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A Mouse Model for Beta Cell-Specific Ablation of Target Gene(s) Using the Cre-loxP System

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The rat insulin promoter (RIP) has been used to drive the expression of Cre recombinase (Cre) specifically in beta cells. Transient transfection was performed in the mouse insulinoma cell line, NIT-1, and control cell lines. RT-PCR was performed using total RNA from pancreas and other tissues of RIP-Cre transgenic mice. In addition, the efficiency and specificity of RIP were further analyzed by cross breeding the RIP-Cre transgenic mice with reporter mice bearing a β-actin-loxP-CAT-loxP-lacZ transgene. In these mice, lacZ is expressed only after excision of the *floxed-CAT* gene by Cre-mediated recombination. Here, we present the data for beta cell-specific expression of lacZ in bigenic mice, as proof of concept in a mouse model for targeting beta cell-specific gene(s). The RIP-Cre transgenic mice will be used as a potential tool for targeting the excision of beta cell-specific gene(s) to study their role in islet cell physiology.

Key Words: **beta cell; Cre recombinase (Cre); Cre-loxP; rat insulin promoter (RIP); β-galactosidase (lacZ); transfection.**

Gene targeting in mouse embryo-derived stem (ES) cells has been widely used to introduce specific mutations into the mouse genome (1–7). Inherent impediments, however, can complicate the use of conventional homologous recombination to elicit the role of the target gene(s) in adult pathophysiology (8). To eliminate the possibility of embryonic lethality, ablation of the target gene(s) has recently been reported to have been confined in a tissue-specific or temporal manner using Cre mediated recombination. Both yeast (9–11) and phage (12–18) site-specific recombinases have been used to achieve tissue-restricted and/or stage specific recombination to induce or inactivate the predetermined genes. Cre recombinase, from the P1 bacteriophage, has proven efficacious in mediating recombination in mammalian cells for tissue restricted ablation of the target gene(s) (19–22). The Cre recombinase, a 38-kDa protein, recognizes a 34 bp sequences known as loxP. DNA sequences, flanked by the loxP sequences are excised by the Cre mediated recombination. Each loxP site contains a dyad of 13 bp and is separated by an 8 bp spacer which determines 5′ to 3′ orientation of the motif. Cre recombinase excises the *floxed*-gene using one of the three forms of Cre mediated recombination and is directed by the orientation of loxP sites relative to the each other. Excision or inversion occurs when the loxP sites exist on the same strand of DNA, whereas insertion occurs, using the loxP sites on separate strands. Cre-mediated recombination can be regulated by controlling the timing or spatial distribution of Cre expression via tissue-specific promoters (21), ligand-inducible promoters (22), and ligand dependent Cre fusion proteins (20). In this study, the expression of Cre recombinase will be controlled in a tissue-specific manner using a tissue-specific promoter, such as the rat insulin promoter, RIP (23).

In this study the specificity of RIP as well as the ability of Cre recombinase to excise the *floxed*-gene has been tested to establish the beta cell specific gene ablation mouse model. 448 nucleotides of RIP sequence was isolated by restriction endonuclease digestion and ligated either with the β-galactosidase gene (lacZ) or Cre coding sequences, having a nuclear localization signal (NLS) and a polyadenylation signal (pA). The RIP-lacZ construct was used for transient transfection into a mouse insulinoma cell line, NIT-1 and control cell lines. The transgene, RIP-Cre, was microinjected into one cell embryo to generate transgenic mice. RT-PCR was used to determine the specificity of Cre expression using total RNA from RIP-Cre transgenic mice. To further verify the specificity of RIP and the ability of the Cre recombinase to excise the *floxed*-gene, the RIP-Cre mice were

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cross bred with loxP reporter mice bearing the β-actin-loxP-CAT-loxP-lacZ transgene (reporter mice were generously provided by Dr. Michael D. Schneider M.D., Department of Medicine, Baylor College of Medicine). In this reporter mouse, the CAT gene is flanked by loxP sequence (*floxed-CAT*) and is driven by the chicken β-actin promoter (24). The *E. coli* β-galactosidase gene (lacZ) has been attached downstream of the CAT gene and is expressed only when *floxed-CAT* sequences are excised by the Cre mediated recombination. Once the *floxed-CAT* sequences are excised, lacZ is positioned adjacent to the β-actin promoter allowing the ubiquitous expression of lacZ. In the absence of Cre recombinase, the CAT gene prevents read-through expression of lacZ. The expression of lacZ was detected by enzymatic β-galactosidase staining resulting in the development of blue color only in the nucleus of the beta cells. This RIP-Cre transgenic mouse line will be used as a potential tool for beta cell specific ablation of the target gene(s) to demonstrate their role in islet cell physiology.

