韓國家畜繁殖學會誌 18(3) : 199~206(1994)
Korean J. Animal Reprod.

Differential Display of mRNA in the Preimplantation Mouse Embryos by Reverse Transcriptase Polymerase Chain Reaction

Kim, J.H., H.D. Park*, H.T. Lee and K.S. Chung
Animal Resources Research Center, Kon-Kuk Univ

역전사 연쇄중합반응에 의한 착상전 생쥐난자에서의 상이한 mRNA의 발현조사에 의한 새로운 유전자의 크로닝법

김진희 · 박흠대* · 이훈택 · 정길생
건국대학교 동물자원연구센터

요 약

본 연구는 생쥐 배 발생과정의 상이한 발현을 RT-PCR법에 의해 무작위 증폭함으로써 새로운 유전자를 손쉽게 크로닝하기 위해 수행되었다. mRNA의 상이한 display법은 Ling 과 Pardee (Science 257, 1992)에 의해 개발되었으며, 최근 Zimermann과 Schultz (PNAS USA 91, 1994)에 의해 재증명되었다. 이 방법은 특정 유전자의 일시적 발현의 변화가 maternal 제어로 부터 접합체 제어로의 이행에 따른 발현전이, 다정자 침입과 단일 정자 침입에 의한 배발생의 기능적 차이, 성공적으로 부화한 배반포기 배와 부화에 실패한 배반포기 배에서의 발현의 차이는 물론 세포주기에 따른 유전자 발현 양식의 변화에 따른 새로운 유전자의 크로닝을 가능케 한다. 이 방법에 의해, 2 세포기 특이 발현 유전자를 크로닝 하였으며, 이 유전자는 EcoRI제한 효소 처리후 Southern blot을 행한 결과 약 15 kb genomic size를 가진 것으로 나타났다. 이 새로운 유전자는 간장 특이적 발현을 나타내었다. 또한, 적어도 2개의 mRNA가 존재하였으며, 이는 RNA splicing에 의한 것으로 추정되었다. (PCR, RT-PCR, cloning, preimplantation, mouse)

I. INTRODUCTION

In multicellular organizations, genetic information passes through specialized cells from embryos to offspring. Among 100,000 different genes in higher eukaryotic organism, about 15% are expressed in any individual cells (Liang and Pardee, 1992). Since introduction of polymerase chain reaction (PCR) in 1985 (Saiki et al), PCR has revolutionized molecular biology (Saiki et al, 1988). The method does not only allow from minute amounts of target DNA to a million-folds amplification between two sequence-specific primers, but also utilize for gene mapping (Olson et al, 1989), cloning (Green and Olson, 1990; Wieland et al., 1990; Miyamoto et al., 1993), and sequencing (Strub and Walter, 1989; Ruano and Kidd, 1991). However, current methods to distinguish mRNAs in comparative studies rely largely on the subtractive hybridization technique (Wang et al, 1989; Lee et al., 1991). The

*대구대학교 생물공학과 (Dept. of Biotec., Tae-Gu Univ.)

use of cDNA libraries to analyze gene expression has a restriction that they do not allow facile analysis of gene expression during developmental stages, but differential mRNA display allow the analysis of differential gene expression during embryogenesis and has provided a valuable resource to analyze expression and clone gene.

The recent development of the reverse transcription-PCR (RT-PCR) method has opened up a major avenue to study differential gene expression and clone gene in the preimplantation mouse embryo (Liang and Pardee, 1992; Liang et al, 1993; Bauer et al., 1993). These identified spots should be usable for identifying and isolating the corresponding genes, or mRNAs, cDNAs (Liang et al., 1993). The general strategy is to amplify partial cDNA sequences from subsets of mRNA by the reverse transcription and the polymerase chain reaction. These short sequences are displayed on a sequencing gel and the change in gene expression during developmental stages of embryos can be observed.

In this report, we will report the use and validation of this method to study differential gene expression in the embryogenesis. We find that a noble gene by using this method is cloned and this gene shows 2 different mRNA expression patterens in liver.

