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≪Research Note≫

Construction of an Insertion Vector for Gene Targeting of Chicken Lens-specific Gene

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Production of gene-targeted chickens is considered to be valuable for studies in both biological science and industry, but it is yet to be achieved. In this study, an insertion vector for gene targeting of chicken lens-specific gene, delta1-crystallin (d1cry), was constructed as a useful tool to evaluate homologous recombination (HR) in chickens. A promoter-less d1 cry-homologous DNA and DsRed with an artificial linker sequence (linkerDsRed) were amplified by PCR from genomic DNA and pCMV-DsRed-Express, respectively. The amplified fragments were then cloned and sequenced. The homologous DNA was 7,402 bp in size and contained no amber and frameshift mutations. The linkerDsRed fragment had accurate sequence without artificial errors. Floxed marker genes composed of enhanced green fluorescent protein (EGFP) gene, internal ribosome entry site (IRES) and puromycin resistance gene (Pac) regulated by CAG promoter (*P*CAGEIP) was constructed using standard genetic engineering methods. Finally, these DNA materials were ligated to pCC1BAC vector. The accuracy of construction was confirmed by sequencing of ligated portions, and a 20.7-kb insertion d1cry-targeting vector was accomplished. By gene targeting in the pluripotent stem cells using this vector and transplantation of the cells into recipient embryos, chicken transformation would be observed rapidly and easily by the mutant gene-derived red fluorescence in the lens at an early stage of embryogenesis.

Key words: chicken, delta1-crystallin, gene targeting, insertion vector

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Introduction

Chickens are good animal models for studies in both biological science and industry. They offer effective experimental systems and many important discoveries have been made about them in the fields of developmental biology, immunology and microbiology (Brown *et al.,* 2003). In addition, the recent completion of the chicken genomic resource has provided the potential for genetic analysis of vertebrates (International Chicken Genome Sequencing Consortium, 2004). Chicken meat and eggs are popular foodstuffs, and large numbers of chickens can be bred and confined in small areas, while at least 300 eggs are produced per hen per year. For these reasons, gene modification of chickens is an attractive subject.

Gene targeting is one of the most powerful techniques in genetic modification. It enables gene-specific mutagenesis to be carried out on the basis of homologous recombination (HR) between cellular DNA and targeting vector DNA. Many kinds of gene-targeted (knock-in and -out) mice are created routinely using mouse embryonic stem (ES) cells because these cells maintained pluripotency and differentiation competency into germ cells during the culture period, a prerequisite for germline transmission of the targeted gene (Evans and Kaufman, 1981; Bradley *et al.,* 1984). The strategy is yet to be achieved in nonmurine species due to long-term absence of germline-competent cell lines such as pluripotent stem cells. In chickens, ES cells have been established (Pain *et al.,* 1996; van de Lavoir *et al.,* 2006), and our research group has been developed germline-competent cells in progress. Therefore, HR of these cells and production of the gene-targeted chickens are next challenges.

On the other hand, chicken delta1-cryctallin gene (d1cry), an excellent molecular marker of lens differentiation, has unique natures. The mRNA expression is accompanied by the initiation of lens formation at stage 13 (Hamburger and Hamilton, 1951) embryos or after 48 h incubation of eggs (Kamachi *et al.,* 1998), and the protein comprises as much as 70–80% of all soluble protein synthesized in

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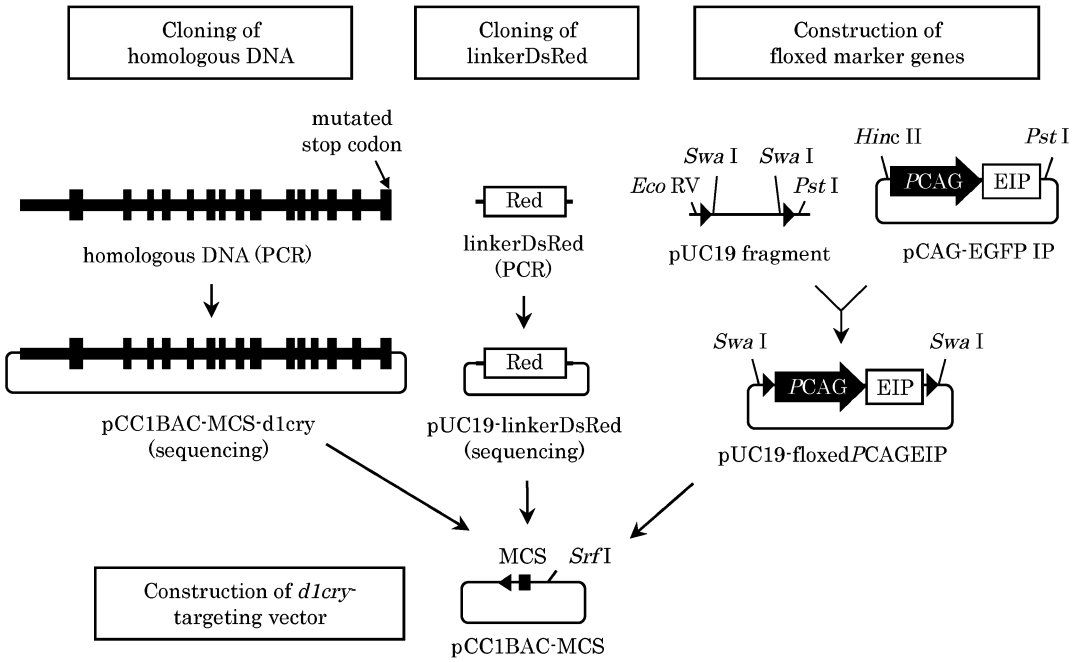

