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Retrovirus Vector-mediated Gene Transfer into the Fertilized Embryos of the Farm Animals

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Retrovirus Vector를 이용한 동물 수정란에의 유전자 전이

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요 약

Retrovirus는 DNA가 아닌 RNA를 유전 물질로 갖고 있는 동물 virus 인데 각 virus는 RNA와 함께 크게 *gag*, *pol*, 그리고 *env* 등의 3가지 단백질로 구성되어 있다. gag 단백질은 virus의 내부구조를 형성하는 단백질이고, pol단백질은 감염을 통해 표적 세포에 도입된 retrovirus의 RNA를 DNA로 逆轉寫시키는 reverse transcriptase의 역할을 하며, env단백질은 virus의 외부를 구성하는 단백질로써 이 단백질에 의해 각 retrovirus의 종류에 따른 감염이 가능한 표적세포의 종류가 결정된다(host cell specificity). 따라서 어떤 retrovirus의 envelope단백질과 표식세포에 있는 retrovirus의 envelope 단백질에 대한 특정 receptor와의 상호 작용에 의해 세포속으로 도입된 virus의 RNA는 reverse transcriptase에 의해 DNA로 逆轉寫된 후 표적세포의 genomic DNA에 삽입되는 특징을 가진다. 이러한 특징을 가진 retrovirus vector system은 형질 전환 동물의 생산에 있어서 현재까지의 주된 방법인 수정란의 pronucleus에의 DNA microinjection방법 보다 여러 가지 면에서 우수함에도 불구하고 쥐 이외의 다른 동물에서는 거의 이용되고 있지 않는 실정이다. 주된 원인으로는 현재 사용되고 있는 대부분의 retrovirus vector system이 쥐의 백혈병 virus를 근간으로 하기 때문에 이 system에서 생산된 virus는 쥐 이외의 다른 동물, 특히 有蹄類의 세포에는 감염성이 아주 약하기 때문이다. 이러한 결점을 해결하기 위하여 최근에 기존의 쥐 백혈병 virus의 envelope protein을 vesicular stomatitis virus의 G protein으로 대체한 hybrid retrovirus vector system이 개발되었다. 이러한 system에서 생산되는 virus는 鳥類를 포함한 거의 모든 종류의 동물세포를 감염시킬 수 있으며 몇몇 특정세포에 대해서는 기존의 retrovirus vector system에 비해 1,000배 이상의 높은 감염도를 나타내는데 그 특징이 있다. 따라서 이러한 새로운 virus vector system을 이용할 경우, 보다 다양한 종에 있어서 형질전환 동물을 효율적으로 생산할 수 있을 뿐만 아니라 형질전환 동물의 생산 방법 자체를 다양화 시킬 수 있다고 본다.

Ⅰ. INTRODUCTION

Transgenic animals are animals carrying a foreign gene introduced at an early developmental stage of the embryo. The foreign gene is generally termed a 'transgene' (reviewed in Palmiter, 1986). For modern biological sciences, in particular, basic developmental biology, production of transgenic animals has offered a powerful experimental tool for investigating gene expression *in vivo*, as well as biological applications such as the use of genetically engineered farm animals as bioreactors to produce pharmaceu-

ticals, etc.

Since the first report describing transgenic animals, which developed from microinjection of SV40 (simian virus 40) DNA into the blastocoel cavity of mouse embryos (Jaenisch and Mintz, 1974), three methods have been applied in most transgenic animal production: microinjection of recombinant DNA into pronuclei of fertilized embryos, infection of early stage embryos with recombinant retroviruses, and introduction of embryonic stem cells into the blastocoel cavity.

Among the three methods, the technique of direct microinjection of recombinant DNA into a pronucleus of an embryo (first reported by Gordon et al. in 1980) is most common. As a consequence of the remarkable success of pronuclear DNA microinjection in production of transgenic mice, this approach was extended to farm animals. Shortly after the first documentation of transgenic rabbits, sheep, and pigs (Hammer et al., 1985), the first transgenic cattle (Biery et al., 1988) and goats (Ebert et al., 1991) were produced by this method. 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 reports of transgenic domestic animals exist as of yet indicating a dismal prospective from an economical point of view for producing transgenic livestock. Disadvantages of this method include technical difficulties in DNA microinjection into the pronuclei of most farm animal species mainly due to the cytoplasmic opacity of ova (reviewed in Pursel et al., 1989), host cellular gene rearrangements including deletion (Covarrubias., 1986) and translocation (Mahon et al., 1988), multiple tandem repeats of introduced DNA (Brinster et al., 1981), damage of embryos during micromanipulation (Walton et al.,1987), and a significant investment for the equipment ($50,000; Camper, 1987).

