

# Construction of novel conditional transgenic vectors for molecular therapy based on the Tet-On system and flanked with insulators in a single plasmid

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**Abstract.** Regulated expression of a gene of interest is crucial for transgenic research, as well as for safe and efficacious gene therapy. The most commonly used conditional expression system requires the generation of two transgenic strains, one carrying an inducible promoter and the other a transactivator. The generation of conditional transgenic models using this method is costly and time consuming. In this study, we report the design and construction of novel simplified gene delivery vectors that integrate both the regulatory and responsive elements in a single vector. The Tet-On system was used and integrated the inducible promoter and transactivator in a single plasmid, between which a copy of an insulator was inserted to minimize interference between the two units. Another copy of an insulator was inserted upstream of the transgene cassette to eliminate transgene silencing and to lower basal expression. The two insulators were in the same orientation. To further decrease basal expression, the most powerful repressor domain containing a 'kruppel-associated box' of the zinc finger protein NK10 was used and fused to TetR in one of the two vectors. The function of this system was confirmed after *in vitro* transient transfection. The two conditional plasmids were successfully constructed, and the expression of the gene of interest was regulated tightly *in vitro*. In conclusion, the vectors described here may be useful for gene therapy applications, as well as for the establishment of conditional animal models.

## Introduction

Over the last two decades, fundamental insight into the function of genes has been efficiently provided through conventional knockout and transgenic experiments. Although genetically modified mice have provided important new information about the function of many genes, there are serious limitations to current animal models for a number of diseases. Introducing genetic changes to the germ line of a mouse may track down the effects of a particular gene, but may also have severe developmental consequences, complicating or even precluding the desired experimental analysis. A good example illustrating these restrictions is embryonic lethality. In gene therapy, an ideal vector should include a regulatory system that is off in the resting state, exhibit tight regulation, and allow for rapid and repeatable induction in response to a clinically approved inducer molecule. To overcome these undesired limitations and to precisely control gene expression, conditional mouse models are becoming increasingly popular. Although there are many types of inducible transgenic expression systems, tetracycline-controlled expression systems have been employed most frequently. The tet system, developed by Gossen and co-workers, includes two basic variants: the Tet-Off system (tTA) and the Tet-On system (rtTA) (1,2). As a positive regulatory system, the Tet-On system has been used more widely in numerous transgenic animal models (3,4). The original tet system contained two transcriptional units, the transactivator and the responder, on two plasmids. Currently available tetracycline-regulated transgenic methods require the generation of two transgenic strains, one carrying the transgene of interest under the control of the tet operator, and the other the reverse transactivator (rtTA). Crossing the lines generates progeny with both transgenes, allowing the regulation of the gene of interest through the administration of tetracycline. However, crossing and analysis of animals transgenic for the individual components of the system is costly and time consuming. Therefore, the generation of a vector containing the two units in a single plasmid may provide advantages for the simplified generation of conditional transgenic animals. The successful use of a single construct containing the two elements has been

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reported (5-7). In addition, improvements to newly created tet-based systems should include the elimination of background expression, increased sensitivity to tetracycline or its derivative, doxycycline (Dox), and increased inducibility.

In the present study, we designed and constructed two novel conditional transgenic vectors combining the two transcriptional units on a single plasmid. In addition, in order to eliminate the background expression of the gene of interest, the most powerful repressor domain containing a 'kruppel-associated box' of the zinc finger protein NK10 (8,9) was used and fused to tetR. The transactivator was placed under the control of the CMV promoter and the tet responsive element, driving the gene of interest, was inserted downstream of the same vector. To minimize any potential interference between the two elements, they were spaced with a 1.2-kb cHS4 insulator. To shield the transgene from chromosomal position effects and to improve transgene expression efficiency, another copy of the cHS4 insulator was inserted upstream of the transgene cassette. The two insulators were placed in the same orientation.

