韓國家畜繁殖學會誌 19(4) : 283~291(1995)
Korean J. Animal Reprod.

Modification of Animal Genotypes for the Regulation of Transgene Expression

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이식유전자 발현조절을 위한 동물유전자의 조작

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요 약

Transgenic animal을 응용할 수 있는 분야에서는 이식유전자의 기능을 정확하게 규명하고 이를 바탕으로 실질적인 유전적인 개량을 이루기 위해서 이식유전자의 발현을 조절할 수 있는 정교한 system이 필요하다. 유전자의 미세주입법에 의해 transgenic animal을 생산할 수 있는데 이용되고 있는 tissue-specific promoter에 의한 이식유전자의 발현조절은 필요로 하는 시기나 양 등을 인위적으로 조절하고자 하는데 한계점을 갖고 있다. 이러한 이식유전자 발현의 문제점을 극복하기 위해 효모의 recombinase나 미생물의 repressor 단백질과 이들의 binding site인 operator sequence를 이용하여 인위적으로 이식유전자의 발현을 조절할 수 있는 system이 개발되고 있다. Cre/loxP system은 site-specific recombination에 의해 DNA sequence를 제거함으로써 이식유전자의 발현을 조절할 수 있다. 이식유전자 발현의 장소와 양을 조절하기 위해서는 미생물이 이용하고 있는 repressor와 이들의 operator sequence를 적용하여 ligand binary system이 개발되었다. Lac repressor system에서는 isopropyl-β-D-thiogalactoside (IPTG)가 이식유전자 발현을 조절할 수 있는 positive regulator로서 작용하고, tetracycline-VP16 system에서는 tetracycline이나 유사물질들이 negative regulator로서 이용할 수 있다. 이러한 binary system은 transgenic animal에서 이식유전자 발현의 장소와 시기 또한 양을 효과적으로 조절하는데 적용할 수 있는 것으로 나타났다. 따라서 기존의 binary system과 함께 새로운 regulatory system의 장점을 이용하여 보다 완벽한 이식유전자의 인위적인 조절 system을 이룩함으로서 transgenic animal technology의 실용화를 앞당길 것으로 기대된다.

I. INTRODUCTION

One of the critical trials in transgenic livestock was the production of transgenic pigs which contained metallothionine promoter fused to human or bovine growth hormone gene (Pursel et al., 1989). Even if transgenic mice with the same construction grew twice as much as control, the net result in transgenic pigs was disappointing. The severe side effects of uncontrolable growth hormone level made some beneficial growth performance worthless for practical application. More accurate regulation of transgene expression as well as specific gene targeting are needed for adaptation of gene transfer into livestock animals. Inducible systems for transgene expression *in vivo* are required for the better control of phenotype by turning transgene expression on or off. Microin-

jection of DNA into pronucleus of 1-cell stage embryos is still most popular method for producing transgenic animals. By improvement of injection condition, consistant efficiency can be obtained. However, this techinque has some obstacles for the gene integration and gene expression. Integration of exogenous DNA occurs randomly in host chromosome. Also, number of integrated gene are variable (few to several hundred copies). Somehow it is very difficult to control gene copy number and gene expression.

There are several ways to regulate gene expression in transgenic animals. Inducible promoter or binary approaches using strong transcription transactivation molecules or transcription repressor can be used. For the control of transgene expresssion, we can use developmental- and tissue-specific promoters which are responsive to various treatments such as heat shock, heavy metal or hormone. These systems have some advantage that is great inducibility of transgenes in some case. However, since such promoters are responsive to cellular transcription factors, induction may be altered by those endogenous factors. Furthermore, these promoters often have relatively high basal activities and various inducibility. One of inducible promoter is metallothionein promoter. Several of reporter gene expression can be induced by supplyment of metal ion. Basal level of expression is high in wide range of tissues. In transgenic pig, expression of transgnene (GH) could not be controllable even after mature age (Pursel et al., 1989). Promoters or regulatory elements for the tissue- and developmental-specific expression of transgenes are required to switch on and off in appropriate time or tissues. Maximum undesirable side effects can be eliminated from the transgenic animals while desirable effects can be maximized.

In this review, I will describe recent techniques of binary systems in transgenic mice for better regulation of transgene expression.

Ⅱ. BINARY SYSTEMS

The target transgene is silent in one transgenic line and can be expressed upon crossing with another transgenic line which carrys either a recombinase gene or a transactivator gene. In these systems, the temporal expression of target gene is dependent on the expression pattern of the effector molecule (recombinase or transactivator). Especially bacterial repressor proteins with their operator sequences offer a distinct advantage as regulatory switches in mammalian cells since the operator sequences are recognized uniquely by the repressor and this repressor binding ability can be modulated by ligand molecules.