The most important features of retroviruses for their use as vectors are technical ease and effectiveness of gene transfer. This is due to the their infectivity for certain target cells resulting in gene transfer. Once the cells are infected by retroviruses, the resultant viral DNA, after reverse transcription and integration, becomes a part of the host cell genome, and the stability of transgene formation and maintenance (Temin, 1989) is far superior to other gene transfer systems. In addition, it is believed that DNase hypersensitive regions are the preferred target for retrovirus integration (Rohdewohld et al., 1987) implying efficient expression of exogenous proviral gene even though the copy number of provirus per each integration site is accurately only one. Unlike DNA microinjection, integration of a viral gene does not seem to induce rearrangements of the host genome except a short duplication at the site of integration (reviewed in Jaenisch, 1988).

Despite the high potency of the retroviral vector system in gene transfer, there is still no report of successful application in domestic mammals. This is caused by little development of retroviral vector systems specific for domestic animals except chickens. In chickens, the DNA microinjection approach is not possible due to the high cell number of freshly layed eggs (more than 10,000 cells/embryo) (Kochav et al., 1980). However, by using retroviral vectors, chickens carrying transgenes in their somatic and germ lines have been reported (Bosselman et al., 1989).

The attractive features of the ES (embryonic stem) cell-mediated approach are: (a) pluripotency of ES cells which can contribute to the germ line of a resulting chimeric animal after introduction into an embryo, (b) ease of foreign gene transfer into ES cells compared to an em-

bryo, and (c) the availability of screening or selection of transformed ES cells with desirable characteristics before injection into an embryo. These advantages seem to be ideal for the production of transgenic animals where modeling on gene targeting is a part of gene therapy (Thomas and Capecchi, 1987). However, lack of availability of stem cells from animal species other than the mouse has limited this approach only to mice so far. Establishments of embryonic stem cells of non-murine species including hamster (Doetschman et al., 1988), pig (Notarianni et al., 1990), sheep (Notarianni et al, 1991), and bovine (Sims and First, 1994) have been reported recently, but the totipotencies of the cells of thses farm animals have not yet been proven.

In addition to the three methods described above for the generation of transgenic animals, sperm cell-mediated DNA transfer has been reported to result in transgenic mice. (Lavitrano et al., 1989). The approach of using sperm cells as vectors for introducing foreign DNA into eggs seems to be very revolutionary but controversial. This has not been independently reproduced (Brinster et al., 1989), even though it is clear sperm can take up DNA without affecting fertility (Bachiller et al., 1991).

The main objective of this review is not to attempt to give a comprehensive review but rather emphasize principles and recent developments in retrovirus vector system.

Ⅱ. RETROVIRUSES

Retroviruses are animal virus with two identical strands of RNA in their virion (reviewed in Temin, 1987). As the word 'retro' indicates, when these viruses infect a host cell, the viral RNA is reverse transcribed in the cytoplasm to make linear double stranded DNA, which is transported into the host cell nucleus and integrates into a chromosome directly without change of its original linear form (Ellis and Bernstein, 1989). The viral genes are expressed from this integrated form of DNA, the provirus, and the progeny viruses are produced from the infected host cell as a result of proviral gene expression.

Among the 4 types (A, B, C and D) of retroviruses classfied on their electromicroscopical morphology (Teich, 1982), C-type retroviruses are the only ones that have been used in vector construction since they have been the most extensively studied. The most commonly used C-type retroviruses are murine leukemia virus (MLV), reticuloendotheliosis virus (REV), and Rous sarcoma virus (RSV). REV and RSV are avian retroviruses.

Ⅲ. STRATEGIES OF RETROVIRAL VECTOR SYSTEMS

Retroviruses have three trans-acting protein-coding genes, *gag*, *pol*, and *env* (Fig. 1). The proteins encoded by the *gag* gene are responsible for virus internal structure. The *pol* gene encodes enzymes including the carboxyl portion of protease for post-translational cleavage of viral proteins, reverse transcriptase to transfer information from RNA into DNA, and integrase for the integration of reverse transcribed viral DNA into the host cell chromosome (Craigie et al., 1990). The *env* gene encodes viral envelope glycoproteins which are part of the outside of the progeny virus and are the primary determinant of retrovirus host range such that, for example, infection is initiated by binding of gp70, MoMLV (Moloney murine leukemia virus) surface envelope protein, to the cellular receptor consisting of 14 transmembrane domains (Albritton et al., 1989).