Fig. 1. **Flow chart of vector construction.** *LoxP* sites shown as triangles pointing in direction of *loxP* sequence. Red, linkerDsRed sequence. Completed vector structure shown in Fig. 3.

embryonic lens fiber cells (Piatigorsky, 1981). Moreover, embryonic chick lens is easy to observe visually, access and manipulate. These genetic and histological properties indicate mutations at d1cry can be easily and rapidly observed and a d1cry-targeting vector will be useful to evaluate HR in chickens. In this study, we constructed an insertion vector for gene targeting of the lens-specific d1cry.

Materials and Methods

General Procedures for Constructions

The construction process was as follows: cloning of a d1cry-homologous DNA and DsRed with an artificial linker sequence (linkerDsRed), sequencing of them, construction of floxed marker genes, and then construction of a d1cry-targeting vector (Fig. 1).

Polymerase chain reaction (PCR) products and DNA fragments digested with restriction enzymes were separated on 1.0% agarose gels and stained with EtBr. Isolation and purification of them were performed with QIAquick gel extraction kit (Qiagen, Hilden, Germany). General procedures of genetic engineering were performed using standard protocols (Sambrook and Russell, 2001) for restriction enzyme digestion, ligation with T4 DNA ligase and transformation of competent *E. coli* DH5α, JM109 and epi300. DNA constructs based on pUC19 and pCC1 BAC (Epicentre, Madison, WI) were kept in DH5α or JM 109 and epi300, respectively. Plasmid extraction was conducted with standard alkaline SDS method (Sambrook and Russell, 2001).

Cloning of a D1cry-Homologous DNA

Multiple restriction sites (5′-*Pme* I-*Asc* I-*Avr* II-*Pme* I-*Avr* II-*Mlu* I-*Bam* HI-*Asc* I-*Pme* I-3′) were inserted into the *Bam* HI-cloning site of the pCC1BAC to produce pCC1 BAC-multicloning site (MCS). A 7.4 kb homologous DNA of d1cry from intron 2 to the stop codon in exon 17 was amplified from genomic DNA of Barred Plymouth Rock (BPR) chickens using KOD Plus (TOYOBO, Osaka, Japan) and the primer pair int2SMF [5′-CTG**ACGCGT**ATTT-AAATCCACAGAGCATCCAGTCGCGTTTG-3′, *Mlu* I site (underline)] and NotstopMR [5′-CTG**ACGCGT**A-GCCTGCTCCTTCTGCTTCTTCAGC-3′, *Mlu* I site (underline) and mutation site in stop codon (bold)] under the following cycling conditions: 2 min at 94℃ and 30-amplification cycles (15 s at 94℃ and 8 min at 68℃). The *Mlu* I fragment of the PCR product was cloned into *Mlu* I site of pCC1BAC-MCS (pCC1BAC-MCS-d1cry) and sequenced by shotgun method.

