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Developmental Stage-Specific Expression Patterns of *c-myc* and *myn* Proto-Oncogenes and a Possible Role of *myn* in Preimplantation Mouse Embryo Development

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The *c-myc* proto-oncogene, one of the immediately early genes, is involved in cellular proliferation and differentiation, and its biological function is regulated by dimerization with a heterodimeric partner, *myn*. In the present study, gene expression patterns of *c-myc* and *myn* during mouse preimplantation embryo development were examined using a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). *Myn* transcripts were rather constitutively expressed throughout embryonic stages with a slight increase only at blastocyst stage. In contrast, expression of *c-myc* transcripts was developmental stage-specific. The *c-myc* transcripts were detected at 1-cell stage, declined abruptly at 2-cell stage and then increased gradually at blastocyst stage. To examine the possible role of *myn* during preimplantation mouse embryo development, two *myn* antisense oligonucleotides spanning the tail of zipper domain (myn2; 20-mer) and the second helix domain (myn3; 20-mer) were microinjected into the fertilized 1-cell embryos. Microinjection of myn2 and myn3 resulted in developmental retardation at morula/blastocyst transition stage, leading to the fragmentation of embryos. Taken together, these results suggest that *c-myc* and its heterodimeric partner, *myn*, are differentially expressed in a developmental stage-dependent manner, and *myn* may play an important role in mouse preimplantation embryo development.

KEY WORDS: *c-myc*, *myn*, Antisense Oligonucleotides, Microinjection, Mouse Embryo Development

The mammalian embryo development is a well-coordinated process of cellular proliferation and differentiation. After several rounds of cleavage, the embryonic cells differentiate into trophectoderm and inner cell mass at the stage of blastocyst, which is the first sign of differentiation. Little is, however, known about the molecular mechanisms underlying proliferation and differentiation during preimplantation embryo development. Several growth factors and proto-oncogenes are evidently involved in this process (Werb, 1990; Rosner *et al.*, 1991; Pal *et al.*, 1993; Wu *et al.*, 1992; Doherty *et al.*, 1994). Among these, the *c-myc* is a nuclear DNA-binding phosphoprotein that acts as a transcription factor activating the downstream genes essential for proliferation and differentiation in a variety of cell lines. The sequence specific DNA binding activity of *c-myc*, a well known proto-oncogene, is executed by dimerization with its heterodimeric

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partner *myn*, a murine homolog of max (Blackwood *et al.*, 1991, 1992; Prendergast *et al.*, 1991). Recently, another novel protein which binds to Myn, Mad was reported (Ayer *et al.*, 1993). The expression of *c-myc* seems to confer proliferation competence to quiescent cells and to switch off during terminal differentiation in leukemic cells (Kelly *et al.*, 1983; Dani *et al.*, 1985). The biological effect of *c-myc* on cellular proliferation and differentiation is likely to induce the re-entry into cell cycle from quiescent stage (Hann *et al.*, 1985; Thompson *et al.*, 1985). In fact, the microinjection of *c-myc* protein or transfection of *c-myc* gene into the cultured cells, induced cell division (Kingston *et al.*, 1984; Kaczmarek *et al.*, 1985).

Recently, it has been reported that *c-myc* may play an important role in the regulation of cleavage and/or differentiation of preimplantation mouse embryos. For intance, treatment of mouse embryos at 1-cell stage with antisense oligonucleotides resulted in the arrest of embryonic development at the eight-cell/morula stage (Paria *et al.*, 1992). Since *c-myc* dimerizes with its heterodimeric partner *myn*, it is of importance to examine the expression patterns of both *c-myc* and *myn* during preimplantation embryo development. In the present strudy, we also tested the functional role of *myn* by a microinjection technique of antisense oligonucleotides.