Cis-acting sequences of a retrovirus are clus-

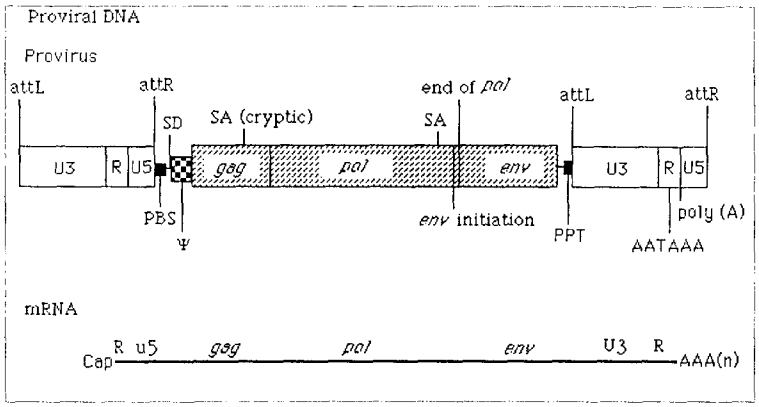

Fig. 1. Structure of MoMLV provirus and its full transcript. (a) Provirus. Hatched areas indicate trans-acting sequences. Each of the two LTR (long terminal repeat) consists of U3, R and U5. Abbreviations are: U3, the sequence unique to 3' end of mRNA (449 bp); R, the sequence of direct repeat (68 bp); U5, the sequence unique to 5' end of mRNA (77 bp); attL and attR, left and right side attachment sites corresponding to 13 bp of each inverted repeat sequence; PBS, primer binding site (18 bp); SD, splicing donor site (AGGT); Ψ, encapsidation site (350 bp fragment just upstream from the initiation codon of *gag*); SA, splicing acceptor site (CACTTACAG); PPT, polypurine track (13 bp); AATAAA, sequence of polyadenylation signal; poly A, polyadenylation site. The real size of the sequences not mentioned above are: the sequence between 5'LTR and *gag*, 475 bp; distance between PBS and SD, 45 bp; *gag*, 1,617 bp; *pol*, 3,600 bp; *env*, 1,998 bp; overlapping sequence of *pol* and *env*, 61 bp; the sequence between *env* and 3'LTR, 41 bp. (b) Transcription of provirus begins at the first base of 5' R and end at the last base of 3'R with Cap or polyadenylation at each end. **Length of each sequence is not drawn to scale.

tered at the ends of the viral genome (Fig. 1): (a) long terminal repeat (LTR) sequences for enhancer/promoter function, transacting factor response (Tong-Starksen and Peterlin, 1990), integration (Colicelli and Goff, 1988), and polyadenylation of viral RNA (Iwasaki and Temin, 1990); (b) primer binding site and polypurine sequence for reverse transcription; (c) posttranscriptional splicing sites including splicing donor and acceptor sites along with two short fragments within the viral intron (Hwang et al., 1984) for *env* mRNA production (reviewed in Coffin, 1982); and (d) Ψ or E signal in case of MLV and REV for encapsidation.

Because many retroviruses are pathogenic, in designing retrovirus-production system for gene transfer, replication-competent retrovirus is divided into two parts (*cis*-acting and trans-acting) so as not to transmit possible disease to the target organisms.

1. Packaging cells

Helper cells (or packaging cells) are usually constructed by the transfection of viral transacting sequences (*gag*, *pol*, and *env*) to an appropriate eukaryote cell line. Since the viral RNAs produced from helper cells are devoid of retroviral *cis*-acting sequence, especially the encapsid-

ation site, the RNAs from helper cells cannot be encapsidated into a retroviral virion. The purpose of a packaging cell is, therefore, to provide *Gag, Pol,* and *Env* proteins to the retroviral vector having no trans-acting sequences. Unlike the construction of a replication-defective retroviral vector, the promoters for *gag, pol,* and *env* gene expression need not be a retroviral LTR promoter. Any promoter that is active in the helper cell can be used because the helper cell does not have to have retrovirus *cis*-acting sequences, even though most helper cell lines available use the LTR promoter due to its strong activity. Most importantly, regardless of promoter, the Ψ sequence (between splicing donor and 5' end of *gag*) should be removed to prevent RNAs of *gag, pol* and *env* from encapsidation.