## Materials and methods

### Plasmid construction

**Construction of regulatory units.** The first step of the study involved the cloning of the cHS4 insulator from chicken genomic DNA. The primers used were forward primer 5'AA AACGCCTCCAGGAAACCACTCT3' and reverse primer 5'CAGCAATATTCCCCCATCTCACT3'. To ensure that the sequence was in frame with the targeting sequence, the pfu enzyme was used for the PCR experiments. PCR conditions were 95°C for 5 min followed by 34 cycles at 94°C for 30 sec, 60.1°C for 40 sec and 72°C for 2 min 40 sec, and then 72°C for 10 min.

The NK10 gene was cloned from brain tissue cDNA. The primers used were forward primer 5'GAATTCGGAATGGC CCGAGACCT3' and reverse primer 5'CTGCAGTCAATGC CCCTGGATAGCGACATT 3'. *EcoRI* and *PstI* restriction site sequences were respectively incorporated into the 5' end of the PCR primers. PCR conditions were 95°C for 5 min followed by 34 cycles at 94°C for 30 sec, 61°C for 40 sec and 72°C for 1 min, and then 72°C for 10 min.

The tetR gene was cloned from the plasmid of Ptet-off. The primers used were forward primer 5'AAACTCGAGCCCGGG ATGGCTAGATTAGATAAAAG 3' and reverse primer 5'AAA GAATTCCTTTCTCTTCTTTTGGCGACCCACTTTCA CATTT3'. The *XhoI* and *EcoRI* restriction site sequences were respectively introduced into the 5' end of the PCR primers. PCR conditions were 95°C for 5 min followed by 34 cycles at 94°C for 30 sec, 61.5°C for 40 sec and 72°C for 1 min, and then 72°C for 10 min.

The 1.36-kb cHS4 insulator was digested by *SacII* and *SspI*, and the *SacII-SspI* fragment (1.2 kb) was subcloned into the *SacII-EcoRV* fragment of plasmid pSK<sup>+</sup> to yield the plasmid pBSHs4. The NK10 and tetR PCR products were respectively subcloned into the T-vector. The pMD-NK10 plasmid was digested by *PstI* and *EcoRI*, and pMD-tetR was digested by *XhoI* and *EcoRI*. The *PstI-EcoRI* fragment, containing the NK10 gene, and the *XhoI-EcoRI* fragment, containing the tetR gene, were subcloned into the *PstI-XhoI* fragment of the pSK<sup>+</sup>

plasmid to yield the plasmid pBSNK10-tetR, in which the NK10-tetR gene could be translated into a fusion protein. The plasmid pBSNK10-tetR was digested by *XhoI*. After the end was blunted and purified, the fragment was digested by *NotI*. The plasmid pIRES-EGFP was digested by *BstXI*. After the end was blunted and purified, the fragment was also digested by *NotI*, cutting out the EGFP gene. The *XhoI-NotI* insert fragment containing the NK10-tetR gene and the *BstXI-NotI* vector fragment were ligated together to generate the plasmid pIRES-NK10-tetR. To build the plasmid pVP16-NK10-tetR, an *EcoRI-PstI* digestion was carried out on the plasmid pIRES-NK10-tetR, generating the 4-kb *EcoRI-PstI* vector fragment. A *BamHI-PstI* double digestion was also performed on the same plasmid to obtain the 1.6-kb *BamHI-PstI* insert fragment. The ptet-on plasmid was digested by *EcoRI* and *BamHI*, isolating the 1.6-kb *EcoRI-BamHI* insert fragment rtTA. The three fragments, *EcoRI-PstI* vector fragment, *BamHI-PstI* insert fragment and *EcoRI-BamHI* insert fragment, which all had sticky ends, were ligated in one ligation reaction, and the pVP16-NK10-tetR plasmid was constructed. The pVP16-NK10-tetR plasmid was digested with *BamHI* and *NotI*, cutting out the IRES-NK10-tetR fragment, and was then itself ligated after its end was blunted, generating the plasmid pVsp-tet-on.

### Construction of responsive elements

**Cloning of the MT gene.** The plasmid pPyMT1 containing the MT gene was digested by *BstXI* restriction enzyme. A 0.8% agarose gel electrophoresis loaded with all the samples was used to isolate the 1.53-kb fragments. After the ends were blunted and purified, the fragments were digested by *HindIII*, and the *BstXI* (blunting)-*HindIII* fragment (1.48 kb) containing an open reading frame of the MT gene was subcloned into the *SmaI-HindIII* fragment of pSK<sup>+</sup>, yielding the plasmid PbsMT. A *BamHI-HindIII* digestion reaction was carried out on the plasmid PbsMT, and the *BamHI-HindIII* fragment containing the MT gene was cloned to the *BamHI-HindIII* vector fragment of the pTRE2 plasmid, yielding the vector pTREMT.