1. Recombinase system

Some baterial and yeast recombinase enzymes cleave DNA st specific target sequences and ligate it to the cleaved DNA of a second site (Kilby et al., 1993). These simple reaction creates precise recombination between two appropriate target sequences. The cre recombinase from bateriophage P1 and FLP recombinase from yeast plamids can catalyze recombination between about 35 base pair DNA target sites. Eukaryotic genomes unlikely have this size of target site at random and the expression of appropriate recombiase is required for the recombination reaction. This site-specific recombination system can be used in any species. The 38 kDa cre (causes recombination) recombinase of bacteriophage P1 recognizes a 34 bp target site called loxP (locus of crossing-over). The 43 kDa FLP (flip) recombinase carried by circular plasmid of budding yeast catalyze recombination between two 34 bp target sites called FRT (FLP recog-

nition site). These recombinase systems have similarity in recombiation mechanism and their target site structures. Deletion can occur when DNA constructs containing loxP-loxP are exposed to a transient source of cre recombinase while FLP recombinase can effectively remove DNA sequences flanked by FRT sites in mammalian cells (O'Gorman et al., 1991). These systems can be used for activating genes by removing a blocking sequences or eleminating gene activities by deletion. Site-specific recombinations can be applicable for the study of development biology by activating or removing genes at particular stages. Such controlled gene expression could mark a clone of cells for lineage studies or allow the effect of lethal or deleterious sequences to be studied in a particular cell type or developmental stage.

The potential of recombination system in this direction was examined by producing transgenic mice expressing cre recombinase by microinjection (Lakso et al., 1992). These were mated with mice carrying a lens-specific αA-cristallin promoter separated from a SV40 T antigen coding sequence by an stop codon sequence flanked by loxP sequences (Fig. 1). In double transgenic animals, cre protein excises stop codon by recombination of the two loxP sequences and activates SV40 T antigen resulting in lens tumors. Advantages of cre /loxP system can extend the control of transgene copy number at integration site in transgenic animals and insertion of DNA sequences to preintegrated loxP site in the mammalian genome (Fushige and Sauer, 1992).

2. Ligands as regulators

The repressor binds to its operator sequences to block the passage of RNA polymerase II (Deuschle et al., 1990). The repressor-operator complex can interfere with eukaryotic gene expression *in vivo* when it placed immediately up-

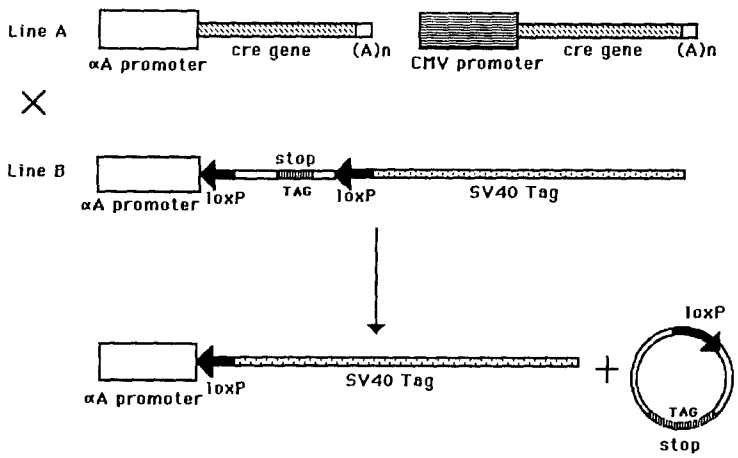

Fig. 1. The cre/loxP system used to activate transgene in transgenic mice. Line A mice contained cre gene fused to tissue-specific αA-crystallin promoter or CMV promoter. Line B mice carried SV40 T antigen gene separated from αA-crystallin promoter by stop codon and two direct repeat loxP sites. In double transgenic mice produced by crossing line A and line B mice, SV40 T antigen was expressed on lens of eyes (Lasko et al., 1992). (A)n : poly A signal, αA promoter : αA-crystallin promoter, Tag : T antigen.

stream or downstream of the transcription initiation site. One example of these binary systems is lac repressor/operator. The lac repressor, the product of lac I gene, has strong binding activity to its operator sequence and block transcription of gene. The lac repressor can inhibit gene expression from SV40 promoter, vaccinia virus promoter or T3 bacteriophage promoter in mammalian cells when its operator was placed in these promoters. However, the non-hydolyzable galactose analogue isopropyl-β-D-thiogalactoside (IPTG) inhibits strongly lac repressor binding activity to its operator. Transgenic animal can be produced by using tissue-specific promoter fused to lac repressor gene and transgene fused to promoter containing lac operator sequence (Fig. 2). In double transgenic animals, lac repressor inativates transgene expression by its binding to operate sequence. IPTG adminstration into tissue can change the conformation of lac repressor to inactive form so that transgene can be expressed from tissuespecific promoter. Transgene expression can be modulated by administration of IPTG in transgenic animals. However, it may be difficult to administrate lactose analogue *in vivo* into tissues to control transgene expression and level of expression or inducibility was not consistant in mammalian cells.

