PCR cycles. The PCR products were separated by 1.4% agarose gel electrophoresis. a: Primer sequences. b: Agarose gel electrophoresis of RT-PCR product. c: Relative levels of *c-myc* transcripts. P1: primer 1, P2: primer 2. C: negative control (yeast tRNA only), 1: Fertilized one-cell embryo, E2: Early two-cell embryo, L2: Late two-cell embryo, Mo: Morula stage, B: Blastocyst, M: Molecular weight marker ($\phi\chi$174DNA-*HaeIII* digest).

5Gal, *tk* promoter was removed by a partial digestion with *BamHI*. The resulted vector was named as pcMYC-lacZ (Fig. 2). The plasmid was digested with *SmaI* and *ApaI* to linearize, separated by agarose gel electrophoresis and then binded to DEAE-cellulose membrane (Schleicher & Schuell). The eluted DNA fragment was once extracted with phenol/chloroform and precipitated with ethanol. For microinjection, DNA was dissolved at a concentration of 2 ng/ml in injection buffer (10 mM Tris-HCl, pH 7.4, 0.25 mM EDTA) (Brinster *et al.*, 1985).

Microinjection

Microinjection of pcMYC-lacZ into fertilized one-cell embryo 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 micromanupulator (Leitz) under microscope. About 1-2 pl of DNA solution was injected into the 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-lacZ injected-embryos

Expression of pcMYC-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 the appropriate stages. The embryos were thoroughly 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 $MgCl_4$ in PBS) for overnight in 37°C. The protocol for β-galactosidase activity detection was described previously (Lee *et al.*, 1994).

Results and Discussion

Expression pattern of *c-myc* mRNA levels in preimplantation mouse embryos

To investigate whether *c-myc* gene expression is activated at a particular stage in mouse preimplantation development, endogenous *c-myc* transcripts were detected by RT-PCR. Total RNAs were isolated from 20 embryos at appropriate stages and subjected to reverse transcription to synthesis the first stranded cDNA. *c-myc* mRNA was then amplified by PCR using *c-myc* specific primers (Fig. 1A). The estimated size of PCR product was 445 bp as expected from *c-myc* cDNA sequence (Stanton *et al.*, 1983). As shown in Fig. 1B, *c-myc* gene was amplified to the expected band in all stages of developing embryo. Southern blot hybridization confirmed that the amplified bands were derived from *c-myc* transcripts (data, not shown).

Relative levels of *c-myc* expression in different stages of embryos was measured by densitometric scanning (Fig. 1C). *c-myc* transcript was detected in fertilized one-cell embryos as a maternal

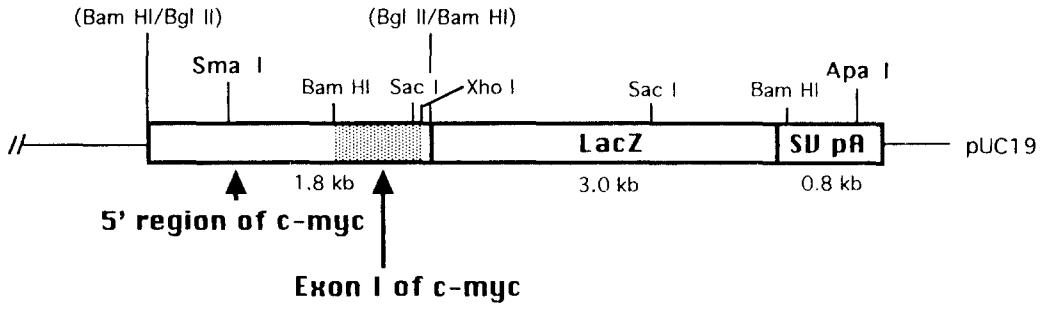


Fig. 2. Map of pcMYC-lacZ. For details, see Materials and Methods.

mRNA. *c-myc* transcript was hardly detected in early two-cell embryo, but reappeared in late two-cell embryos and continued to increase its synthesis when embryonic development proceeded. This result is well consistent to the previous report that *c-myc* expression is initiated from embryonic genome after the first cleavage, and heightened during differentiation of morula to blastocyst (Paria *et al.*, 1992). Because the first embryonic gene expression occurred in the late two-cell embryo stage (Pico and Clegg, 1982; Rothstein, 1992), *c-myc* mRNA detected in the one-cell embryos seems to be originated from oogenesis as a maternal transcript. It is likely that synthesis of *c-myc* mRNA during oogenesis is preparative for the first cleavage before embryonic genome is activated after fertilization. It also appears that *c-myc* is clearly involved in regulation of cell division related to the differentiation and cleavage of embryos.