MATERIALS AND METHODS

Generation of the insulin-Cre recombinase (RIP-Cre) construction. Cre coding sequences having a nuclear localization signal (NLS) and a polyadenylation signal were isolated from plasmid pOG231, by digesting with *Sal*I and *Bgl*II restriction enzymes. The reaction mixture was subjected to low melt agarose gel (GIBCO-BRL, Bethesda, MD) electrophoresis to separate the insert from the pOG231 vector. The Cre DNA fragment was excised and isolated from the gel using a QIAEX gel extraction kit following the procedure supplied by the manufacturer (QIAGEN, Santa Clarita, CA). The plasmid INS-pKS$^-$ (448 nucleotides of rat insulin promoter in BlueScript) was digested with SalI and BamHI restriction enzymes. The isolated Cre DNA fragment was ligated with a restriction endonuclease treated RIP-BlueScript vector at room temperature for four hours. The ligation mixture was subsequently used for transformation using maximum efficiency DH5α cells (GIBCO-BRL, Bethesda, MD) following the procedure supplied by the manufacturer. The resulting RIP-Cre transgene was isolated by digesting with SalI and NotI using a QIAEX gel extraction kit.

Generation and identification of the RIP-Cre transgenic mice. The RIP-Cre transgene was isolated from plasmid sequences by digestion with SalI and NotI restriction enzymes. DNA was purified using a QIAEX gel extraction kit and the isolated fragment was diluted to 2 μg/ml in a modified TE buffer (10 mM Tris, pH 7.5, and 25 mM EDTA). Transgenic mice were produced at the Baylor College of Medicine using the Transgenic Core facility following a modified procedure described by Hogan *et al.* (25). The isolated RIP-Cre DNA fragment was microinjected into ICR × B63F1 embryos. The transgenic founder mice were identified by polymerase chain reaction (PCR) and the PCR data further verified by Southern blot analysis (26) using mouse tail DNA and suitable random primed [α-^{32}P] dCTP-labeled probes from the Cre coding sequence.

Generation of insulin-β-galactosidase (RIP-lacZ) construction. The plasmid pD46.21 having a β-galactosidase gene with a polyadenylation signal and a nuclear localization signal was digested with *Hin*dIII restriction endonuclease. After complete digestion the reaction mixture was subjected to DNA polymerase large fragment (Klenow, GIBCO-BRL) treatment to generate blunt ends. At the completion of the reaction the DNA was purified and was digested with *Bam*HI restriction endonuclease. The insulin promoter was isolated from BlueScript KS$^+$ (Stratagene) by treatment with *Sst*II restriction endonuclease and subsequent treatment of the reaction mixture with T4 polymerase (Promega, Madison, WI) to generate blunt ends. Blunt ended DNA was purified using PCI (phenol:chloroform: isoamyl alcohol = 25:24:1) and was treated with BamHI restriction endonuclease. Finally, the reaction mixture was subjected to low melt agarose gel (GIBCO-BRL) electrophoresis to separate the insert from the BlueScript vector. The DNA fragment was excised from the gel and isolated using a QIAEX gel extraction kit. The isolated insulin promoter was ligated with the restriction endonuclease treated pPD46.21 vector.