The pBSHs4 plasmid was first digested with *ClaI*, and then *SacI* digestion was carried out on the same fragment after the *ClaI* ends were blunted. The 1.2-kb *ClaI* (blunt)-*SacI* insert fragment containing the Hs4 insulator was ligated with the *SacI-EcoRI* vector fragment of the pN2-EGFP plasmid for which the *EcoRI* ends were also blunted. This plasmid was labeled pHs4-N2EGFP. The *XhoI-BamHI* fragment of the pTRE2 plasmid containing the TRE gene was cloned to the *SalI-BamHI* vector fragment of the pHs4-N2EGFP plasmid. As *XhoI* is an isoschizomer of *SalI*, this ligation reaction was sticky-ended, and the plasmid was named pHs4TRE-N2EGFP. The next step was to replace the EGFP gene of the pHs4TRE-N2EGFP vector with MT. The experiments were carried out as follows: pHs4TRE-N2EGFP was digested with *BamHI* and *NotI*, the *NotI* ends were blunted, and the vector fragment was ligated with the *BamHI-EcoRV* insert fragment of the pTRE-MT plasmid containing the MT gene. This plasmid was named pN2-Hs4TRE-MT.

**Ligation of regulatory units and responsive elements.** The *SspI-VspI* insert fragment of the pVP16-NK10-tetR plasmid (2.5 kb) and pVsp-tet-on (1.9 kb) were respectively ligated with the *NheI-VspI* vector fragment of pN2-Hs4TRE-MT, whose

*NheI* ends were blunted. Thus, the plasmids pVP16NK10-Hs4-TREMT and pVP16-Hs4-TREMT were constructed.

In order to insert another Hs4 insulator upstream of the transgene cassette, a plasmid was constructed. First, a pair of primers was designed: forward primer 5'AAAATCGATCATA TGGCGGAGCGAGGAACTGAG3', having *ClaI* and *NdeI* restriction site sequences, and reverse primer 5'AAACTC GAGGATATCCATATGCCGCCCTGGGAA3', having *XhoI*, *EcoRV* and *NdeI* restriction site sequences. The template DNA was the 1.5-kb MT gene, originated from the PbsMT plasmid. PCR was carried out to amplify a 500-bp fragment. The product as a spacer was digested with *ClaI* and *XhoI* and cloned to the *ClaI*-*XhoI* vector fragment of pBSHs4. Thus, the pBSHs4-Nde plasmid was constructed. Upstream of the spacer was the *NdeI* restriction site, and downstream were *NdeI* and *EcoV*. Therefore, the *VspI*-*SspI* insert fragments of pVP16NK10-Hs4-TREMT (6.9 kb) and pVP16-Hs4-TREMT (5.4 kb) could theoretically be cloned to the pBSHs4-Nde's *NdeI*-*EcoRV* vector fragment (4.2 kb). However, despite numerous attempts, we did not succeed in achieving this, and thus changed our strategy. The pBSHs4-Nde plasmid was first digested with *VspI* and *NdeI*. The 1.4-kb fragment containing two types of DNAs was extracted, and *SspI* digestion was carried out. Thus, two types of DNAs were divided, and the 1.4-kb fragment containing the 1.2-kb insulator flanking the sticky ends of *VspI* and *NdeI* was respectively cloned to the *VspI* site of the plasmids pVP16-NK10-Hs4-TREMT and pVP16-Hs4-TREMT, yielding the final plasmids pHs4-VP16NK10-Hs4-TREMT and pHs4-VP16-Hs4-TREMT. *BstXI* digestion was performed to determine the insert directions.