An alternative approach would combined the unique specificity of bacterial repressors with the powerful induction system provided by certain mammalian transactivator (Labow et al., 1990; Gossen and Bujard, 1992). Eukaryotic transcription factors are often composed of separate, independent DNA binding and transcriptional activator domains. The independence of the domains has allowed for the creation of functional fusion proteins consisting of the DNA binding and transactivating domains of heterol ogous proteins such as lac repressor or tetracycline repressor and virion protein 16 (VP16) transactivator of herpes simplex virus type 1 (Fig. 3). In this system, ligand serves as a negative regulator for the transgene expression. The development of a regulatory circuit based

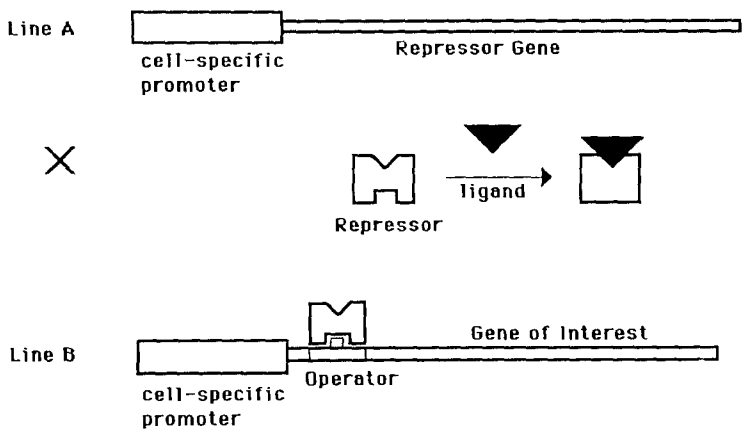

Fig. 2. Ligand-positive binary system. Line A mice were generated by microinjection of lac repressor gene under the control of tissue-specific promoter. Line B mice contained gene of interest fused to lac operator sequences and tissue-specific promoter. In double transgenic mice ligand changes the conformation of repressor molecules and blocks binding of repressor to its operator sequences and activates transgene expression.

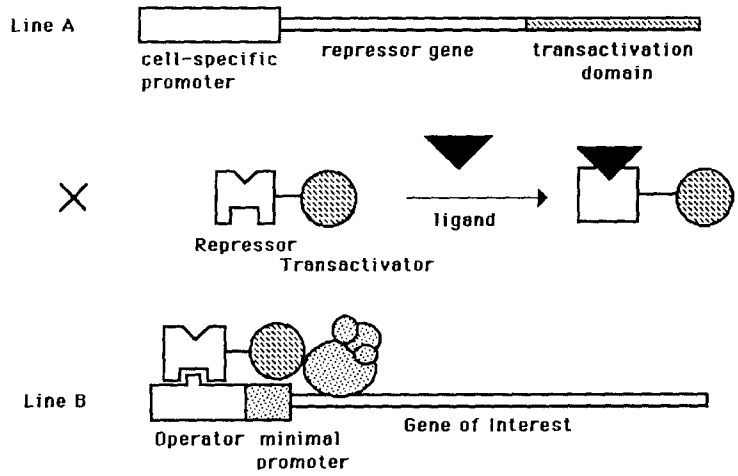

Fig. 3. Ligand-negative binary system. Line A mice carried fusion genes encoding repressor-eukaryotic transactivator complex under the control of tissue-specific promoter. In line B mice, gene of interest was placed with operator sequences and eukaryotic minimal promoter. Ligand inhibits binding of repressor to its operater sequences and inactivates gene expression in double transgenic mice while repressor-transactivator complex is bound to its operator and activates gene expression without ligand.