Cell culture, transient transfection and detection of β-galactosidase gene expression. The mouse beta-cell specific cell line (NIT-1, 27), cultured in F-12 medium containing serum plus 100 units of penicillin and 0.1 mg/ml streptomycin was used for transient transfection analysis using approximately 0.4 pmol (1.5–2 μg of DNA). DNA was mixed with 20 μl of lipofectamine (Life Technologies, Inc.) in 1 ml of Dulbecco's modified Eagle's medium (without serum). Cells growing in logarithmic phase (50–70% confluent) on 100 mM dishes were washed twice with phosphate-buffered saline and were fed with 5 ml of Dulbecco's modified Eagle's medium (without serum). One milliliter of plasmid DNA-lipofectamine mixture was added to the 5 ml DMEM and mixed very gently. The cells were incubated for 24 h at 37°C. Cells were washed with phosphate-buffered saline, fed with the F-12 media containing serum and incubated for another 24 h at 37°C. At the end of incubation period the cells were washed twice with cold PBS and incubated with 0.5% glutaraldehyde at room temperature for 5 min. The cells were washed twice with cold PBS and X-gal staining solution (1 M $MgCl_2$, 5 M NaCl, 0.5 M Hepes, pH 7.3, 30 mM potassium ferricyanide, 30 mM potassium ferrocyanide, 2% X-gal) was added. Color was allowed to develop at 37°C for 6–24 h.

Reverse-transcriptase polymerase chain reaction (RT-PCR). Tissues were removed from euthanized RIP-Cre transgenic mice and were homogenized in RNAzolB (Tel Test, Friendswood, TX) The RNA was isolated following the procedure described by Chomczynski *et al.* (28) and consisted of treatment with RNase-free DNase, RQ1 (Promega), for 15 minutes, extraction twice with PCI and CI (chloroform: isoamyl alcohol = 24:1) and precipitation in ethanol. Suitable primers for Cre recombinase as well as GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal control for the RNA samples, were designed for RT-PCR amplification. For each reaction, 1 μg of RNA template, 0.1 μg poly $(dT)_{12-15}$, 9 units of RNasin (Promega), 1 mM of each dNTP, 10× PCR buffer (500 mM KCl, 100 mM Tris 8.4), 30 mM $MgCl_2$, and 10 units of SuperscriptII reverse transcriptase (GIBCO-BRL) were added and incubated at 37°C for 30 min. The enzyme was inactivated by incubating at 90°C for 5 min. The reaction mixture was subjected to PCR amplification using suitable primers following the standard PCR protocol.

RESULTS

Transient transfection of RIP-lacZ construction into NIT-1 cells and expression of the reporter gene. To determine the specificity of the insulin promoter a RIP-lacZ construct was generated (Fig. 1a) and used for transient transfection. As shown in Fig. 1b, this reporter construct can successfully drive the expression of the β-galactosidase gene specifically in pancreatic beta cells (NIT-1) as evidenced by the presence of a chromogenic substrate, for X-gal, having blue color, at the nucleus of these cells. The control cell line used for this experiment, CV1, was negative

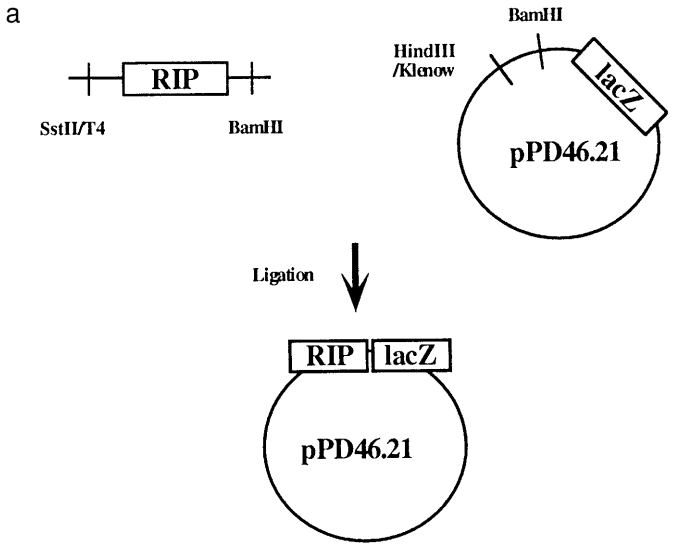

FIG. 1. Transient transfection of the RIP-lacZ construct into NIT-1 cells. The rat insulin promoter was ligated to the coding sequence of b-galactosidase gene and used for transient transfection (a). b represents the transfection data using mouse insulinoma cell line, NIT-1, and a control cell line, CV1. The expression of lacZ in the NIT-1 cells was evidenced by the presence of blue color in the nucleus of these cells.

for the expression of β-galactosidase gene as evidenced by the absence of blue color in these cells.