Among retrovirus production systems for gene transfer in mammalian cells, NIH_3T_3 cells transformed with appropriate MLV genes are the most popular due largely to the relatively simple structure and good characterization of MLV, as well as the permissiveness of NIH_3T_3 cells for MLV gene expression. There are 3 kinds of MLV: ecotropic, amphotropic, and xenotropic (Weiss, 1982). Ecotropic MLV is able to infect only cells of mice and rats, amphotropic MLV can infect not only murine cells but also other cells, whereas xenotropic MLV can infect only non-murine cells. The host range difference is mainly due to the difference of envelope protein which interacts with specific receptors on host cell membrane. Compared to amphotropic MLV, xenotropic MLV seems to be more infectious to bovine, pig, and horse cells (Delous et al., 1990; Kim et al., 1993a).

2. Retroviral vectors

To construct a virus that can be used as a vector, only the cis-acting DNA sequences should be retained along with a gene of interest to transfer. Consequently, the resultant virus is replication-defective, but with the coordination of trans-acting functions provided from another source, i.e., a helper cell or packaging cell, the virus becomes infective to target cells. In the target cell, there are no trans-acting elements of the retrovirus. Therefore, the infected virus cannot produce its progeny, and no spread of pathogenic viruses is expected from the target cells.

Generally most retroviral vectors contain all the cis-acting sequences described above. However, removal of some *cis*-acting sequences is possible without sacrificing retroviral vector characteristics: Firstly, attL and U3 (except promoter /enhancer) of the 5' LTR and attR of the 3' LTR can be deleted. Secondly, the sites related to mRNA splicing can be removed unless the vector is designed to exploit a posttranscriptional splicing mechanism. Finally, U3 (except attL) of the 3' LTR can also be omitted when virus-producing cells are to be constructed through the transfection (not infection) of recombinant viral DNA to the packaging cells (discussed in next section). Unlike the optional deletion of retroviral *cis*-acting sequences, the foreign DNA inserted inside a retroviral vector should not contain a poly (A) signal, otherwise full-length transcription of the retroviral vector can be hampered (Temin, 1987).

3. Vector virus-producing cells

Vector virus-producing cells are constructed by either transfection of retroviral vector plasmid to helper cells or infection of the helper cells with the viruses produced from other virus-producing cells. In the former approach, most of the U3 region in the 3' LTR can be deleted to prevent mutual suppression of two adjacent promoters (this will be discussed in section IV) in a retroviral vector sequence. The advantage of the latter approach over the former is higher

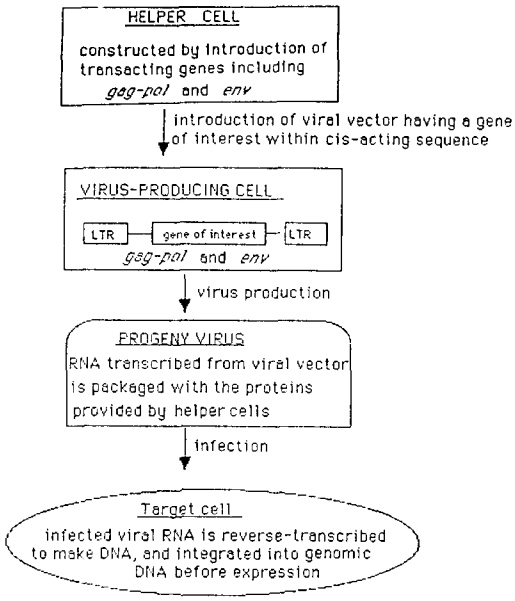

Fig. 2. Strategy for the construction of replication-defective retrovirus-producing cells and introduction of genes in target cells.

virus productivity in general because of lower susceptibility to methylation of the provirus in host celluar genome (Hwang and Gilboa, 1984). A summarized strategy for the construction of replication-defective retrovirus-producing cells is shown in Fig. 2.

Ⅳ. PROBLEMS IN THE USE OF RETROVIRAL VECTORS IN GENE TRANSFER

As well as some superiority of retroviral vectors over others, there are some drawbacks including size limitation, high rate of recombination, low titer, etc. Among them, the most significant limitation of the retroviral vector systems is low titer of the viruses. Hence the following description focuses mainly on titer.

