Expression of Gal4-VP16 and Gal4-DNA binding domain under the control of the T lymphocyte-specific lck proximal promoter in transgenic mice

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Thymocyte-specific transcriptional regulatory systems can be used to better understand the relationship between transcription and V(D)J recombination during early T cell development. In this study, we generated transgenic mice expressing the transactivator Gal4-VP16 or the Gal4 DNA binding domain (Gal4-DBD) under the control of the lck proximal promoter, which is only active in immature thymocytes. From these studies Gal4-VP16 and Gal4-DBD expression was shown to significantly alter thymic cellularity and differentiation without significantly changing the CD3+ thymocyte distribution. Furthermore, the presence of Gal4-VP16 or Gal4-DBD in the transgenic thymocytes retarded the mobility of the Gal4 DNA binding motif as determined by a gel mobility shift assay, suggesting that the developmental alteration did not affect the functional property of the transgenic proteins. These results indicated that lck promoter-driven Gal4-VP16 or Gal4-DBD expression did not affect CD3+ mature thymocytes, thus this system can be applied to study transcriptional regulation of transresponder genes in bigenic mouse model thymocytes. [BMB reports 2008; 41(8): 575-580]

INTRODUCTION

Transgenic mouse technology has significantly impacted in vivo functional gene analyses, but uncontrolled and deleterious effects of the expressed transgenes can make it difficult to interpret their physiological role in the transgenic mice. Furthermore, unique copy numbers and unpredictable sites of integration in every transgenic mouse result in variable transgene expression levels, and thus variable phenotypes.

Binary transgenic systems, such as the Gal4-UAS method, overcome such problems (1) by generating transgenic lines that carry both a “transactivator” and a “transresponder” component. The parent transactivator line expresses the yeast transcription factor Gal4 (2), which is under the control of the desired tissue-specific promoter. The transresponder line contains the gene of interest linked to upstream activator sequences (UAS) (3) that correspond to the area where the Gal4 DNA binding motif lies. Crossing these two lines generates the bigenic mice, where expression of the transactivator activates expression of the transresponder gene in a tissue-specific manner.

In the system developed in this study, the Gal4-VP16 system, the transactivator component is assembled by the fusion between the DNA binding domain (DBD) of Gal4 and the transactivation domain of VP16, a herpes simplex virus (HSV) protein, which is typically under the control of a tissue-specific promoter. In previous reports where the Gal4-VP16 transactivator system was used the system was under the control of the mouse mammary tumor virus long terminal repeat (1) or hoxc (4) promoters in mouse tissues. However, when expressed at high levels the Gal4-VP16 transactivator was shown to inhibit transcription by binding and titrating out transcription factors (5). Furthermore, the VP16 protein has been shown to be toxic to pre-implantation mouse embryos (6). Therefore, achieving a balance between transactivation and toxicity will be required to attain optimal results from this system.

The lck gene encodes the lymphocyte-specific tyrosine kinase p56\(^{lck}\). Two distinct promoter elements, a proximal and a distal element, have been shown to regulate lck expression (7). The proximal element of the lck promoter is more active in immature thymocytes, whereas the activity of the distal promoter dominates in mature thymocytes (8). Analyses of the lck proximal promoter in transgenic mice revealed that its activity was limited to immature T cell subsets; it did not function in other cell types, including B lineage cells (9-11).

V(D)J recombination is a developmentally-regulated process during early T cell development in which germline variable (V), diversity (D) and joining (J) gene segments are assembled...
to form the variable regions of the T cell receptors. The correlation between transcription and V(D)J recombination has been extensively documented (12), although there are some isolated studies which have reported that these two processes are dissociated (13, 14). In addition, it is still not clear whether transcriptional activation promotes recombinational accessibility or directs V(DJ) recombinase targeting.