on the tetracycline-resistant operone from *E. coli* transposon Tn10 opened a new approach for controlling transgene expression (Passman and Fishman, 1994; Efrat et al., 1995; Furth et al., 1995; Shockett et al., 1995). In the presence of the antibiotic tetracycline, its repressor (tetR) does not bind to its operators located within the promoter region to allow the transcription of gene. By combining tetR with the carboxy-terminal domain of VP16 from HSV, a hybrid molecule can activate minimal promoter fused to tetracycline operator (tetO) sequence. One of the most efficient eukaryotic transactivator is the carboxy-terminal 100 amino acids of VP16. The VP16 transactivation domain has been previously shown to function efficiently when fused to heterogenous DNA binding domian such as GAL4 (Sadowski et al., 1988). The tetR-VP16 fusion protein is tetracycline-controlled transactivator (tTA) by binding to tetO sequence and strongly activates transcription from a minimal promoter. The tTA binds to the tetO sequence in the absence of tetracycline but not in its presence (Fig. 4). The introduction of tetracycline results in suppression of gene expression. One of application for this system in transgenic mice was the study of β-cell tumorigenesis (Efrat et al., 1995). Two separate transgenic mouse lines were produced ; simian virus 40 (SV40) large tumor T antigen (Tag) fused to cytomegalovirus (CMV) minimal promoter containing tandem array of tetO sequence and the tet repressor gene fused to the VP16 (tTA) with rat insulin promoter. These separate lineages of mice were crossed to produce double transgenic mice. In double transgenic mice, the expression of tet repressor-VP16 fusion protein in β-cells stimulated transcription of SV40 T antigen without tetracycline and resulted in development of β-cell tumors. Tetracycline inhibited β-cell proliferation

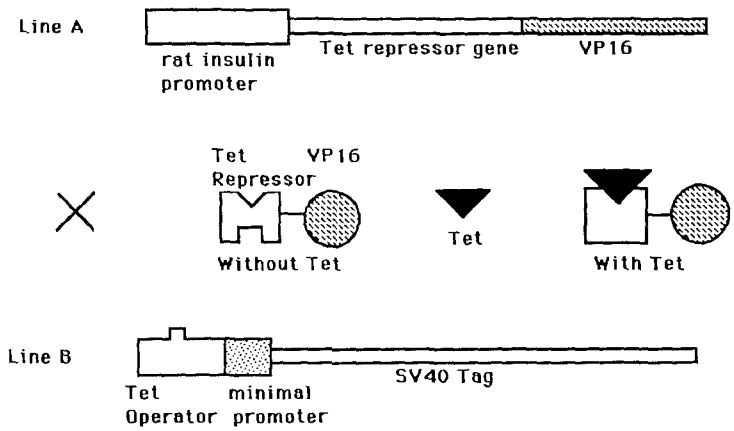

Fig. 4. Tetracycline-responsive binary system. Line A mice carried tetracycline repressor gene and VP6 transactivator gene under the control of rat insulin promoter. Line B mice contained SV40 T antigen fused to tetracycline operator sequence and CMV minimal promoter. Tetracycline or its derivatives inhibit binding of repressor to its operator sequence to inactivate transgene expression in double transgenic mice (Efrat et al., 1995).

through inactivation of tet repressor. Teracycline derivatives such as doxycline or anhydrotetracycline are readily absorbed in the animal and broadly distributed to different tissues with minimal toxicity at the concentration needed to regulate the activity of the synthetic promoter (Gossen and Bujard, 1993). Administration of tetracycline derivatives in the drinking water or with slow release pellets can inhibit transgene expression *in vivo*. Through the withdrawal of tetracycline derivatives, reversible effect can be obtained. This kind of regulatory system will provide better control of transgene at various stage of development.

3. Conditional nuclear localization system

Transport of repressor proteins or transcription factors from cytoplasm to nucleus for facilitating their binding to specific DNA sequences is an essential step to control gene expression. Inhibiton or activation of nuclear localization of effector molecules can provide another regulatory system for the regulation of transgene expression. One of the putative candidates is steroid hormone receptor-heat shock protein 90 complex system (Fig. 5). Steroid hormone receptors are involved in direct signal transduction system as a hormone-activated transcriptional regulator by binding cognate responsive elements of regulated genes (Lindquist and Craig, 1988; Pratt, 1993; Smith and Toft, 1993; Rutherford and Zucker, 1994). Hormones induce nuclear translocalization of the receptors to recognize elements of target promoters. The receptors have separate domains responsible for hormone binding and DNA binding. Both hormone and heat shock protein 90 (hsp 90) seem to be bound to the same domain of receptors. Hsp 90 is an abundant protein in cells and believed to be bound to hormone binding domain of steroid hormones including estrogen receptor, progesterone receptor or glucocorticoid receptor. When hormone binding domain of certain receptor is fused to transcription factors or repressors, hormone or