Materials and Methods

Preimplantation embryo collection and culture *in vitro*

F1 (CBAxC57BL) mice were obtained from the Animal Breeding Center in Seoul National University and maintained under controlled photocycle (light on at 06:00) with water and food supplied *ad libitum*. Six- to eight-week-old female mice were superovulated by an intraperitoneal injection of 5 iu pregnant mare's serum gonadotropin (PMSG; Sigma), followed by 5 iu human chorionic gonadotropin (hCG; Sigma) 48 hr later. Animals were subsequently mated with fertile male mice of the same strain (Hogan *et al.*, 1986). In the following morning, the fertilized 1-cell stage embryos with a prominent male pronucleus were collected by flushing oviducts of plugged females. Adherent cumulus cells were removed by treatment with 0.1% hyaluronidase (300 μg/ml) in culture medium for 10 min, and embryos were cultured after several rounds of washing in fresh medium. Various stages of preimplantation embryos were obtained by cultivating 1-cell stage embryos according to the method described by Brinster (1965) under a paraffin oil at 37°C in a humidified atmosphere of 5% CO_2 in air. Embryo culture medium was modified standard egg culture medium (SECM) containing 0.1 mM EDTA (m-SECM+EDTA) supplemented with 60 μg/ml penicillin G, 50 μg/ml streptomycin, and 4 mg/ml BSA (fraction V, Sigma).

Total RNA isolation from mouse embryos

After cultivation, embryos at various stages of development were collected. Embryos were transferred into microfuge tubes containing solution D (50 μl of solution D per 20 embryos) with 10 μg of carrier yeast tRNAs, and 10 ng of *in vitro* transcribed chloramphenicol acetyltransferase (CAT) mRNA for an external control. Solution D consists of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. Samples were homogenized by repeated freezing and thawing. Homogenates were vortexed in the presence of 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of water-saturated phenol and 0.2 volume of chloroform-isoamyl alcohol mixture (49:1) for 10 min on ice. After centrifugation at 12,000 rpm for 20 min at 4°C, RNAs in aqueous phase were precipitated with 2 volumes of ice-cold ethanol. RNA precipitates were collected by centrifugation at 12,000 rpm for 10 min at 4°C, washed twice with 75% ethanol and dissolved in 9.75 μl of diethyl pyrocarbonate-treated water.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as described previously (Saiki *et al.*, 1988; Rappolee *et al.*, 1988). Briefly, RNAs were incubated with 100 pmol of

random hexamer (Boehringer Mannheim) for 10 min at 70°C followed by cooling on ice for 10 min. RNAs were reverse transcribed in 20 μl of reaction buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM dNTPs, 0.5 U RNase-inhibitor (Promega) and 200 U RNase H-MMLV-reverse transcriptase (Promega) for 1 hr at 37°C. After the reverse transcription, the reaction mixture was heated at 95°C for 10 min and cooled on ice for 10 min. For PCR amplification, 2 μl of RT reaction product was added to PCR master mixture (a final volume of 50 μl) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 pmol of each primer, 0.2 mM dNTP, 3 μCi of α[^{32}P]-dCTP (3,000 Ci/mmol, NEN) and 1.25 unit of AmpliTaq DNA polymerase (Perkin Elmer Cetus). The mixture was overlaid with 50 μl of mineral oil, and cDNA was amplified on Hybaid heating block (Pharmacia LKB), where each cycle included pre-denaturing at 95°C for 5 min, denaturation at 95°C for 1 min, primer annealing at 56°C for 1 min and primer extension at 72°C for 1.5 min, and post-elongation at 72°C for 10 min. After RT-PCR amplification, resulted PCR products were analyzed by an electrophoresis on 5% polyacrylamide gel. Gels were dried and exposed to X-ray film (Fuji). PCR primer sets specific for *c-myc* and *myn* are shown in Fig. 1. Control primers for a specific amplification of β-actin and CAT mRNAs were designed on the basis of published cDNA sequences; 5′-primer, 5′-GATTACT-GCTCTGGCTCCTA-3′ and 3′-primer, 3′-CAGTAACAGTCCGCCTAGAA-3′ for β-actin (Tokunaga *et al.*, 1986), and 5′-primer, 5′-AACCAGACCGTTCAG-CTGGA-3′ and 3′-primer, 5′-GTAACACGCCAC-ATCTTGCG-3′, for CAT, respectively (Alton and Vapnek, 1979). The RT reaction mixture from culture media was used as a negative control for a possible follicular cell contaminants. RNA from blastocyst that had not been reverse transcribed, was also used for a negative control for a possible genomic DNA contaminant during PCR reaction.

