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Expression of *E. coli* LacZ Gene in Bovine Morulae or Blastocysts after Microinjection of Retrovirus Vector-Producing Cells into the Perivitelline Space of One- to Four-Cell Embryos

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體外生産된 牛受精卵으로부터 形質轉換牛의 生産性 提高를 위한 Retrovirus Vector System의 利用性 檢討

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요 약

본 연구는 형질전환우의 생산성 제고를 위한 일환으로서 새로운 기법인 retrovirus vector system의 이용성을 검토하고자 실시하였다. Retrovirus-producing cell은 미세주입법을 이용하여 체외생산된 1.5일(1~4-세포기) 수정란의 위란강에 주입(5~10 cells/embryo) 되었으며, 이때 사용된 retrovirus-producing cell line은 Gibbon ape leukemia virus (GaLV) envelope protein에 encapsidation되어 replication-defective retrovirus를 분비하도록 제작되었다. 주입된 유전자의 표지유전자로서 *E. coli* LacZ 유전자를 사용하였으며, X-gal 염색법은 발달이 유도된 상실배와 배반포 단계에 실시하여 LacZ 유전자의 발현 유무를 확인하였다.

이 실험의 결과를 요약하면 다음과 같다.

1. Virus의 infectivity를 높이기 위해 사용된 polybrene의 최저농도는 5μg/ml 이었다.
2. Retrovirus-producing cell이 주입된 1.5일 수정란의 상실배기와 배반포기로의 발달율은 29%였다.
3. 이 때의 LacZ$^+$ 발현율은 21%였다.
4. 본 실험에 사용된 retrovirus-producing cell은 replication-competent retroviruses를 생산해 내지 않는다는 것을 확인할 수 있었다.

I. INTRODUCTION

Since the first report describing transgenic animals (Jaenisch and Minz, 1974), most of the transgenic animals have been produced by microinjection of DNA into a pronucleus of a zygote. Unlike mouse models, however, pronuclear microinjection does not seem to work well in the production of transgenic livestock. Despite immense investment of time and money, few (Ebert et al., 1991; Wright et al., 1991) reports of transgenic domestic animals exist as of yet indicating a dismal prospective from an econ-

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omic point of view for producing transgenic livestock.

One of the alternative methods of producing transgenic animals is transfer of an exogenous gene of our interest into the embryo by means of retroviral infection, rather than physical microinjection of the gene into the pronucleus. Advantages of retrovirus vector system approach in gene transfer over conventional DNA microinjection method are technical ease and effectiveness of gene transfer, stability of transgene formation and maintenance in terms of high fidelity of inheritance and low mutation rate (Temin, 1989), preferable integration of the transgene in DNase hypersensitive region of the chromosome (Rohdewohld et al., 1987) implying efficient expression of the transferred gene, and low incidence of rearrangements of the host genome except a short duplication at the site of integration (reviewed in Jaenisch, 1988).

Despite the great utility of retrovirus vector systems in rodent gene transfer, there is still no report of successful application to domestic animals. This is because most murine virus vector systems work pooly with cells from other speices and insufficient attention has been directed at domestic viral vectors except chickens (Bosselman et al., 1989a, b). Finally, approval for field trials of transgenic animals constructed with viral vectors will be difficult.

In a previous report, we have demonstrated retrovirus-mediated gene transfer to the bovine embryos (Kim et al., 1993). By using PG13 packaging cell line (Miller et al., 1991) and the E. coli β galactosidase (LacZ) gene as a marker, we can successfully detect LacZ positive ICM (inner cell mass) cells from the embryos co-cultured with virus-producing cells. However, unlike mouse, we have found that co-cultured approach is not applicable to cattle because of low infectivity of the viruses to the bovine embryonic cells and difficulty in in vitro culturing of zona-free pre-morular stage bovine embryos. PG13-based retrovirus-producing cells are one of the best systems currently available, but infectivity of the viruses produced from that system was far lower than that of our expectation when the target cells were derived from cattle. In vitro culture of zona-free pre-morular stage bovine embryos resulted in dissociation of blastomeres.

In this study, we asked whether direct injection of virus-producing cells into the perivitelline space of the early embryos could increase infectivity without significant sacrifice of embryo viability. The rationale is based on the assumption that continuous production of the virus-producing cells with the blastomeres for a long period might increase infection efficiency.

Ⅱ. MATERIALS AND METHODS

1. Virus-producing cells

PG13-LNBβZ c6 virus-producing cell line was constructed by infecting GaLV-based PG13 (Miller et al., 1991) packaging cell line (Miller and Buttimore, 1986), and pLNβZ is the plasmid containing retrovirus vector sequence (Fig. 1). Detailed methods for constructions of the retrovirus-producing cell line and the vector are described in one of our previous report (Kim et al., 1993).