**In vitro experiments with the two plasmids.** To confirm the function of the two plasmids, transient transfection was carried out. COS7 cells were cultivated in DMEM for 72 h and transfected by electroporation with the same amount of the two plasmids. The electroporation conditions were: voltage, 220 V; electrical capacity, 975  $\mu$ F; no. of pulses, 1. After electroporation and the addition of 1.5 ml DMEM to the cuvette, equal amounts of cells were carefully transferred to two culture dishes. Dox (15  $\mu$ g/ml DMEM) was added to one dish 3 h after electroporation, and the other dish was used as the control.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was applied to measure the marker gene expression of the two plasmids. After 72 h of incubation, total RNA was isolated from the cells by the TRIzol method according to the manufacturer's instructions (Invitrogen, Cergy-Pontoise, France). In order to resolve the problem of genomic DNA contamination, 2  $\mu$ g RNA was digested with RNase Free DNase I (New England BioLab, UK). RT-PCR was carried out with 1  $\mu$ g of DNase-digested RNA. cDNA was generated using the First-Strand Synthesis System for RT-PCR (Takara, Dalian) followed by PCR amplification of the marker gene. Expression of  $\beta$ -actin, a housekeeping gene which is assumed to be expressed at roughly equal levels in different cell types and under different conditions, was examined as an internal control. The primers and templates used the same amount of substances in different reactions. The primers used were the MT forward primer 5'AAAGAATTCCCATTTTTT GGCTGGGCAGATATCGCGGAGCGAGGAACTGAG3' and MT reverse primer 5'AAAAGCTTCCATTTTTTGGAT

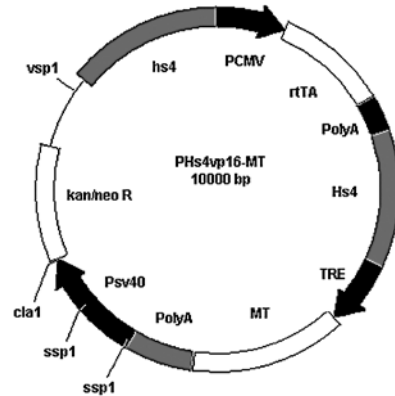


Figure 1. Map of the plasmid pHs4vp16-MT.

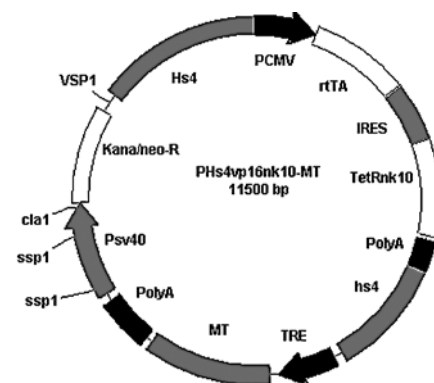


Figure 2. Map of the plasmid pHs4vp16nk10-MT.

GGCGAGATATCCGCCCTGGGAATGATAG3'.  $\beta$ -actin PCR primers were kindly provided by Dr Fei (IBCB, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) and were designed to span an intron of the genomic sequence, resulting in a PCR product from genomic contamination that was larger in size than the product generated from the cDNA. The PCR conditions were 95°C for 5 min followed by 27 cycles at 94°C for 30 sec, 60°C for 1 min 30 sec and 72°C for 1 min, and then 72°C for 10 min. Agarose gel (0.8%) loaded with the same volume of PCR products was run to test the differences in marker gene expression levels.

## Results

For the construction of the vectors, the following plasmids were used: pTet-On, pTet-Off, pTRE2, PBSsk, pIRES2-EGFP and pEGFP-N2, and the backbone of the final plasmid was pEGFP-N2. The Tet-Off and Tet-On systems were developed to regulate transgene expression, respectively providing negative and positive control of transgene expression. Although the Tet-On system produces high basal expression in the absence of an inducer, as a positive regulation system it is widely applied. In order to eliminate the basal expression, we cloned the 'kruppel-associated box' silencing domain of the zinc finger protein NK10 gene, a powerful transcriptional repressor, and ligated it with tet repressor to translate a fusion protein. The fusion pro-



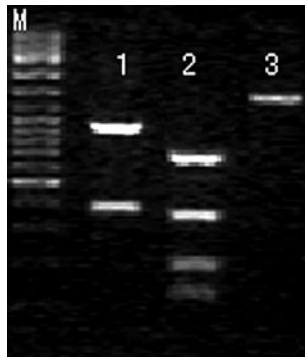


Figure 3. cHS4 insulator restriction enzyme digestion. Lane M, DNA ladder; 1, *Hind*III digestion; 2, *Pst*I digestion; 3, cHS4 PCR products.