Microinjection of antisense oligonucleotides

Antisense and sense oligonucleotides against *myn* transcript were designed as shown in Fig. 4B. Oligonucleotides were obtained from OPERON Inc. Oligonucleotides at the concentration of 100 pmol/μl in TE buffer (pH 7.4) were directly microinjected into the cytoplasm of 1-cell stage embryos. For microinjection, fifteen to twenty eggs were transferred to a depression slide in M2 medium (Hogan *et al.*, 1986) overlaid with mineral oil, and held firmly in place using holding pipette. The microinjection pipette filled with oligonucleotide solutions was inserted into cytoplasmic portion of embryos with Leitz M manipulator and oligonucleotide solution was slowly discharged until the size of difused solution is about two-fold (about 10 pl) the pronucleus. The injected eggs were washed in modified SECM, and incubated in culture dish (35 × 10 mm, Falcon) containing microdrops of m-SECM+EDTA under oil at 37°C in a humidified atmosphere of 5% CO_2 in air. Developing embryos were observed under a phase contrast microscope.

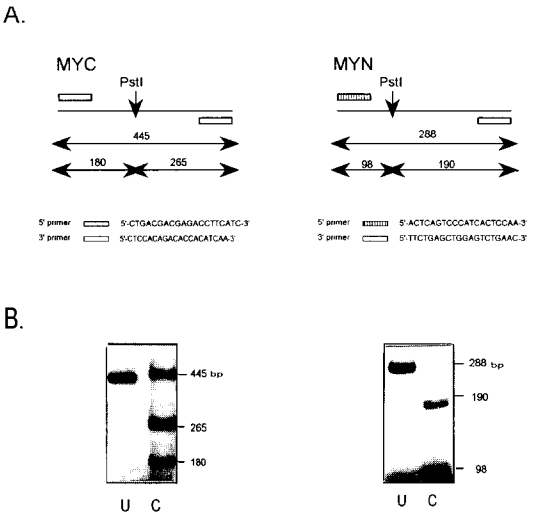

Fig. 1. Primer sequences used for RT-PCR amplication of *c-myc* and myn transcripts (A), and validation of PCR products by restriction enzyme analysis (B). Embryos at blastocyst stage (20 embryos in each group) were collected and total RNA was isolated as described in Materials and Methods. Total RNA was reverse transcribed and resulted cDNAs were subsequently amplified with *myc*- and *myn*-specific primer sets in the presence of [^{32}P] dCTP. RT-PCR products (U) were digested with Pst I (C), and visualized on X-ray film after size fractionation by 5% polyacrylamide gel electrophoresis.

Results and Discussion

Expression patterns of *c-myc* and *myn* transcripts during preimplantation mouse embryo development

The endogenous expression patterns of *c-myc* and *myn* genes during preimplantation mouse embryo development were examined using RT-PCR technique. Total RNAs isolated from various stages of preimplantation embryos were reverse transcribed and subjected to PCR amplification. The estimated size of *c-myc* and *myn* PCR products were 445 bp and 288 bp in size, respectively. To validate the RT-PCR products, diagnostic restriction analysis was performed. Pst I treatment produced two fragments of 265 bp and 180 bp for *c-myc*, and 190 bp and 98 bp for *myn*, as predicted from the mouse cDNA sequences (Fig. 1). After restriction analysis, the intact *c-myc* PCR products were still remained due to suboptimal time condition. The optimal PCR cycle condition was established when the PCR