The promoter of *c-myc* gene is active in preimplantation mouse embryo

Whether *c-myc* transcript detected in early mouse embryos is due to the activation of zygotic genome, we have examined the functionality of the promoter of *c-myc* gene. We have constructed pcMYC-lacZ vector, which consists of about 1.8 kb sequence including 5′ flanking region and the first exon of *c-myc* genomic DNA and *E. coli* lacZ gene as a reporter (Fig. 2). pcMYC-lacZ was microinjected into the pronucleus of one-cell embryo and cultured *in vitro*. Embryos were collected at appropriate stages and stained with X-gal to detect b-galactosidase activity.

Total 379 embryos were microinjected with pcMYC-lacZ. Among them, 263 embryos (69.4 %) were developed to two-cell embryos, whose morphology is normal when compared with control embryos without microinjection. Over half of these two-cell embryos was developed to blastocysts (data, not shown). Note that development of injected embryos was delayed than normal embryos. The delayed development is largely due to the mechanical damage of the embryo, since buffer injection showed similar results (data, not shown). The expression of *lacZ* gene in microinjected embryos is summarized in Table 1. β-galactosidase activity was detected only in blastocyst stage. Twenty one out of 33 embryos developed up to blastocyst stage from one-cell embryo were positively stained into blue color when stained with X-gal. No embryos in other stages except blastocyst did exhibit μ-galactosidase activity. The degree of staining in blastocysts was very intense and the blue color was observed in the whole embryo including inner cell mass and trophectoderm (Fig. 3). Note that one thirds of blastocysts were negatively stained and it may be due to loss of exogenous DNA during development following disintegration of pcMYC-lacZ into their genomic DNA after microinjection as previously suggested (Burdon and Walls, 1992).

It seems likely that pcMYC-lacZ gene expression is initiated at blastocyst stage. However, it cannot be ruled out the possibility that low amounts of lacZ gene expression might occur at earlier stages, which cannot be detected by our X-gal staining method. It would be then better to use the fluorescent-activating device (Nollan *et al.*, 1988) for the measurement of a weak expression of β-galactosidase or in situ hybridization method to detect *lacZ* mRNA.

It is also of importance to note that the activation of exogenous promoter might be different from the onset of endogenous gene expression due to a deficiency of the regulatory elements for initiation of transcription in a exogenous DNA construct. For instances, the promoter of methallotheionein and mouse β-actin gene are active at two-cell stage, while the

Table 1. Expression of *lacZ* gene in microinjected preimplantation mouse embryos.

Stage/dpc*	1.5	2.5	3.5	4.5
1-cell	0/10	0/15	0/3	0/11
2-cell	0/31	0/18	0/5	0/2
4-cell	0/6	0/38	0/13	0/2
morula	-	0/13	0/59	0/9
blastocyst	-	-	-	21/33
Total	0/44(0)#	0/74(0)	0/80(0)	21/57(36.8)

*day post coitum

#Number of embryos expressing *lacZ* gene/number of embryos examined (%)