In order to dissect the roles of various transcriptional regulatory elements within the T cell receptor β locus, we generated T cell receptor β enhancer (Eβ) and β13 promoter replacement mice, in which the original enhancer and promoter regions were replaced with the Gal4 DNA binding motifs (15-17). Introducing the Gal4-VP16 component into the replacement mice by selective breeding was shown to transactivate the Eβ and β13 promoter regions, which allowed us to study the relationship between transcriptional activation and V(DJ) recombination in unprecedented detail. To complement this replacement strain, we also constructed a suitable transactivator strain. More specifically, we generated and characterized transgenic mice in which the Gal4-VP16 transactivator was driven by the lck proximal promoter. This is the first report that has achieved thymocyte-specific expression of Gal4-VP16. As a control, Gal4-DBD transgenic mice that were missing the corresponding VP16 transactivator domain were also generated.

RESULTS AND DISCUSSION

Expression of Gal4-VP16 and Gal4-DBD proteins in a pro-T cell line

In order to establish a bigenic transgenic system with reproducible transgene expression in immature thymocytes, we subcloned the Gal4-VP16 transactivator coding sequence into the BamHI site of the pLck-neo expression vector, which contains the lck proximal promoter (Fig. 1A). The coding sequence of Gal4-DBD was also subcloned into the same site of the same vector to use as a negative control. In addition, a neomycin resistance gene (neo), for selection of cells containing the transgene, and the human growth hormone gene (hGH) 3′ tail, to provide a polyadenylation signal, were included in the expression vector (9, 18).

We subsequently transfected the pro-T cell line P5424 with linearized Gal4-VP16 and Gal4-DBD expression constructs and selected for stable transfectants. To evaluate the expression pattern of the Gal4 gene constructs, we isolated genomic DNA from stable transfectants, digested them with PvuII, and subjected them to Southern blot analysis. From these experiments, we confirmed that the stable transfectants harbored the Gal4-DBD gene constructs (around 2.4 kb) with a 32P-labeled Gal4 DNA probe; representative data are shown in Fig. 1B (lanes G4-1, G4-2, and G4-3). We also confirmed the presence of the Gal4-VP16 gene constructs (around 2.5 kb; Fig. 1B, lanes G4-VP16-1 and G4-VP16-2).

Immunoprecipitation and Western blot analysis of the nuclear extracts (G4-1, G4-3, G4-VP16-1, and G4-VP16-2) using a single Gal4 antibody revealed that the transfectants expressed the Gal4-DBD and Gal4-VP16 proteins. In an electrophoretic mobility shift assay (EMSA), these nuclear extracts retarded the mobility of oligonucleotides containing the Gal4 DNA binding motif (data not shown), indicating that the transfectomas expressed functionally active Gal4-DBD and Gal4-VP16 proteins.

Generation and characterization of transgenic mice

Previous studies have demonstrated that the lck proximal promoter is active exclusively in immature thymocyte subsets (9-11). The lck promoter was shown to direct thymocyte-specific expression of pertussis toxin S1 subunit, SV40 T antigen, and green fluorescence protein in transgenic mice (9-11). To isolate Gal4-VP16 transactivator expression to immature thymocytes, four Gal4-VP16 transgenic founder lines were generated by microinjection of the linearized pLck-Gal4VP16 DNA fragment. A single Gal4-DBD transgenic founder line was also generated as a control.