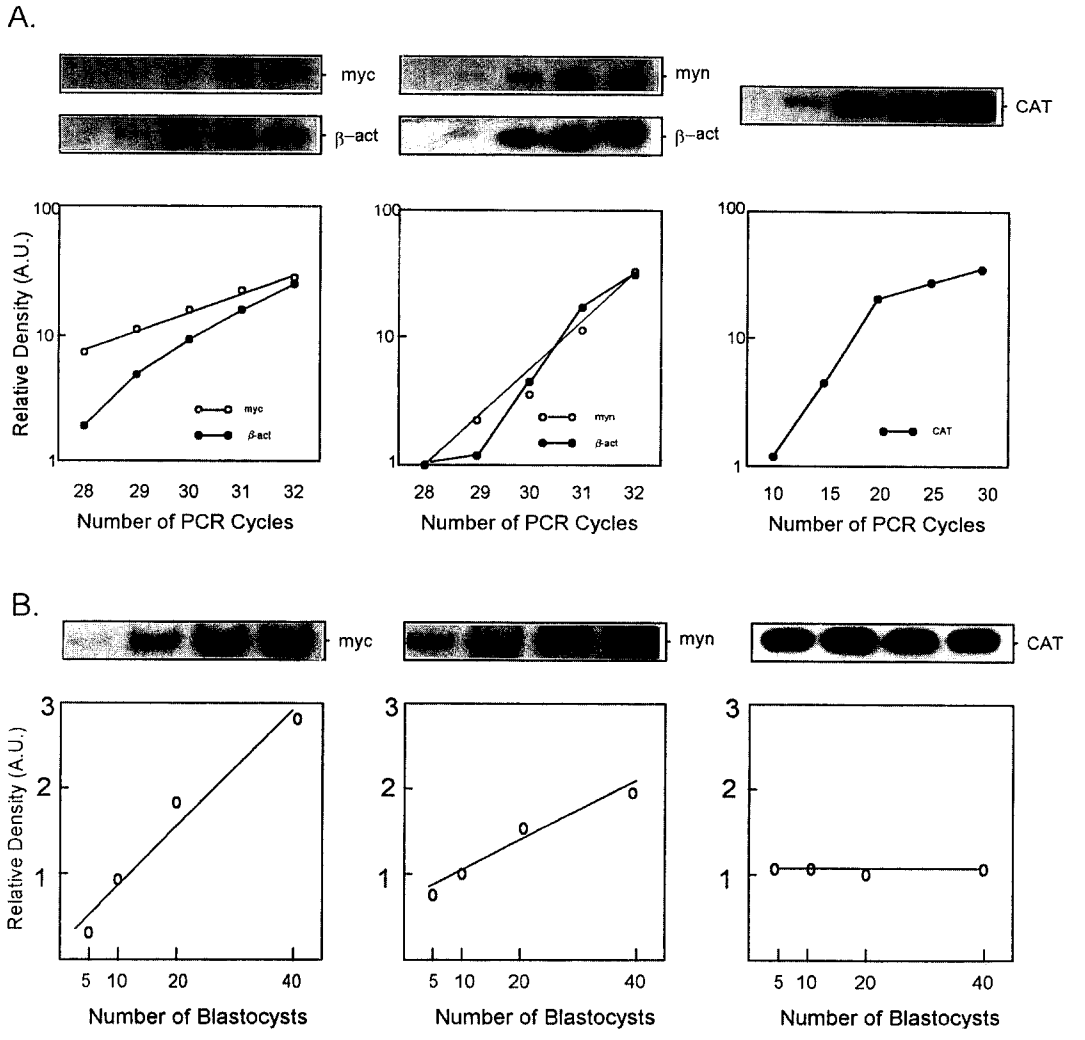

Fig. 2. Optimization of RT-PCR procedure with regard to number of PCR cycles (A), and amount of RNA input (B). Total RNA isolated from blastocysts was reverse transcribed and then subjected to PCR amplification in the presence of [^{32}P] dCTP. RT-PCR products were analyzed by 5% polyacrylamide gel electrophoresis and then quantified by a densitometric scanning of autoradiogram.

products were in the log phase increment (30 cycles for *c-myc*, *myn*, and β-actin; 18 cycles for CAT; Fig. 2A). Amount of RT-PCR products at these PCR cycles showed a good linearity as a function of input RNA (Fig. 2B). These results showed that our RT-PCR procedure was sensitive enough to detect extremely minute amount of mRNAs in embryos and reproducible to examine the changes in mRNA quantities. *c-myc* and *myn* transcripts were detected in all stages of embryo development (Fig. 3A and B). *c-myc* transcripts were found in unfertilized eggs and 1-cell stage embryos, and its level was then declined at 2-cell stage (Fig. 3A). Thereafter, a prominent increment in *c-myc* transcripts was observed in embryos at morula and blastocyst stages. These data are in