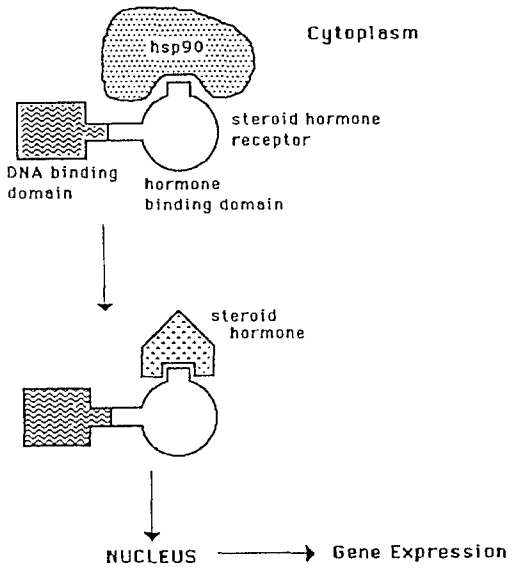

Fig. 5. Steroid hormone receptor signaling system. Steroid receptors are associated with hsp 90 in the inactive form. Upon hormone binding, the receptors are converted into active state and moved in nucleus to be bound to its resposive elements of genes. The receptors are consisted of distinct DNA binding and hormone-binding domain. Transcription factors fused to receptor hormone-binding domain can serve as modulators for steroid hormone-responsive binary system.

its analogue as a ligand can be used for transport of these molecules to nucleus. In the same manner as binary system, the expression of transgene fused to operator or promoters which have responsive elements can be modulated by the administration of hormone in tissues expressing its receptor-repressor or receptor-transcription factor complex. Or endogenous hormone-related transgene expression can be obtained so that this system can be applicable in the field of reproductive physiology. This system is currently being tested.

Ⅲ. DISCUSSION

Binary system approaches allow for the modulation of transgene expression to be tissue- and time-specific in double transgenic animals. The cre/loxP system provide the precise recombination of DNA for either gene activation or disruption. The amount of cre recombinase in double transgenic animals seems to be critical for the efficiency of recombination at loxP sites. The promoters used to drive cre expression should be transiently strong at certain stage or tissue to accomplish conditional reconsititution of transgene in cre/loxP transgenic animals. The application of cre/loxP system can also be aimed at efficient production of transgenic animal as well as precise reduction of the transgene copy number at any integration site by insertion of DNA sequences at preestablished chlomosomal loxP site in the genome (Fukushige and Sauer, 1992). The cre/loxP system has already been used in gene knock-out experiments for tissue- or developmental-specific gene disruption which is superior to all knock-out system (Gu et al., 1994).

The ideal system for the control of transgene expression should provide on/off switch function as well as the regulation of expression level at a defined manner. The well-characterized regulatory systems from *E. coli* has been designed for these purposes. The lac repressor/operator complex interferes RNA polymerase Ⅱ elongation. The operator sequences can place immediately upstream or downstream of transcription initiation site to block eukaryotic gene expression (Deuschle et al., 1990). IPTG is known to be a very effective regulator for inhibition of lac repressor binding activity. Both lac repressor and tetracycline repressor have high binding activity to their operator sequences with

specificity in mammalian genome. However, IPTG has been shown to exhibit slow inducibility and inefficient kinetics *in vivo*. The lac repressor/operator system requires improvement of optimum condition of IPTG regulation and development of other ligands for better inducility. The tetracycline repressor/operator system without eukaryotic transactivator can be used as a negative effector. However, to achieve complete inhibition of transgene inhibition, strong promoter has to be used for repressor molecule expression. Otherwise, basal activity of gene expression would be extremely high. Using high affinity of repressor to its operator sequence, eukaryotic transactivator molecules such as GAL4 or VP16 can be fused to repressor molecules to serve as a positive regulator for operator-driven gene expression. The tetracycline repressor-VP16/operator system has shown great inducibility and quantitative effect on transgene expression of transgenic mice. The concentration of tetracycline required for complete blocking of gene is less than 0.1 μg/ml with linear regression (Gossen and Bujard, 1992). Tetracycline derivatives are broadly penetrated in the tissue with minimal toxicity for regulation of transgene expression. However, this system may have difficulty in expression of tetracycline repressor-VP16 fusion protein probably due to VP16 transactivator domain causing cell death. This problem can overcome by placing the tetracycline repressor-VP16 gene under the control of a promoter containing tetracycline operator sequences (Shockett et al., 1995). Such modification of these binary systems or combination with new regulation system (eq. steroid hormone receptor/hsp 90) can contribute to complete regulation of transgene expression in transgenic animals.

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