Detection of the message for Cre recombinase by RT-PCR. To detect the message for Cre recombinase in pancreas, total RNA from RIP-Cre transgenic mice was subjected to reverse transcriptase polymerase chain reaction along with total RNA from liver as a negative control. Forward and reverse primers were selected from Cre recombinase coding sequences to amplify the correct message size for Cre. As shown Fig. 2, the PCR amplified product was detected in the pancreas RNA of the RIP-Cre transgenic mice and not in the control liver RNA.

Breeding of the RIP-Cre transgenic mice line with the loxP reporter mice. To further characterize the specificity of the insulin promoter and to determine the efficiency of the system, two lines of RIP-Cre transgenic mice were cross bred with the loxP reporter mice. The strategy of generating the bigenic mice is demonstrated in Fig. 3a. Mice generated from cross breeding of the RIP-Cre transgenic mice with the reporter mice

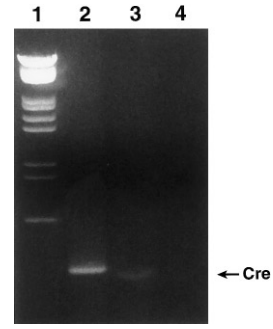

FIG. 2. Detection of Cre recombinase transcripts in the RIP-Cre transgenic mice. Total RNA was isolated from different organs of the RIP-Cre transgenic mice and subjected to RT-PCR. The message for Cre recombinase was detected only in the pancreas (lane 3), where as liver was negative for the presence of Cre recombinase (lane 4). Lane 1 is the DNA ladder and lane 2 is the positive control for PCR.

were allowed to develop to six weeks of age and were screened by PCR analysis using their DNA, isolated from tail digestion. As shown in Fig. 3b, we have developed three independent bigenic lines bearing both β-actin-loxP-CAT-loxP-lacZ and RIP-Cre transgenes.

Beta cell specific expression of lacZ in the bigenic mice. To determine the efficiency and specificity of the RIP promoter the bigenic mice having RIP-Cre and β-actin-loxP-CAT-loxP-lacZ transgenes were sacrificed at 8 weeks of age and different tissues were analyzed for lacZ expression. As shown Fig. 4, the lacZ expression was detected only in the pancreas. The reporter mice alone were analyzed for leaky expression of lacZ in the pancreas, and as expected, no lacZ expression

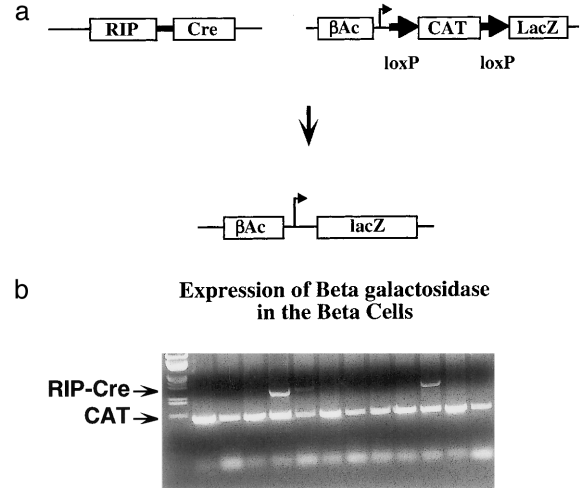

FIG. 3. Generation of bigenic mice by crossbreeding the RIP-Cre mice with the reporter mice. As shown in b, we have developed three independent lines of bigenic mice bearing both the RIP-Cre and b-actin-loxP-CAT-loxP-lacZ transgenes. PCR was conducted on isolated tail DNA to screen the mice using suitable probes for Cre and CAT coding sequences. a represents the strategy for generating the bigenic mice.