The virus-producing cell (PG13-LNβZ c6) and EBTr (bovine embryonic trachea) target cell line (American Type Culture Collection, CCL44) were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l of glucose (Sigma Chemical Co., St. Louis, MO) supplemented with FCS (10%), amphotericin B (2.5 mg/l, gentamycin (50 mg/l; Gibco). All cells were grown in a 37℃, 5% CO_2 incubator. To maximize virus-production, the PG13-LNβZ c6 cells were maintained in the medium containing G418 (600

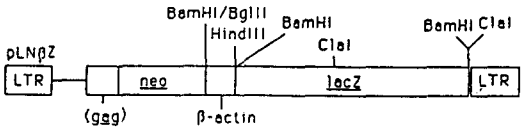

Fig. 1. Structure of pLNβZ retrovirus vector. LTR, long terminal repeat; (gag), 5'portion of Moloney murine leukemia virus (MoMLV) gag gene; neo, G418 resistant gene; β-actin promoter; LacZ, E. coli β -galactosidase gene.

μg /ml) until microinjection (Emerman and Temin, 1984). Mitotic arrest of the injected PG13-LNβZ c6 cells in the embryo was accomplished by culturing the cells in the medium supplemented with mitomycin C (10μg /ml) for 4~6 hours, followed by three washed before proceeding to subsequent steps of the experiment.

Determination of virus titer was done by counting LacZ$^+$EBTr cells after infection with virus-containing medium (Kim et al., 1993).

2. Microinjection of the PG13-LNβZ c6 cells and embryo culture

5~10 cells were microinjected into each of day 1.5 (1.5days of post-fertilization) embryos prepared by following the procedures previously detailed in Sirard et al. (1988) and Kim et al. (1993). Injected embryos were cultured in CR1 aa (Rosenkrans et al., 1991, 1993) supplemented with 10% heat treated FCS until embryos reached day 7 or 8. Medium was changed at day 2.5 and 4. To help virus absorb to the blastomere, polybrene (5μg /ml; Aldrich) was added to the culture drop for 2.5 days (from day 1.5 to day 4).

3. β-galactosidase assay and second infection test.

Expression of the transgene evaluated by X-gal staining as described in Kim et al. (1993). Second infection test was performed to check whether the virus vector system used in this study was safe in terms of biosafety. Briefly, EBTr target cells were infected (first infection) with 1ml of medium harvested from PG13-LNβZ c6, then selected with the medium containing 600μg /ml of G418. 1ml of the medium harvested from the resulting confluent G418-resistant EBTr cells was added to freshly plated EBTr cells (second infection), followed by G418 selection. No G418 resistant cell colony is to be found from the second infection if the viruses produced from our PG13-LNβZ c6 cells are replication defective.

Ⅲ. RESULTS AND DISCUSSION

1. Determination of minimum concentration of polybrene

Polybrene is generally used in retrovirus infection study because the polycation molecule is believed to help virus adsorption to the target cell membrane. However, it is not known whether low concentration of polybrene is detrimental to the bovine embryos. Under the assumption that polybrene must be toxic to the embryo, we tried to determine the minimal concentration of polybrene without sacrificing virus infectivity. To answer this question, we infected EBTr target cell with the virus-containing medium supplemented with various concentration of polybrene. As shown in Table 1, addition of polybrene more than 5μg /ml did not increase the titer.

2. Injection of virus-producing cells into the embryos

We injected 5~10 mitomycin C-treated PG 13-LNβZ c6 cells into the perivitelline space of

Table 1. Determination of minimum concentration of polybrene

Polybrene	0 μg /ml	5 μg /ml	10 μg /ml	20 μg /ml	50 μg /ml	100 μg /ml
1st experiment	4×10^4	6.2×10^4	6.4×10^4	5.8×10^4	4.7×10^4	4.6×10^4
2nd experiment	3×10^4	5.8×10^4	4.6×10^4	5.3×10^4	6.7×10^4	4.6×10^4
average	3.5×10^4	6×10^4	5.5×10^4	5.6×10^4	5.7×10^4	4.6×10^4

The unit of each number is $LacZ^+$ Transforming Unit /ml

each embryo (day 1.5) and cultured until the embryos reach day 7 or 8. From 673 embryos injected at day 1.5 (one-to four-cell stage), 193 (29%) embryos developed to morulae or blastocysts, and 6% of the microinjected embryos or 21% of morulae or blastocysts were LacZ positive (Table 2).

As expected, development frequency was somewhat lower than that of our routine work. Usually, we could score about 40% of development frequency from day 1 embryos of good quality to morulae or blastocysts. Microinjectional stress, existence of the exogenous cells inside of the embryo and, and other factors including chromosomal disturbance by retrovirus gene integration and expression of foreign proteins inside of the embryos might be main causes for lower development rate.