Previous studies demonstrated that Gal4-VP16 expression was detrimental to early mouse development (6), and SV40 T-antigen expression driven by the lck proximal promoter induced thy-
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As expected, introducing Gal4-VP16 reduced the average number of transgenic thymocytes from $3 \times 10^8$ in wild-type mice to $7 \times 10^7$ in the transgenic mice, which represents an approximate 77% decrease in cellularity. The transgene also significantly altered thymocyte distribution, as indicated by the approximate 50% decrease in the proportion of CD4^{-} CD8^{-} thymocytes and the concomitant 50% increase in the proportions of CD4^{+} and CD8^{+} thymocytes (Fig. 2, first line of the panels). In addition, the Gal4-VP16 transgene slightly increased the proportion of CD4^{+} CD8^{+} thymocytes (Fig. 2, first line of the panels). We also observed a substantial increase in the proportion of mature CD4^{+} and CD8^{+} T cells in the spleens of the transgenic mice (Fig. 2, third line of the panels). Importantly, we did not observe significant changes in the proportion of B220^{+} cells (i.e., the B lymphocytes) between the four different Gal4-VP16 transgenic founder mice and wild-type mice, indicating that the alterations were T lymphocyte-specific (Fig. 2, fourth line of the panels, and data not shown). Interestingly, we observed similar alterations in the T cell distribution in the Gal4-DBD transgenic mice that were missing the potent transactivator VP16 (Fig. 2, far right row of the panels). This result suggested that thymic developmental alterations in the Gal4-VP16 and Gal4-DBD transgenic mice might result from the additional insertion of the lck proximal promoter, not from any harmful effects of VP16 (see discussion below).

Next, we examined the proportion of CD3^{+} thymocytes, an indicator of T cell maturity and normal T cell receptor signaling. The proportion of CD3^{+} thymocytes was not significantly different between wild-type and transgenic mice, although it was slightly higher in transgenic spleens (Fig. 2, second and fourth lines in the panels). Taken together, these results indicate that even though the transgenic mice exhibit decreased cellularity and thymic developmental alterations, the mature B and T lymphocytes within their spleens were normal or slightly increased, respectively.

**The transgenic proteins are functionally active**

To evaluate the tissue-specific expression of the transgene products, we prepared protein extracts from the thymus, spleen, heart, liver, and lung tissues of the transgenic mice (by sonication) and subjected them to Western blotting using Gal4 antibodies. In this analysis no detectable levels of the Gal4 fusion product was observed in any of the transgenic organs except for the thymus, where extremely low amounts of Gal4 protein was detected (data not shown). Because Gal4 proteins are nuclear proteins, we subsequently prepared nuclear extracts of transgenic and wild-type thymocytes, and subjected equal amounts of these extracts to immunoprecipitation using Gal4 antibodies. As shown in Fig. 3A, this procedure identified Gal4-VP16 and Gal4-DBD in transgenic thymocytes but not wild-type thymocytes (Fig. 3A), suggesting that transgenic thymocytes expressed the Gal4 fusion proteins.

To confirm that Gal4 fusion proteins bind to the Gal4 DNA binding motif, nuclear extracts from Gal4-DBD transgenic and wild-type thymocytes were subjected to EMSA (Fig. 3B). The mobility of the Gal4 DNA binding motif was retarded in nuclear extracts prepared from the thymocytes of a Gal4-DBD founder mouse (Fig. 3B, lanes 2 and 3). Unlabeled competitor sequences abolished this mobility shift (Fig. 3B, lane 4), suggesting that the transgenic gene product could bind directly to the Gal4 DNA binding motif. In addition, adding the Gal4 antibody to the complex containing the Gal4-DBD and Gal4 DNA binding motif further retarded the mobility of the Gal4 DNA binding motif (note the supershift in Fig. 3B, lane 5), confirming that the Gal4 DBD specifically interacted with the Gal4 DNA binding motif.