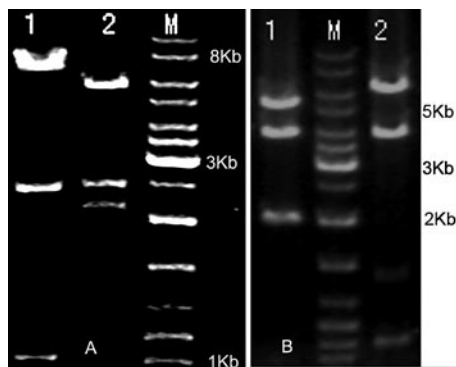


Figure 4. Results of *Bst*XI digestion for the plasmids pHs4vp16-MT (A) and pHs4vp16nk10-MT (B). (A) Lane 1, positive cloning; 2, negative cloning; M, DNA ladder. (B) Lane 1, negative cloning; 2, positive cloning; M, DNA ladder.

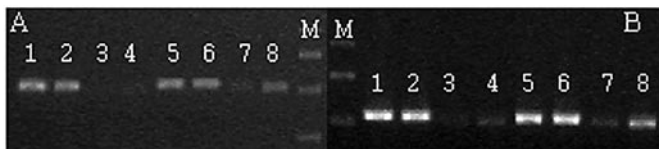


Figure 5. The results of semi-quantitative RT-PCR of the plasmids pHs4vp16nk10-MT and pHs4vp16-MT (lane 7 and 8). Cycles 22 (A) and 24 (B) are shown. No Dox was added in lanes 1, 3, 5 or 7; Dox was added in lanes 2, 4, 6 and 8. No difference was observed in  $\beta$ -actin expression before and after the addition of Dox (lanes 1, 2, 5 and 6). M, DNA ladder.

tein binds to the tet operator sequences in the response element only in the absence of Dox, and reduces leaked gene transcription. When Dox is added, the silencer dissociates from the tet operator, relieving transcriptional inhibition, while rtTA binds to the TRE and activates gene expression. To compare the efficiency and effectiveness of diverse operating systems, two different Tet-On-based inducible expression systems carried in a single cassette were constructed (Figs. 1 and 2).

In this study, three genes were successfully cloned using the PCR method. The sequences were verified using sequence-specific identification and restriction enzyme digestion (Fig. 3).

Restriction digestions were carried out to identify the final two transgene vectors, and analysis of the gel showed that the two plasmids were successfully constructed (Fig. 4).

The relative quantification of MT target transcripts was conducted using semi-quantitative RT-PCR in mRNA samples by comparison with the mRNA abundance of  $\beta$ -actin. The results showed that the plasmids pHs4vp16nk10-MT and pHs4vp16-MT had certain levels of transgene expression in the absence of tetracycline. The inducibility of pHs4vp16-MT was higher than that of pHs4vp16nk10-MT (Fig. 5).

## Discussion

Transgenic models have provided a wealth of information regarding the function of specific genes. However, the limitations of promoter-controlled transgene expression have become apparent. The constitutive system has no control over the time of expression, which depends entirely on the properties of the promoters used. The promoters in this setting were constitutively active, many from early in the embryonic stages. The target gene is activated as soon as the promoter becomes active. If the transgene product happens to be toxic to the organism, particularly during gestation, it can be detrimental to the developing embryo. This results in the failure to generate live progeny carrying the transgene, and the transgene cannot be studied past embryogenesis. If the gene product is not toxic to the embryos, then the animals are viable and may demonstrate an interesting phenotype. However, it is uncertain whether the phenotype is the result of transgene expression or the mutation of endogenous genes. To address these limitations, a number of investigators have established transgenic systems in which the expression of the transgene can be externally regulated. Although a variety of approaches have been utilized, tetracycline-controlled expression systems have been employed most frequently (10).