Ⅱ. MATERIALS AND METHODS

1. Collection of mouse embryos

Embryos of C57BL/6J mice were used through this study. Superovulation and embryo recovery were done as described by Hogan et al. (1988). After injection of human chorionic gonadotrophin (hCG), 1-cell fertilized embryos were recovered at 22 hr; 2-cell embryos from the reproductive tract of mated females, at 44hr; 48-50 hr for 4 cell stages; 66-68 hr for 8-cells; 92 or 120 hr for early and hatched blastocysts.

2. Oligonucleotide synthesis

Primers were synthesized according to sequences determined in our laboratory or published elsewhere (Ling and Pardee, 1992;Vincent et al., 1994).

3. RNA isolation and RT-PCR

Total RNA was isolated from 200 embryos containing 20 μg Escherichia coli ribosomal RNA by acid guanidinium thiocyanate-phenol-chloroform method (AGPC; Chomczynski and Sacchi, 1987).

The cDNA template for RT-PCR is synthesized from total RNA by using molony murine leukemia virus (MMLV) reverse transcriptase (ClonTec.), dNTP, and random hexamers as previously described (Gerard GF., 1987). Briefly, RNA was incubated at 37℃ for 60 min with a mixture of 100 U of MMLV-RT and the following reagents: each 0.2 μg oligonucleotide, 3 mM $MgCl_2$, 10mM Tris-HCl, pH 8. 3 75 mM KCl, 1 μg acetylated bovine serum albumin, 0.5 mM dNTP, and 5 U of RNAsin in 10 μl volume. The RT can be repeated by addition of 50 U of fresh MMTV-RT after a 93℃, 5 min denaturing step, followed by flash cooling to 4℃.

For RT-PCR, 1~5 μl of cDNA were added to 50 μl of PCR mixture and covered with mineral oil. The mixture was consisted of 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM of ^{32}P-labeled dNTP with activity of 250 μCi, 1 μM of each primers (5'-TTTTTTTT-TTTTGC-3' and 5'-CTGATCCATG-3' or 5'-CTTGATTG CC), and 1.5 unit of Taq polymerase (AmpliTaq polymerase, Takara). Amplification was repeated for 40~60 cycles. The first cycle is denatured at 95℃ for 5 min, annealing at 45℃ for 1 min, and extended at 72℃ for 3

min. Following 40 to 60 cycles, the PCR reaction was terminated at 95℃ for 1 min, 45℃ for 2 min, and at 72℃ for 10 min. The PCR products were separated in an 15% sequencing gel and photographed by X-ray film.

4. Reamplification

Complementary DNA of an identified mRNA species from a dried gel was recovered by using the methods recommended by Farin (personal communication). Brifely, a cDNA band from the sequencing gel was electroeluted with Hoefer's gel eluter and precipiated with ethanol to remove contaminants such as urea. The recovered DNA for cloning was reamplified in the presence of 20 μM dNTP to achieve optimal yields and specificity as above conditions.

5. Southern and Nothern blot analysis

Genomic DNA and/or RNA extracted from pools of embryos or tissue was subjected to electrophoresis, transferred to hybond nylon membrane, and hybridized with the Dig-labelling PCR product as described by our previous data (Kim et al., 1994). Standard curves for total RNA levels were quantitated by using a purified mouse actin mRNA.

Ⅲ. RESULTS and DISCUSSION

1. General strategy

The strategy of the mRNA differential display at the preimplantation embryos is illustrated in Fig. 1. The first steps in the RNA phenotyping analysis was to isolate the total RNA by a micromethod from 1 to 200 embryos as described previously (Graves et al., 1985; Chomczynski and Sacchi, 1987; Rapporee et al. 1988; Michelle and William, 1991). The resulting RNA was then reverse transcribed to prepare cDNA.