Nuclear extracts from the thymocytes of the two Gal4-VP16

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**Fig. 2.** Flow cytometric analyses of wild type and transgenic lymphocytes. Thymocytes from wild-type, Gal4-VP16A, Gal4-VP16B, and Gal4-DBD mice were stained with anti-CD4-PE and anti-CD8-FITC (first line of the panels), or anti-CD3e-FITC alone (second line of the panels). Splenocytes from wild-type, Gal4-VP16A, Gal4-VP16B, and Gal4-DBD mice were stained with anti-CD4-PE and anti-CD8-FITC (third line of the panels), or with anti-CD3e-FITC and anti-B220-PE (fourth line of the panels). The percentage of cells in each quadrant is indicated.
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Fig. 3. Expression and characterization of transgenic Gal4-VP16 and Gal4-DBD proteins. (A) Nuclear extracts were prepared from wild-type, Gal4-DBD, Gal4-VP16A, and Gal4-VP16B thymocytes and then subjected to immunoprecipitation followed by Western blotting with Gal4 antibodies. The immunoprecipitated Gal4-DBD and Gal4-VP16 proteins are indicated with arrows. (B) DNA binding activities of Gal4-DBD (DBD), Gal4-VP16A (VP16A), and Gal4-VP16B (VP16B) proteins. Gel mobility shift assays were performed with nuclear extracts from wild-type (WT) and transgenic thymocytes using a 32P-labeled oligonucleotide containing a Gal4 DNA binding motif. Unbound oligonucleotides migrate to just above the bottom of the gel (lanes 1 and 2). The shifted DNA-protein complexes are indicated with arrows (lanes 3, 6, and 8). To check the specificity of the interaction between transgenic proteins and the Gal4 DNA binding motif, unlabeled oligonucleotide competitors were added to the DNA-protein complexes (lanes 4, 7, and 9). Gal4 antibodies were also added to the DNA-protein complexes; the supershifted DNA-protein complexes are indicated with arrow on the right side (lanes 5 and 10).

founder mice (VP16A and VP16B) were also subjected to EMSA. Similar to the results obtained for Gal4-DBD, the Gal4-VP16 transgenic proteins also retarded the mobility of the Gal4 DNA binding motif (Fig. 3B, lanes 6 and 7) and adding unlabeled competitor abolished this mobility shift (Fig. 3B, lanes 8 and 9). Furthermore, including the Gal4 antibody supershifted the Gal4 DNA binding motif (Fig. 3B, lane 10). These results indicated that both of the Gal4 fusion proteins from transgenic thymocytes are functionally active.

Transgenic mouse models carrying tissue-specific promoters provide valuable tools in which to target gene expression to relevant areas and to regulate promoter activities during different stages of growth and development. Transgenic mouse models also facilitate studies of transcriptional silencing or of the toxic effects caused by potent transcriptional activators, as is the case for VP16. In this study, we generated five transgenic founder mice harboring Gal4-VP16 and Gal4-DBD DNA constructs that were driven by the T cell-specific Lck proximal promoter. We consistently observed alterations in thymic development in all five transgenic lines. However, in order for the binary transgenic system to work properly the trans-activator component must exclusively regulate the trans-responder/target transgene; it should not, by itself, alter endogenous gene expression (1). The results of our study suggest that the Gal4-VP16 and Gal4-DBD transactivators by themselves are sufficient to alter thymic development.

There are two plausible explanations for these alterations; 1) the additional insertion of the Lck proximal promoter may reduce endogenous p56Lck expression by titrating out transcription factors responsible for endogenous lck promoter activity and 2) the alterations could stem from direct effects of the transgenes themselves. Based on our results and previous data, we believe the first explanation is more likely because these developmental alterations consistently occur in the thymi from other transgenic lines in which the lck proximal promoter drives the expression of a variety of seemingly unrelated transgenes (9, 10). However, we can not entirely rule out the second explanation because in the plck-GFP-Tg mice, introducing a lck promoter-driven green fluorescence protein (GFP) did not induce these significant alterations in thymic development (11). Furthermore, introduction of the Gal4-DBD and Gal4-VP16 proteins was not toxic to the more mature CD4+ T lymphocytes in this study (Fig. 2), contrary to the results of a previous report (9). The exact reason behind the thymic developmental alterations in lck promoter-driven transgenic mice remains to be determined and will require further exploration.