In the present study, we successfully constructed a single plasmid containing a tet-responsive transactivator together with the transgene responder. This strategy circumvents the need to generate two independent transgenic lines, and greatly facilitates mouse breeding as the effector and responder will not segregate.

A recent development in gene transfer approaches in plants and animals has revealed that transgenes undergo silencing after integration in the genome (11-13). Gene expression is influenced not only by the presence or absence of nearby transcription factors, but also by the structure of surrounding chromatin. Variable repression of transcription is often associated with heterochromatin and frequently manifests itself as position effect variegation. In an extensive study of this phenomenon, Cerutti *et al* (12) concluded that in *C. reinhardtii* the local heterochromatin structure rather than DNA methylation has a role in transgene inactivation. Insulators are DNA sequence elements that function to block the action of an enhancer and shield a transgene from a chromosomal position effect in transgenic animals and cell lines (14). Flanking a transgene with two copies of an insulator can shield it from a chromosomal position effect following stable integration. In a preliminary study, we attempted to insert two copies of 1.2-kb insulators into the flank of the transgene cassette (three copies of direct repeat sequences in one plasmid might cause it to be unstable). Despite numerous attempts, we did not succeed, even though STBL4 competent cells capable of stabilizing direct repeat sequences were used. Transgenes are usually

found inserted into a single chromosomal location as multiple copies forming head-to-tail concatemers (10), while insulators can be effective when placed to only one side of the transgenes in multicopy arrays. This strategy was successfully employed in this study and others (15-17). Theoretically, only the end copy of the transgene is susceptible to a position effect after the concatemers are integrated with germ line genomic DNA. This not only reduces cloning time and decreases plasmid size, but also improves transgene expression efficiency.

Tetracycline-inducible transgene expression systems should have negligible basal expression levels and high inducibility. However, these goals were not achieved in the present study. Background expression remained in the two vectors, though the background expression in pHs4vp16nk10-MT was much lower than that of pHs4vp16-MT, while the inducibility of the pHs4vp16-MT was much higher than that of pHs4vp16nk10-MT. The reasons are as follows: first, there was an enhancer/promoter of the neo gene in the two plasmids, which also acts on the CMV minipromoter of TR E, promoting the expression of the target gene from a distance. In *in vivo* experiments, this factor will be excluded after the linearization of the plasmid with *VspI* and *SspI* digestions. Second, in the plasmid of pHs4vp16nk10-MT, the products rtTA and tetR-NK10 can form heterodimers that do not function appropriately and compromise the overall inducibility of this system. On the other hand, competition exists between rtTA and tetR-NK10 for the DNA binding sites of TRE, which also lowers Dox inducibility. Basal expression was also associated with the site(s) of integration of the transgenic constructs, and enhancer sequences near the target gene have been repeatedly demonstrated to increase basal target gene expression, thereby compromising the desired tight regulation (18,19). To eliminate basal expression in our study, an insulator was used to block the effect of the enhancer near the transgene integration sites on TRE. Although the results of transient transfection testing showed a degree of leakage expression, in some cases the leak that resulted was acceptable and did not negate the ability of the experimental system to appropriately address the investigated hypothesis. Upstream of each of our transgene cassettes was a neo gene. When the vectors were only digested with *SspI*, this gene, being a selective marker, was included in the transgene cassette. This benefited the stable transfection of the two vectors *in vitro*.

In summary, we combined two expression units of the Tet-On system on a single plasmid by constructing two conditional transgenic vectors, one of which contained the NK10 gene fused to TetR. cHS4 insulator elements were utilized in the construction of the vectors to eliminate basal expression and transgene silencing. Our *in vitro* study demonstrated that the two 'two-in-one' systems were functional. This may minimize the breeding and genotyping time required for phenotypic characterization in the construction of conditional animal models. Moreover, when the vectors were digested only with *SspI*, a neo gene as a selection marker was included in the transgene cassettes. Therefore, the Tet-On system presented here should be easy to use and widely applicable to the study of gene function in transfected cells and molecular therapy, as well as for the creation of conditional transgenic animal models to study disease and development *in vivo*.

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