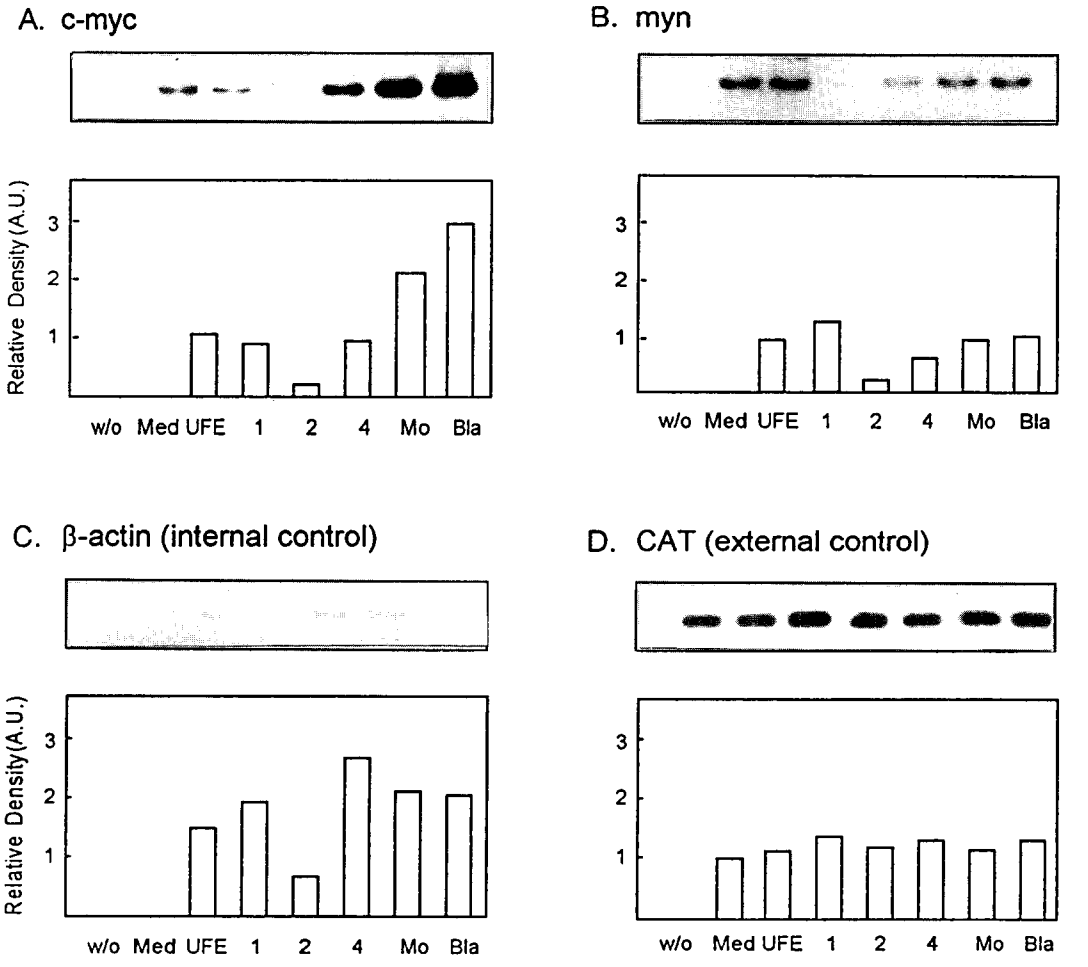

Fig. 3. Expression patterns of *c-myc* (A) and *myn* transcripts (B) during preimplantation mouse embryo development as determined by RT-PCR. RT-PCR was performed with total RNA isolated from 20 embryos at different developmental stages (UFE; unfertilized egg, 1; 1-cell, 2; 2-cell, 4; 4-cell, Mo; morula, and Bla; blastocyst). After 30 cycles of PCR amplification, resulted PCR products were analyzed by 5% polyacrylamide gel electrophoresis. As an internal control, endogenous β-actin mRNA level was examined (C). To verify the possible technical errors during RNA preparation and RT-PCR amplification procedure, *in vitro* transcribed CAT RNA transcripts (10 ng) were externally added into the sample-containing tubes and amplified (18 PCR cycles) in a same tube (D). Relative changes in signal densities on X-ray film were analyzed by densitometric scanning. To verify genomic contamination, RNA without RT step (w/o) was directly subjected to PCR amplification. Washing medium (Med) was also used as a control for cellular contaminant.

agreement with the recent findings (Paria *et al.*, 1992; Pal *et al.*, 1993; Jeong *et al.*, 1995). However, the expression pattern of *myn* transcripts was quite different from that of *c-myc*. *Myn* transcripts were also found in unfertilized eggs and 1-cell stage embryos, and declined to 2-cell stage embryos. However, its level was rather constitutive from 4-cell to blastocyst stage (Fig. 3B). In mice, maternal-to-zygotic transition occurs at the 2-cell stage of development and is characterized by a precipitous decline in maternal mRNA content, and transcriptional activation of the embryonic genome (Kidder, 1993). Expression pattern of β-actin gene, one of the typical structural genes, was similar to the previous result of Taylor and Piko (1990), verifying that the gene expression is under the control of zygotic clock in our experimental condition, and can be shown as a positive internal marker gene expression in regards its own fructuating expression pattern.