1. SIZE

The maximum permissible size for efficient encapsidation or reverse transcription of each retroviral vector is approximately 10 kb (reviewed in Temin, 1987). Because introns of foreign genes carried by retrovirus vectors are spliced out during replication, the insert does not have to contain introns. Considering an insert of permissible size (around 8 kb) can code for a protein as big as 300 kd, maximum size limitation does not seem to be a significant obstacle in most cases.

The determination of minimum size of a retrovirus has some experimental difficulty due to the required size of a marker gene, however, it is believed that the lower limit is less than 2 kb as RNA form (reviewed in Temin, 1987). Considering the size of required *cis* acting sequences for a retroviral vector, low size limit is unlikely to be a problem.

2. Recombination

Genetic stability of retroviruses is intrinsically very poor, and the genetic variations including deletion, base pair substitution, insertion, recombination, etc. increase as the number of replication cycles increase (Temin, 1989). However, the provirus is as stable as the cellular genome because it is a part of chromosome. Consequently, the genetic variations in retroviral vector stock harvested from proviral transcripts in virus-producing cells are very low and tolerable. The exception to this is recombination.

The most serious effect of recombination is production of replication-competent retrovirus from virus-producing cells. To decrease this possibility, two strategies are generally applied: (a) Reduction of homologous sequences between the DNAs for packaging cells and vector

(Miller and Rosman, 1989) and (b) use of different plasmids to separate *gag-pol* and *env* genes (Markowitz et al., 1988).

3. Titer

Because, in most cases, titer of viruses is measured by the expression level of progeny virions on the target cells, factors affecting titer are reviewed in the following four sections.

4. Virus productivity

In determining virus productivity from packaging cells, at least three factors are involved: (a) Permissiveness of a specific cell line to be constructed as a virus-producing cell line for the expression of *gag, pol,* and *env* genes as well as the genes of the retroviral vector. (b) Non-viral negative *cis*-acting sequences in the retroviral vector.

An example of permissiveness is the poor expression of the avian spleen necrosis virus LTR in rat cells compared to that in dog or chicken cells (Emerman and Temin, 1984). In the case of *gag, pol* and *env* genes, their expression can be optimized by using appropriate promoters that are active in the cells to be used as a packaging cell line. Likewise, transcription of the provirus can be optimized by substituting an appropriate promoter for the enhancer/promoter region of U3.

The best example of a non-viral negative cis-acting sequence is the *E. coli* neomycin resistance gene (Artelt et al., 1991), which is one of the most frequently used reporter genes in eukaryotic cells. Artelt and his colleagues reported that the neomycin resistance gene acts as a silencer on proximal promoters of SV40 and retroviral myeloproliferative sarcoma virus in a location and orientation-independent manner.

One of the novel ways to increase virus productivity is overcoming superinfection interference. Retroviruses cannot infect cells producing envelope proteins of the same class because the proteins block the interaction between cellular receptors and specific retroviral envelope proteins before infection (Temin, 1988). However, co-culture of two different virus-producing cell lines, each of which produces replication-defective progeny virions infectious to the other cell line in co-culture, can overcome this superinfection interference barrier. For example, co-culture of amphotropic and ecotropic virus-producing cells can amplify the copy number of proviruses in the chromosome of each cell line to high levels by continuous mutual infections. Even though the relationship between gene copy number and expression level is not well understood, successful amplification of virus titer by using this approach has been reported (Kozak and Kobat, 1990).

5. Infectivity of virus

Because the susceptibility of host cells to retroviral infection is determined by specific interactions between the virion envelope glycoproteins and cell-surface receptors, it is sometimes very difficult to find a suitable *env* gene whose product is recognized favorably by cellular receptors. For example, to bovine target cells, retroviruses encapsidated with xenotropic MLV or Gibbon Ape monkey leukemia virus envelope protein are more infectious than those packaged by amphotropic MLV proteins. (Kim et al., 1993a, b).

6. Integration into the host cell genome

It is generally believed that productive infection by retrovirus requires integration of reverse transcribed retroviral DNA. This integration occurs only in mitotically active cells (Stevenson et al., 1990) during S phase (Savatier et al., 1989). Therefore, to maximize stable expression

of the genes introduced by a retroviral vector, it is reasonable to maintain the target cells in an active state of mitosis by treating with mitogenic agents, *i.e.*, PHA (phytohemagglutinin), if necessary (Stevenson et al., 1990).