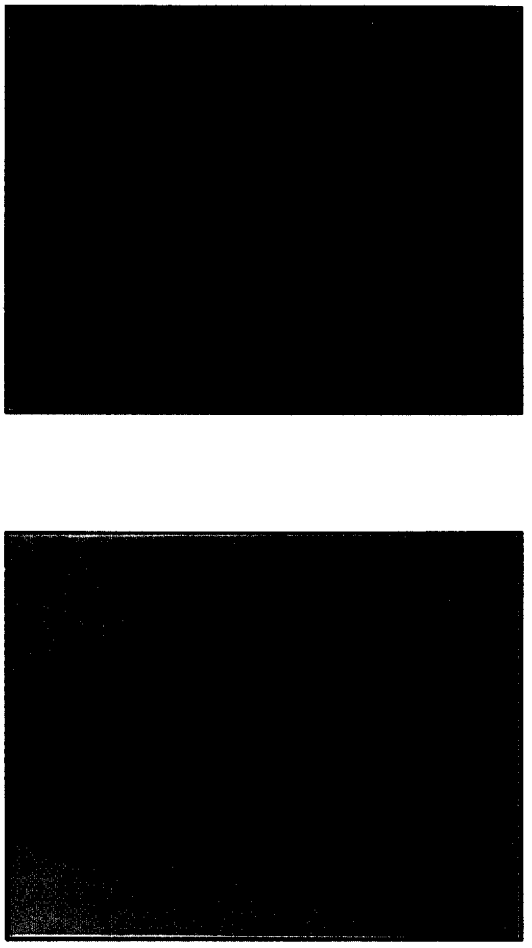


Fig. 3. Expression of β-galactosidase activity in pcMYC-lacZ microinjected blastocysts. pcMYC-lacZ was micriinjected into the pronucleus of one-cell embryo and cultured with M16 medium. After culture, embryos obtained at different stages were stained with X-gal to detect β-galactosidase activity. Only embryos at blastocyst stages were positive to X-gal staining.

promoter of heat shock gene is active at the blastocyst stage when fusion DNA with these promoters are microinjected into mouse one-cell embryos (Stevens *et al.*, 1989; Bonnerot *et al.*, 1991; Nilson *et al.*, 1992).

In spite of the notions discussed above, expression of μ-galactosidase in microinjected embryos suggests that the 5′ flanking region including the first exon of *c-myc* could function in the early embryonic stage. The mammalian *c-myc* genomic DNA consists of three exons, the first of which has a large untranslated region (Stanton *et al.*, 1983). The first exon contains two promoters (P1 and P2) (Yang *et al.*, 1986) and several enhancer elements (Moberg *et al.*, 1992). The first exon segment including the promoter and intron has been known to play a functional role in expression of *c-myc* gene in a variety of cell lines (Yang *et al.*, 1986). Then, the developing mouse embryos may contain putative trans-acting regulatory elements to activate *c-myc* gene. The molecular regulation of *c-myc* transcription in mouse embryos remains, however, to be resolved. In conclusion, our results demonstrate that the transcriptional activation of *c-myc* gene is occured after two-cell stage and the 5′-flanking region including the first exon of *c-myc* is sufficient for the activation of *c-myc* gene at least after blastula stage.

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착상전 생쥐배아에서 *c-myc* 유전자의 발현

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c-myc 유전자는 다양한 세포들에서 발현되며, 세포의 분화와 증식에 중요한 역할을 한다. 착상전 생쥐의 초기배아에서 내재적인 *c-myc* 유전자의 발현을 조사하기 위해 RT-PCR로써 mRNA를 측정하였다. *c-myc* mRNA는 수정란에서는 모계유전자로 존재하였으며, 초기 2세포기 배아에서는 거의 측정되지 않은 반면에 후기 2세포기부터 다시 나타나 발생이 진행됨에 따라 증가하였다. 이러한 *c-myc* mRNA가 배아 게놈으로부터 전사되는가를 알기 위해, *c-myc* 유전자의 promoter의 기능을 조사하였다. *c-myc* 유전자에서 promoter를 포함하는 5′ 부위를 절단하여 *E. coli*의 *lacZ*유전자와 결합시켜, pcMYC-*lacZ*를 만들어 생쥐의 수정란 전핵에 미세주입한 후, 배 발생에 따른 β-galactosidase의 활성을 측정하였다. β-galactosidase활성은 상실기까지는 없었으나, 포배기에서는 뚜렷이 나타났다. 본 연구결과는 착상전 생쥐의 초기배아에서 발현되는 *c-myc*유전자는 배아게놈의 전사적인 활성화에 의한 것임을 시사한다.