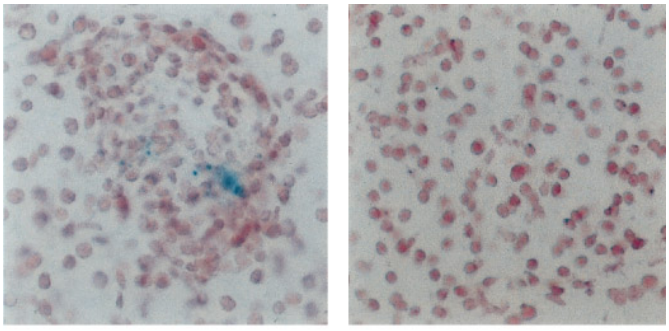

FIG. 4. Beta cell specific expression of lacZ in the bigenic mice. The lacZ expression shown is from a 8-week-old bigenic mouse. Tissues were isolated and subjected to enzymatic staining of b-galactosidase expression with color development. Liver was used as a control tissue for lacZ expression in the bigenic mice.

was detected by RT-PCR using the total RNA isolated from the loxP reporter mice pancreas.

DISCUSSION

We have generated a tissue-specific transgenic mouse line in which the rat insulin promoter has been used to drive the expression of a reporter gene in a tissue-specific manner. To determine the specificity of the rat insulin promoter, *in vitro* and *in vivo* studies have been conducted. In transient transfection analysis, the expression of lacZ is detected only in the pancreatic beta cells as evidenced by the presence of blue color in the nucleus of these cells. RNA isolated from the RIP-Cre transgenic mice detects Cre recombinase transcripts only in the pancreas. Furthermore, cross-breeding of the RIP-Cre mice with reporter mice (β-actin-loxP-CAT-loxP-lacZ), drives the expression of the β-galactosidase gene specifically in the islets.

In recent years, the Cre-loxP system has been widely used for tissue-specific ablation of a particular gene. The expression of Cre recombinase is controlled either by using a tissue-specific promoter or in a ligand dependent manner. Cre recombinase recognizes the loxP sequence, a 34 nucleotides long DNA sequence, and deletes the DNA sequence flanked by the loxP sequences. This Cre-loxP system is of particular importance when the deletion of a gene whose product serves an essential function in embryogenesis can result in early lethality and precludes the investigation of the protein's function in any adult context. Under this situation the ablation of the gene can be restricted in a particular cell type by tissue-specific expression of the Cre recombinase.

We have successfully developed two lines of RIP-Cre transgenic mice by microinjection of the transgene into one cell embryos. The specificity of the rat insulin promoter has been tested *in vitro*, by transient transfection of the RIP-lacZ construct into a mouse insulinoma cell line, NIT-1. LacZ expression was detected in the NIT-1 cell line whereas the control cell line, CV1, was negative for the expression of the β-galactosidase gene. Tissue-specific expression of Cre recombinase was also detected by RT-PCR using total RNA from pancreas of the RIP-Cre transgenic mice. Total liver RNA was negative for Cre recombinase transcripts. The tissue-specificity of RIP will allow us to use this RIP-Cre transgenic mouse line toward developing a mouse model for targeting the beta cell specific genes. The feasibility of this system to recognize the loxP sequences and to ablate the *floxed-gene* by Cre mediated recombination has been tested by cross breeding with the loxP reporter mice and as shown in Fig. 4, lacZ expression was detected in the islet cells.

The data presented here for lacZ expression was obtained from an 8 weeks old bigenic mice. A time kinetic study will be conducted on these bigenic mice at 4, 6, 8, and 10 weeks of age to determine the optimum level of expression of the β-galactosidase gene. This time study will determine the optimum time required for maximum expression of Cre recombinase to efficiently excise the *floxed-CAT* gene. This information will be useful to determine the time required for Cre recombinase to efficiently excise the *floxed-gene* (target gene) in the beta cells.

In conclusion, we have established a mouse model for beta cell specific expression of Cre recombinase. This line of mice could potentially be used for beta cell specific ablation of any gene of interest. The ability of Cre recombinase to recognize the loxP sequence and to ablate the *floxed*-gene has been tested. We can use these RIP-Cre transgenic mouse lines to target beta cell specific genes in order to determine their role in islet cell physiology as well as to explore the pathophysiological consequences of this genetic alteration.

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