Considering the expression patterns of *c-myc* and *myn* during embryo development, it is reasonable to presume that *c-myc* may be a key regulator in Myc:Myn function in developing mouse embryos, as suggested previously (Paria *et al.*, 1992). This idea is, in part, partially supported by the previous report that *c-myc* and *myn* genes were not co-regulated following serum stimulation in Rat-1 cells (Prendergast *et al.*, 1991), and that the basal level of *myn* is sufficient for the transformation of C3H10T1/2 and avian fibroblast cells (Davenport and Taparowsky, 1992; Tikhonenko *et al.*, 1993). In proliferating cells, newly synthesized c-Myc is found to be associated with Myn (Blackwood *et al.*, 1992), suggesting that Myc synthesis is rate-limiting in the formation of Myc:Myn heterodimers. Induction of *c-myc* by mitogenic stimuli rapidly shift the equilibrium from Myn:Myn to Myc:Myn. Conversely, withdrawal of mitogenic stimuli and the rapid decline of Myc leads to a shift of equilibrium toward Myn homodimer, suppressing Myc:Myn function.

Recently, a new binding partner for *myn* (*max*), called *mad*, was reported (Ayer *et al.*, 1993). Human Mad protein binds to Max, forming a sequence-specific DNA binding heterocomplexes which antagonize the function of Myc-Max heterocomplex. Mad protein is rapidly induced when the myeloid cells are differentiated, and the half life of Mad is extremely short ($t_{1/2}$=15-30 min) like that of Myc protein (Ayer and Eisenman, 1993). The Myc-Max-Mad constitute a transcriptional factor network controlling cell cycle progression, cell differentiation and cell death. Through highly regulated synthesis and degradation of Myc and Mad, the heterodimers with Myn(Max) constitutes a very sensitive switch controlling the entry into and exit from cell cycle in response to extracellular stimuli (Amati *et al.*, 1993; Amati and Land, 1994). Myn(Max), as a partner for formation of functional heterodimer molecules, needs to be expressed constitutively for the monitering of two molecules which function oppositely. Without the expression of Myn(Max) protein, the Myc-Max-Mad transcription factor