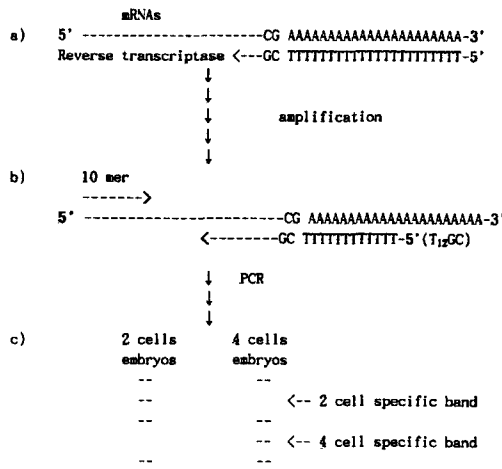

Fig. 1. Systematic representation for cloning of specific cDNAs by the differential mRNA display. a) reverse transcriptase (RT). b) RT-PCR, c) is depicted the mobility of the new gene during gel electrophoresis.

The primers were added to the cDNA preparation with Taq polymerase and ^{32}P labelled dNTPs. The cDNA was then thermally denatured and afterward cooled to allow annealing of primers, and then increased to the optimum for primer extension by Taq polymerase. In the first PCR cycles, 5' primers were annealed to the cDNA from RT. After primer extension, the various size of cDNAs extending from the 5' primer to the 3' end were obtained. In subsequent cycles, these various size cDNA become dominant and then, the cDNAs after 40 or 60 cycles were visualized by X-ray film and showed several prodominent bands as shown in Fig. 3, 4, and 5 when the 5' 10 oligonucleotide primer was used in conjunction with 3' primer $T_{12}GC$.

In the PCR precedure, the amplification is exponential with a theoretical doubling of each strand at each cycle of amplification. In fact,

the efficiency is less than 100% and the extent of amplification (Y) is given by the formular: $Y=A(1+R)^n$ where A is the initial amount DNA/cDNA obtained in the first step, which depends on the amount of initial mRNA present in the sample, R the efficiency and n the number of cycles (Saiki et al., 1985). It is possible to estimate Y with a reasonable accuracy from the extent of incorporation of 5'-labeled primers into the amplified products. The increase of Y was calculated after each cycle by counting the amount of ^{32}P incorporated into amplified products. Figure 2 shows that the amount of amplified fragment after 60 cycles ceased to increase exponentially and reached a plateau. The R value was constant for a given amplified fragment, but the number of cycles after which the amplification rate ceased to be exponential depended on the abundance of the starting specific mRNA.

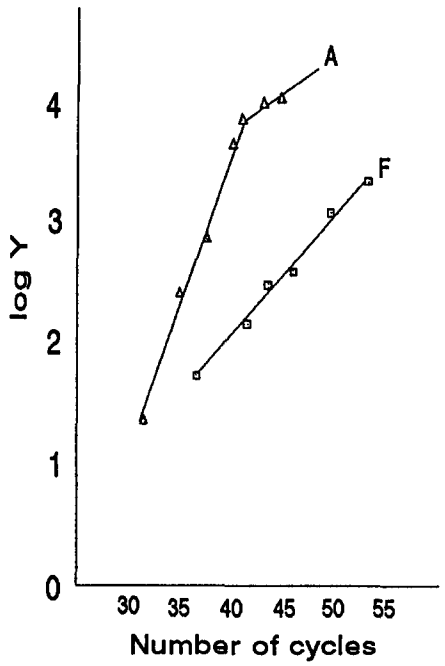

Fig. 2. Quantation of mRNAs in the mouse preimplantation embryos.
Total RNAs were extracted from 10 to 50 embryos. Aborbance of RT-PCR were counted and then converted to absolute amounts by using the standard curve.(A and F indicate the levels of amplification of cDNA by RT-PCR)

2. Effect of length of arbitray primers

To investigate the possibility of using short G/C rich oligonucleotides as primers in RT-PCR, the 5' primers were selected 8 or 10 nucleotides length in order to anneal fairly near the end of a cDNA strand. Selection of 3' primers used the poly $(A)^+$ tail sequence containing 2 additive 3'base to anchor the primer at the 3' end of the mRNA. As shown in Figure 3, we obserbed positive amplification of with 8-mers and $T_{12}GC$, but limited to conditions of approximately 45℃ annealing temperature. However, we can not find the different size of PCR products between 2 cell and 4 cell embryos when PCR products were compared within 500bp because cDNA upto 500dp can not display by limit-