More importantly, these developmental alterations did not affect the functional properties of the transgenic proteins in the thymocytes. Gel mobility shift assays demonstrated that Gal4-DBD and Gal4-VP16 expressed in transgenic thymocytes specifically interacted with the Gal4 DNA binding motif and retarded its mobility (Fig. 3B), indicating that the transgenic proteins, expressed in vivo, were functionally active. Furthermore, the transgenic mice generated in this study exhibited normal developmental behavior and remained tumor-free for over one year, in contrast to previously-characterized mice harboring lck promoter-based transgenes (9, 10). This significant improvement may stem from the fact that the Gal4-VP16 and Gal4-DBD constructs driven by the lck proximal promoter element restricted thymic alterations to immature thymocytes. Levels of functionally-mature thymocytes and T lymphocytes therefore remained normal or slightly increased.

In summary, we developed thymocyte-specific transgenic mouse models carrying the Gal4-VP16 and Gal4-DBD
transactivators. Although the Gal4-VP16 and Gal4-DBD transactivators did alter thymic development, the effects were mild and restrictive. For this reason, our transgenic lines are likely to be useful in regulating thymocyte-specific expression of the transresponder genes.

**MATERIALS AND METHODS**

**Expression constructs**

The expression vector, pLckneo (Fig. 1), was prepared from a 1.9-kb EcoRI-HindIII fragment from pKJ-1, containing the PGK promoter, a neo resistance cassette, and a PGK polyA cloned into the EcoRI site of the human growth hormone gene of the p1017 expression vector (9). pLck-Gal4VP16 was generated by cloning a BgIII-BclI fragment containing the Gal4-VP16 sequence from pMV1VP16 into the BamHI site of pLckneo (19). pLck-Gal4DBD was also generated by cloning a BgIII-BamHI fragment containing Gal4-DBD from pM1 into the BamHI site of pLckneo.

**Transfections, PCR, and Southern blotting**

To prepare linear transgenes for transfection and microinjection, pLck-Gal4VP16, pLck-Gal4DBD, and pPGKPuro were digested with NotI, and the linearized plasmids were purified by agarose gel electrophoresis. The pro-T cell line P5424 was derived from p53−/− mice deficient in Rag-1 and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine (20). Thirty million cells were transfected in 0.5 mL RPMI-1640 medium with 30 μg/ml. This work was supported by the faculty research fund of Sejong University in 2006. The expression vector, pLckneo-Gal4VP16 construct, and one transgenic founder line (Gal4-DBD) was generated from pLck-Gal4DBD. The presence of Gal4 genes in the transgenic founders was confirmed by PCR amplification of genomic DNA from tail biopsies, using the same oligonucleotide primers used to generate the Gal4 DNA probe.

**Flow cytometry**

Antibodies specific for CD4, CD8, CD3ε, and B220 were direct conjugates purchased from BD Pharmingen (San Diego, CA). Flow cytometry was performed on a FACScalibur apparatus (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) as described previously (17, 21).

**Immunoprecipitation, Western blotting, and gel mobility shift assay**

Nuclear extracts were prepared from 1-3 × 10⁶ cells from transfectants or transgenic thymocytes as described (22). Equal amounts of the nuclear extracts (200-230 μg) were subjected to immunoprecipitation with anti-Gal4 (Santa Cruz Biotechnology, Santa Cruz, CA), and the immunoprecipitated proteins were analyzed by Western blotting using the same Gal4 antibody and horseradish peroxidase-conjugated anti-mouse IgG, as previously described (23). To study whether or not the Gal4 proteins bound to the Gal4 DNA binding motif, two oligonucleotides containing the Gal4 DNA binding motif, 5'-AGCTTA and 3'-GGGAAATTTGTCGAGGAC CACAAATTCCGGCTA-3', were annealed and labeled with T4 polynucleotide kinase (Roche, Basel, Switzerland) (24). The purified oligonucleotide was used in a gel mobility shift assay, as previously described (25).

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**REFERENCES**


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