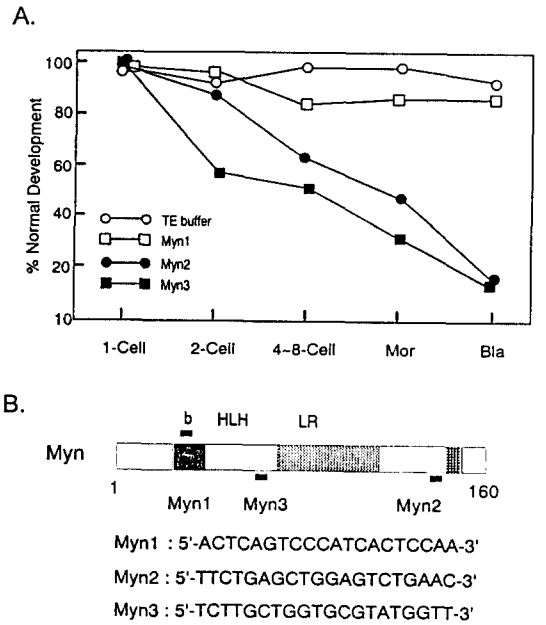

Fig. 4. Effect of *myn* antisense oligonucleotides on preimplantation mouse embryo development. After microinjection of 10 pl of myn2 or myn3 (100 pmol/μl) into the cytoplasm of 1-cell embryos, embryos were cultured *in vitro*, and embryonic development was observed under a phase contrast microscope (A). As a control, myn1 sense oligonucleotides (100 pmol/μl) and TE buffer (injection vehicle) were microinjected. Oligonucleotide sequences used are shown (B). The *myn* antisense oligonucleotides were designed based on the paper reported by Prendergast *et al.* (1991).

network does not work. As a gating molecule, Myn(Max) plays an important role in regulating the down-stream genes.

A possible role of *myn* in preimplantation mouse embryo development

Paria *et al.* (1992) recently showed that addition of oligonucleotides against *c-myc* into culture medium caused a prominent developmental arrest

Table 1. Effects of myn antisense oligonucleotides on preimplantation mouse embryo development

1) myn1 sense

stage \ dpc	0.5	1.5	2.5	3.5	4.5
1	81	1			
2		76	6	4	
4~8			68	2	
Morula			2	69	
Blastocyst					68
D,F†		4	5	5	10
Development (%)‡	81/81(100)	76/81(93.8)	68/81(84.0)	69/80(86.3)	68/78(87.2)

2) myn2 anti-sense

stage \ dpc	0.5	1.5	2.5	3.5	4.5
1	76	8	4	4	4
2		67	17	15	9
4~8			46	10	5
Morula			1	35	2
Blastocyst					9
D,F			2	6	36
Development (%)	76/76(100)	67/75(89.3)	46/70(65.7)	35/70(50.0)	9/65(13.8)

3) myn 3 anti-sense

stage \ dpc	0.5	1.5	2.5	3.5	4.5
1	29	8	7	7	5
2		16	3	3	1
4~8			14	5	
Morula				9	2
Blastocyst					4
D,F		5	4	4	16
Development (%)	29/29(100)	16/29(55.2)	14/28(50.0)	9/28(32.1)	4/28(14.3)

† D, F ; dead, fragmented embryos

‡ Percentage of normal embryonic development

of mouse embryos at the 8-cell/morula transition stage. It is of importance to note that the effect of antisense oligonucleotides added directly into culture media may be hampered by degradation of oligonucleotides and its their poor absorption rate into the cell (Green *et al.*, 1986; Baerschi, 1994). One way to overcome this problem, the structural modification of antisense oligonucleotides was commenly employed, but, this modification often produced toxicity and diminished susceptibility of the [mRNA:oligo-nucleotide] complexes to RNase H cleavage (Toulme, 1992). Thus, the direct microinjection technique seems to be more plausible.

To test the possible role of *myn* in preimplantation mouse embryo development, microinjection of antisense oligonucleotides against *myn* mRNA was performed. Microinjection of myn2, spanning the tail of leucine repeat region (Fig. 4B), significantly inhibited the normal embryo development (13.8% of blastocyst formation). Inhibitory effect of myn3, spanning the second helix region, was also similar to that of myn2 (4.2% of normal blastocyst formation). Notice that microinjection of two antisense oligonucleotides produced remarkable delayed development of embryo, whereas that of TE buffer as a control failed to alter the normal development (90.9%; Fig. 4A). The microinjection of sense oligomer (myn1) did not alter the normal blastocyst formation, indicating that the blockade of normal development was achieved by the specific action of the *myn* antisense oligonucleotides. Transition from morula to blastocyst was most effectively inhibited, and then embryos were fragmented at this stage (Table 1). Fig. 5 represents the embryonic fragmentation by microinjection of the antisense myn2, but not the sense myn1.