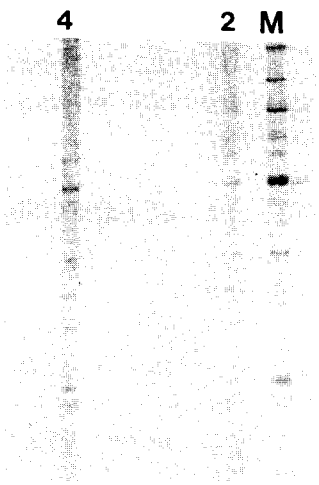

Fig. 3. Location of an autoradiogram of RT-PCR products obtained from total RNA using 5'8 oligonucleotide and 3' T12GC primers.

ing of size on a sequencing gel. Even though such 8 nucleotide primer can give specific DNA amplifications by RT-PCR, no reverse transcribed cDNA species would be amplified a cDNA with different size between 2 cell and 4 cell mouse embryos, hatched blastocysts and blastocyst embryos with intact zona pellucida.

As shown in Fig. 4, primer $T_{12}GC$ in combination with a 10-mer was found to give specific DNA amplification. The specificity of DNA amplification dramatically increased with 5' 10 mer as described by Liang and Pardee (1992).

3. Changes in levels of mRNA during embryo development

The expression patterns of any specific gene to the total mRNA populations can be measured by RT-PCR. The different fragments and relative amount of expression at the preimplantation embryos by RT-PCR were detected as shown in Fig. 3, 4 and 5. The efficiency to generate a series of expression from 2-cell and 4-cell, 8-cell and 16-cell by RT-PCR was depended on PCR conditions and primer combinations. As shown in Fig. 4 and 5, RT-PCR products showed many developmental changes in gene expression. A gene was constant at each of stages, but some genes are expressed in 2-cell, 4-cell, 8-cell, and 16-cell embryos. This fact suggested that some genes were expressed in a stage-specific manner. The relative changes of mRNA by RT-PCR are like to reflect real difference of endogenous mRNA according to embryo development. The relative changes of mRNA are reproducible and detectable appropiate temporal changes during embryo development. Figure 4 shows the four genes with specific expression in only mouse 1-cell embryos. The differences in mRNA content observed are not due to variable losses during RNA extraction procedures because the amount of total RNA did not vary significantly

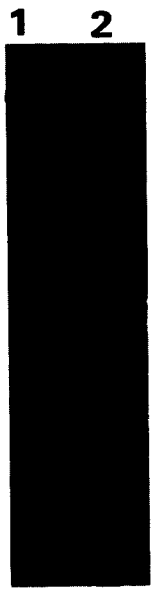

Fig. 4. Levels of differential mRNAs in 1-cell and 2-cells.

The isolated total RNA were reverse transcribed as described in Materials and Methods. The amount of specific RNAs were determined by RT-PCR and were subjected to sequencing gel. Panels A and B show mRNAs amplified by RT-PCR at 1-cell and 2-cells embryos. Films were exposed for 24 hr at −80℃

from one preparation to another. These genes will be members of the same family. Thus, we estimated that these genes were expressed during early mouse egg stages when maternal control mRNA lost and reaccumulated by zygotic derived transcription. Figure 5 shows different expression patterns of some genes extracted from 2-cell, 4-cell, 8-cell, and 16-cell embryos. In each case, the levels of mRNA were markedly reduced in 2-cell embryo, but increased progressively upto the 16-cell.

4. Identification of a mRNA with specific expression at 2-cell embryos

To investigate whether or not an gene con-

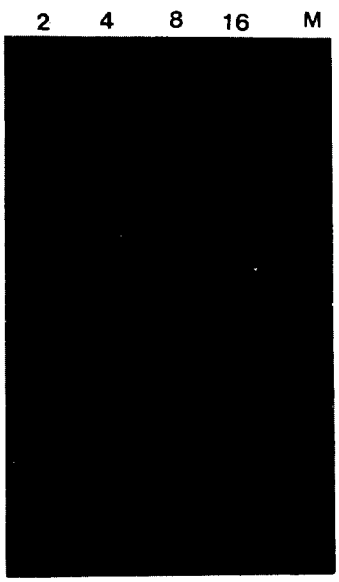

Fig. 5. Expression patterns of differential mRNAs by RT-PCR during embryo development. Lane M: mark, 2, 4, 8, 16 means 2-, 4-, 8-, 16-cells stages embryos, respectively.