7. Expression of MoMLV LTR in mouse embryos

Even though the initial evidence on the inactivity of MoMLV LTR in the preimplantation stage mouse embryo dates back as early as first transgenic mice (Jaenisch et al., 1975), the first report presenting the exact developmental stage at which the MoMLV LTR becomes active was available just recently (Savatier et al., 1990). By infecting mouse embryos of different developmental stages with replication-defective MoMLV based vectors followed by one or two days of expression time, Savatier and his colleague demonstrated that (a) embryos infected with retrovirus at blastula stage did not show any evidence of MoMLV LTR activation and (b) initial activation timing of the LTR varied, depending on the differentiation time table during embryo organogenesis.

After the LTR becomes inactivated due to the introduction of retroviral genes into preimplantation embryos by either infection or pronuclear microinjection, the destiny of the repressed retroviral genes seems to be either persistently repressed (Rubenstein et al., 1986) or *de novo* activated at a low frequency as the embryo develops (Jaenisch et al., 1975). Jaenisch and colleagues have suggested that the chromosomal position of the provirus determines the expression timing of the inactive methylated provirus genes through *de novo* demethylation (Jaenisch et al., 1981; Jahner et al., 1982). However, the role of methylation is obscure because, unlike the differentiated EC (embryonal carcinoma) cells, the undifferentiated cells are still non-permissive for retroviral gene expression even after demethylation by treatment of cells with 5-azacytidine (Niwa et al., 1983).

Since then, further studies have demonstrated that several factors other than methylation are involved in the repression of MoMLV expression. For example; (a) chromosomal position where 5' flanking sequence affects expression of a provirus (Taketo and Tanaka, 1987), (b) 5' untranslated region of the retrovirus including primer binding site where stem cell specific repression factors interact (Petersen et al., 1991), and (c) low level of transcription-activating −353 to −150 region binding protein and high level of transcription-inhibitory −352 to −346 region binding protein in EC cells (Tsukiyama et al, 1989). Among the four factors enumerated above, the trans-acting elements of (c) are controversial (Feuer et al., 1989).

The most common solution for this problem is to construct a retroviral vector with one internal promoter controlling the expression of a gene of interest (Wagner et al., 1985). However, this approach also has one limitation mutual suppression of two adjacent promoters (Emerman & Temin, 1984). Possible solutions for this might be; (a) designing of a SIN (Self-Inactivating) vector by deleting U3 promoter /enhancer region in the 3' LTR (Hawley et al., 1989), (b) substitution of a picornavirus 5' nontranslated region for an internal promoter (Adam et al., 1991), (c) modifying the LTR to be functional in EC and ES cells (Grez et al., 1990), or (d) selection of an appropriate internal promoter that can be functional in target cells with minimal interactions with LTR promoter /enhancer (Soriano et al., 1991).

V. FUTURE ASPECTS

Even though we understand that retrovirus-mediated gene transfer is far superior to conventional DNA microinjection approach, reports about successful application to retrovirus-mediated transgenic livestock production is very few: Demonstration of the first successful gene transfer to bovine blastocysts was done by our group (Kim et al., 1993a, b). By using the virus vectors encapsidated with xenotropic MLV or Gibbon ape leukemia virus envelope protein, we could confirm expression of the transferred *E. coli* LacZ gene in bovine blastocysts. Following our works, Haskell and Brown (1995) reported incorporation of proviruses DNA in the bovine fetuses. In these three successful reports, however, one of the biggest problems to be solved before main application for trangenic cattle production, was low infectivity of the viruses encapsidated with xenotropic MLV or Gibbon ape leukemia virus envelope protein. One possible solution might be use of the retrovirus vector packaged by vesicular stomatitis virus G protein (Burns et al., 1993). The most significant advantage of this hybrid vector system is that the virus stock is ultracentrifugally concentratable without significant loss of infectivity. Most of currently availble retrovirus envelopes are known to be very unstable and concentration of the viruses without loss of infectivity is very difficult. Burns and her colleagues reported that titers of $>10^9$ colony-forming units/ml was possible in certain target cells, and the host cell range extends even to the fish cells. Most importantly, this highly concentrated virus stock can reduce mosaicism of retrovirus-mediated transgenic animals, and also facilitate direct delivery of exogenous genes to the specific organ of the adult animal (Archer et al., 1994). Expression of the foreign gene in a specific part of the animal can dramatically reduce the time of the transgenic animal production and alleviate physiological side effects of the transgenic animal due to expression of the foreign gene in every part of an animal body (Ebert et al., 1988).

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