Discrimination of $LacZ^+$ embryos from LacZ was done on the basis of stained ICM size (Fig. 2). Because the virus-producing cells were pretreated with mitomycin C to prevent the cells from proliferation, only proliferation of infected blastomeres can make a bigger blue region in the ICM than the region consisting of only 5~10 virus-producing cells. Considering the remaining steps involved in transgenic cattle production including embryo transfer and maintenance of pregnancy until the transgenic calf is born, 6% of expression rate assayed at morular or blastular stage seems to need further development before practical application. Concurrently, Haskell and Bowen (1995) reported 65% of the bovine fetuses generated from the approach similar to ours were transgenic, but in that study they evaluated transgenics only by southern analysis. It is generally accepted that the frequency of transgene expression is much lower than that of transgene integration.

Table 2. Infection of bovine embryos by microinjection of PG13-LNβZ c6 cells into perivitelline space

# of embryos (day 1.5)injected	# of morulae or blastocysts	# of $LacZ^+$ morulae or blastcysts
673	193 (29%)	41 (6%)

In the last experiment, we tested whether the viruses produced from PG13-LNβZ c6 are replication defective. Second infection test described in Materials and Methods confirmed no production of replication-positive viruses from the PG13-LNβZ c6 cells (data not shown), eliminating a possible biohazard problem involved in retrovirus-mediated gene transfer approach.

In conclusion, we have demonstrated that retrovirus-mediated approach is one of the alternative way to the DNA microinjection approach in transgenic cattle production. Considering that the frequency of the transgenic cattle production using pronuclear microinjection is less than 0.1% (Krimpenfort et al., 1991; Hill et al., 1992; Bowen et al., 1994), the combined data of this report and of Haskell and Bowen (1995) strongly indicate that the approach of virus-producing cell injection is more efficient than microinjection approach at least in transgenic cattle production. The only problem to be solved is

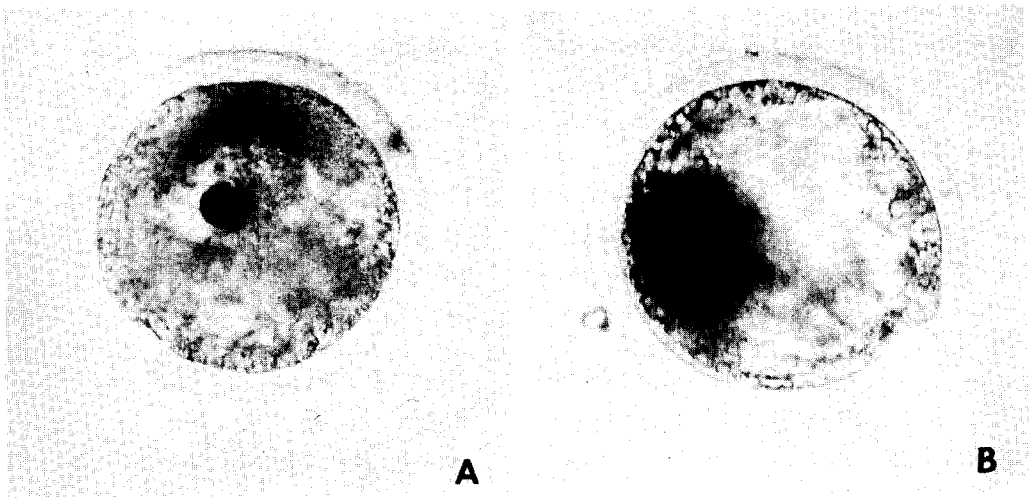

Fig. 2. Blastocysts stained with X-gal.

A: Blastocyst without ICM cells expressing *E. coli* LacZ gene. The small blue area is due to virus-producing cells stained with X-gal.

B: Blastocyst with ICM cells expressing *E. coli* LacZ gene.

possible mosaicism of the transgenic calves as predicted in Fig. 2. The best solution might be development of better retrovirus vector system which produces more infectious retroviruses to bovine cells.

Ⅳ. SUMMARY

In this study, we have tested whether the retrovirus vector system is applicable in transgenic cattle production. To overcome low infectivity of currently available retrovirus vector system we have directly microinjected retrovirus-producing cells into the perivitelline space of the day 1.5 embryos. The virus-producing cell line was designed to release replication-defective retrovirus encapsidated with Gibbon ape leukemia virus (GaLV) envelope protein. *E. coli* LacZ gene was used as a marker gene to facilitate evaluation of the transgene expression and X-gal staining at morula or blastocyst stage resulted in expression of *E. coli* LacZ gene.

The results in these experiments were summarized as follows :

1. The lowest concentration of polybrene necessary for efficient virus infection was 5μ g/ml.
2. Development rate from day 1.5 embryos microinjected with virus-producing cells to the morulae/blastocysts was 29%.
3. 21% of the morulae/blastocysts were $LacZ^+$.
4. There was no evidence that the retrovirus-producing cells used in this study produced replication-competent retrovirus.

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