cerning zygotic control could be cloned, we have investigated the relative changes of mRNA in 1-cell and 2-cells. A series of RT-PCR products were investigated and then we are identified a gene as shown in Fig. 6. Reaccumulation of this gene was detected in low amounts upto 8-cell stages. By Southern blot analysis, this gene digested with EcoRI showed approximately 15 kb (Fig. 7) and expressed in both adult mouse liver (Fig. 8). In addition, this gene is likely to have two kinds of mRNA by alternative splicing.

Ⅳ. CONCLUSION REMARKS

The major advantage of the mRNA differential display at the preimplantation embryos will

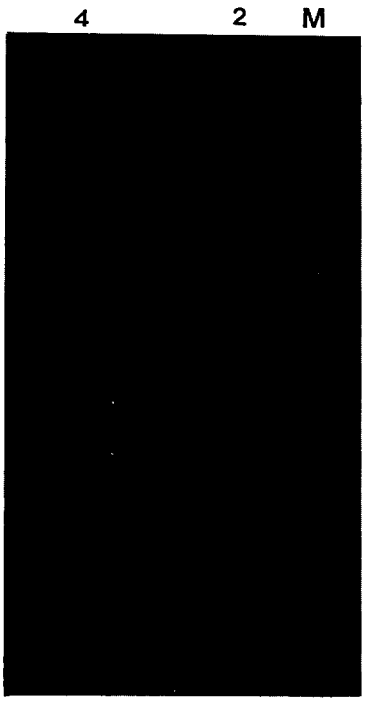

Fig. 6. Location of a specific cDNA by RT-PCR at 2 cells and 4 cells.

Fig. 7. Southern blot analysis of mouse genomic DNA by fragment of Fig 6.

High molecular DNA of mouse genomic DNA were extracted according to the methods recommended by Hogan et al. DNA was digested with EcoRI and were transferred on 1% agarose gel. For blot analysis, see Meterials and Methods.

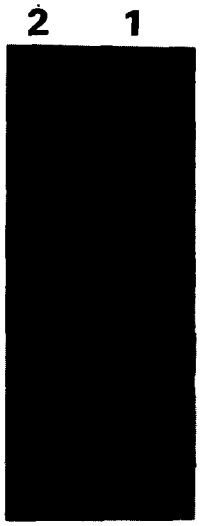

Fig. 8. Expression patterns of novel genes in liver by using fragment of Fig. 6. Lane 1, neonatal liver; lane 2, adult liver

be readily detect the different gene expression at different stages according to embryo development. Thus, this study demonstrates that it is possible to detect the expression of a particular genes expressed in stage specific manner as well as clone noble genes in eggs and embryos. Furthermore, it should be possible to detect and clone relatively rare mRNA by increasing PCR cycles and exposure time of X-ray film. The different-size partial cDNA fragments created by locating many specific mRNA in embryos can potentially be exploited another way.

V. SUMMARY

We present here a new PCR-based cloning technique that allows the different PCR products during mouse embryogenesis. Recently, mRNA differential display described by Liang & Pardee (Science 257, 1992) and re-confirmed by Zimermann & Schultz (PNAS 91,1994). This method will detect the appropriate changes in the temporal patterns of expression or in the transition from maternal control to zygotic control as well as the functional difference of embryo with polyspermy or monospermy, the difference of expression between successfully hatched blastocyst and blastocyst failed to hatching, response to agents, and cell cycle regulation. By this methods, we have cloned an cDNA, which showed mouse 2 cell specific expression. Genomic DNA digested with EcoRI showed approximately 15 kb and then showed higher expression in fetal liver rather than adult liver. Furthermore, this gene is likely to have 2 mRNA by alternative splicing.