Antisense oligomers myn2 and myn3, but not sense oligomer myn1, were equally active in the inhibition of normal development of embryos. Although we did not examine the translational block by antisense microinjection, embryonic fragmentation caused by antisense (myn2 and myn3), but not by sense (myn1) oligonucleotides indicated, in part, the reliability of our antisense microinjection experiment. And in our experiment, about 10^7~10^8 copies of oligonucleotides were injected into the cytoplasm of a single fertilized 1-cell embryo. Therefore it cannot be ruled out the possibility that the excess copies of oligonucleotide might interfere the "zygotic clock" of development. The exhaustion of *myn* transcripts may lead to inhibition of Myc:Myn heterodimer formation.

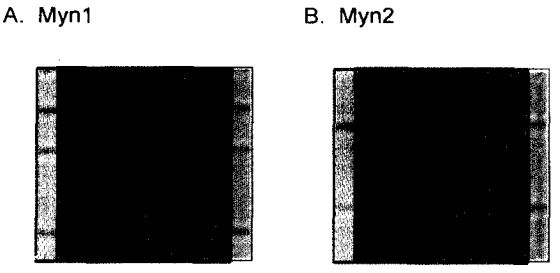

Fig. 5. Microscopic morphology of sense- or antisense-injected embryos 72hr after microinjection. Microinjection of antisense myn2 produced embryonic fragmentation (B), deriving the blockade of blasocyst formation, while that of sense myn1 failed to alter the normal development of blastocyst (A).

In conclusion, *c-myc* and its heterodimeric partner, *myn*, are differentially expressed during preimplantation mouse embryo development. Zygotic expression of *c-myc* and *myn* may be under the control of "zygotic clock", but do not coincide with each other. In the actively proliferating zygotes, c-Myc protein serves as a key molecule directing transcriptional activation, and its heterodimeric protein Myn(Max) act as a consitutive mediator molecule forming active complex. Functional analysis of *myn* by microinjection experiment indicates that Myn(Max) may play an important role as a gating molecule of c-Myc transcription factor network in preimplantation mouse embryo development.

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착상전 생쥐 초기배아에서 *c-myc*과 *myn* 유전자의 발현과 기능에 관한 연구

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내인성 암원유전자인 *c-myc* 유전자는 세포의 증식과 분화에 밀접히 연관되어 있으며, 그의 생물학적인 기능은 이형결합체인 *myn*과의 결합을 통해서 이루어진다. 본 연구에서는 착상전 생쥐 배아에서의 내인성 *c-myc* 유전자와 *myn* 유전자의 발현을 조사하기 위하여 RT-PCR 방법을 이용하였다. *myn* 유전자 산물은 배아 발생시기를 거쳐서 균일하게 발현된 반면, *c-myc* 유전자의 발현은 1세포시기에 측정된 후, 2세포기에 들어 현저하게 줄었으나, 차후 포배기 시기까지 상당한 양으로 증가하였다. 이러한 *c-myc*과 *myn* 유전자발현의 비균등한 조화가 중요한 역할을 할 것으로 사료된다. 착상전 생쥐 초기배아 발생과정에서의 *myn* 유전자의 기능을 알아 보고자, *myn*에 대한 antisense oligonucleotides(Myn2, Myn3)를 1세포기의 수정란에 미세주입하였다. Myn2와 Myn3의 미세주입에 의해 상실기/포배기의 변이 시기에 비정상적인 발생이 야기되었다. 이상의 결과로, *c-myc*은 그의 이형결합체인 *myn*과 함께 착상전 생쥐 초기배아에서 중요한 역할을 할 것으로 사료된다.