VI. REFERENCES

1. Bauer D., Muller H., Reich J., Riedel H., Ahrenkiel V., Warthoe P., and Strauss M. (1993). Identification of differentially expressed mRNA species by an improved display technique. Nucleic. Acids Res. 21:4272-4280.
2. Chomczynski P. and Sacchi N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate -Phenol- chloroform extraction. Anal. Biochem. 162, 156-159.
3. Douglas H. J. and Winistorfer S. C. (1993). A methods for the amplification of unknown flanking DNA:Targeted inverted repeat amplification. Biotechniques 15:894-904.
4. Erlich H. A., Gelfand D. H., and Sninsky J. J. (1991). Recent advances in the polymerase chain reaction. Science 252, 1643-1660.
5. Gaudett M. F. and William R. C. (1991). A simple method for quantifying specific mRNA in small numbers of early mouse embryos. Nucleic. Acids Res. 19:1879-1884.
6. Gerard G. F. (1988). BRL Focus 10:12-13.
7. Graves R. A., Marzluff W. F., Giebelhaus D.

H., and Schultz G. A. (1985). Quantitative and qualitative changes in histone gene expression during early mouse embryo development. Proc. Natl. Acad. Sci. USA. 82:5685-5689.

8. Green E. D. and Olson M. V. (1990). Systematic screening of yeast artifical chromosome libraries by the use of the polymerase chain reaction. Proc. Natl. Sci. USA. 87:1213-1217.
9. Lee S. W., Tomasetto C., and R. Sager. (1991). Positive selection of candidate tumor-suppressor genes by subtrative hybridization. Proc. Natl. Acad. Sci. USA. 88:2825.
10. Ling P. and Pardee A. B. (1992). Differential display of eukaryotic messanger RNA by means of the polymerase chain reaction. Science 257:967-971.
11. Ling P., Averboukh L., and Pardee A. B. (1993). Distribution and cloning of eukaryotic mRNA by means of differential display: refinements and optimization. Nucl. Acids. Res. 21:3269-3275.
12. Miyamoto H., Matsushiro A., and Nozaki M. (1993). Molecular cloning of a novel mRNA sequence expressed in cleavage stage mouse embryos. Mol. Reprod. Devel. 34:1-7.
13. Olson M. V., Hood L., Cantor C., and Botstein D. (1989). A common language for physical mapping of the human genome. Science 245:1334-1335.
14. Rappolee DA., Mark D., Banda MJ., and Werb Z. (1988). Wound macrophage express TGF-α and other growth factors in vivo: analysis by mRNA phenotypiy. Science. 241, 708-712.
15. Saiki RK., Scharf S., Faloona F., Mullis KB., Horn G., Erich HA., and Arnheim N. (1985). Enzymatic amplification of the β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230, 1350-1354.
16. Saiki RK., Gelfand DH., Stoffel S., Scharf SJ., Higuchi R., Horn GT., Mullis KB., and Ehrich HA. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487-491.
17. Strub K. and Walter P. (1989). Isolation of a cDNA clone of the 14-kDa subunit of the signal recognition particle by cross-hybridization of differently primed polymerase chain reactions. Proc. Natl. Acad. Sci. USA. 86:9747-9751.
18. Vincent J., Gurling H., and Melmer G. (1994). Oligonucleotides as shorts as 7-mers can be used for PCR amplification. DNA and Cell Biology 13:75-82.
19. Wang A. M., Doyle M. V., and Mark D. F. (1989). Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA. 86:9717-9721.
20. Welish J., Chada K., Dalal S. S., Cheng R., Ralph D., and McClelland M. (1992). Arbitrarily primed PCR fingerprinting of RNA. Nucleic Acids Res. 20:4965-4970.
21. Wieland I., Bolger G., Asouline G., and Wigler M. (1990). A method for difference cloning:Gene amplification following subtractive hybridization. Proc. Natl. Acad. Sci. USA. 87:2720-2724.
22. Zimmermann JW. and Schultz RM. (1994). Analysis of gene expression in the preimplantation mouse embryos: Use of mRNA differential display. Proc. Natl. Acad. Sci., 91, 5456-5460.