Shotgun Sequencing Method

The d1cry sequence was re-amplified from the cloning plasmid using KOD Plus and the primer pair int2SMF and NotstopMR. The PCR product was sonicated and blunt ends repaired using T4 DNA polymerase (TaKaRa, Ohtsu, Japan), Klenow (New England Biolabs, Ipswich, MA) and T4 polynucleotide kinase (TaKaRa). Fragments 800–1,000 bp in size were recovered after PAGE separation and subcloned into the *Sma* I site of pUC19. The inserted fragment was amplified from a suspension of transformed *E. coli* clones using Ex Taq (TaKaRa) and the primer pair LF3 (5′-GTGCTGCAAGGCGATTAA-GTTGG-3′) and LR3 (5′-TCCGGCTCGTATGTTGT-GTGGA-3′) under the following cycling conditions: 9 min at 94℃, 35-amplification cycles (1 min at 94℃, 1 min

at 50℃ and 2 min at 72℃) and a final extension of 10 min at 72℃ followed by 1 h incubation at 37℃ with Exonuclease I (New England Biolabs) and shrimp alkaline phosphatase (Roche, Basel, Switzerland). PCR products were sequenced with an automated ABI PRISM 3100 genetic analyzer (Applied Biosystems, Tokyo, Japan) using BigDye Terminator (Applied Biosystems) and M13R primer (5′-GGAAACAGCTATGACCATG-3′). Sequencher program (Gene Codes, MI, USA) was used to analyze the sequences.

Cloning of LinkerDsRed

A 1.0 kb linkerDsRed fragment was amplified from pCMV-DsRed-Express (TaKaRa) using Advantage-GC 2 Polymerase Mix (TaKaRa) and the primer pair *Bam* HI-linker-DsRedF [5′-TAT**GGATCCGGCGGTGGCTCGGGTGGTGGGTCGGGCGGCGGATCT**ATGGCCTCCTCCGAGGACG-3′, *Bam* HI site (underline) and linker sequence (bold)] and *Bam* HI-DsRedpAR [5′-TATGGATCCACGCCTTAAGATACATTGATGAGTTTGGAC-3′, *Bam* HI site (underline)] under the following cycling conditions: 3 min at 95℃, 30-amplification cycles (30 s at 95℃, 30 s at 55℃ and 1 min 30 s at 68℃) and a final extension of 10 min at 68℃. The *Bam* HI fragment of the PCR product was cloned into the *Bam* HI site of pUC19 (pUC19-linkerDsRed) and sequenced with automated ABI PRISM 3100 genetic analyzer using BigDye Terminator.

Construction of Floxed Marker Genes

Enhanced green fluorescent protein (EGFP) gene-internal ribosome entry site (IRES)-puromycin resistance gene (Pac) regulated by CAG promoter (*P*CAGEIP) was used for visualization and positive selection of transfected cells. A 3.9 kb *Hin*c II-*Pst* I fragment containing *P*CAGEIP was isolated by enzymatic digestion from pCAG-EGFP-IP (a kind gift from Dr Niwa, CDB, RIKEN, Kobe, Japan). A 2.6 kb pUC19 fragment bearing two *loxP* sites (Stemberg and Hamilton, 1981) was amplified from pUC19 using KOD Plus and the primer pair loxP-pUC19F [5′-GCG**CTGCAGATAACTTCGTATAATGTATGCTATACGAAGTTAT**GCGATCGCATTTAAATCGCTCACAATTCCACACAACATAC-3′, *loxP* sequence (bold), *Pst* I site (dotted-underline) and *Swa* I site (underline)] and loxP-pUC19R [5′-GCG**GATATCATAACTTCGTATAGCATACATTATACGAAGTTATATTTAAAT**AATCGCCTTGCAGCACATCC-3′, *Eco* RV site (dotted-underline), *loxP* sequence (bold) and *Swa* I site (underline)] under the following cycling conditions: 2 min at 94℃ and 30-amplification cycles (15 s at 94℃, 30 s at 60℃ and 3 min at 68℃). The inverse PCR product was digested with *Eco* RV and *Pst* I. Former *Hin*c II-*Pst* I fragment was ligated to later *Eco* RV-*Pst* I fragment to generate pUC19-*Swa* I-floxed*P*CAGEIP-*Swa* I.

Construction of an Insertion D1cry-Targeting Vector

A 4.0 kb *Swa* I fragment of pUC19-*Swa* I-floxed*P*CAGEIP-*Swa* I was transferred into the *Srf* I site of pCC1BAC-MCS, and the resulting pCC1BAC-MCS-floxed*P*CAGEIP was used as a backbone of the d1cry-targeting vector. A *Bam* HI fragment of pUC19-linkerDsRed was re-isolated and subcloned into the *Bam* HI site of pCC1BAC-MCS-floxed*P*CAGEIP to generate pCC 1 BAC-MCS-floxed-*P*CAGEIP-linkerDsRed. A *Mlu* I fragment of pCC1 BAC-MCS-d1cry was transferred to pCC1BAC-MCS-floxed*P*CAGEIP-linkerDsRed to produce the insertion d1 cry-targeting vector.

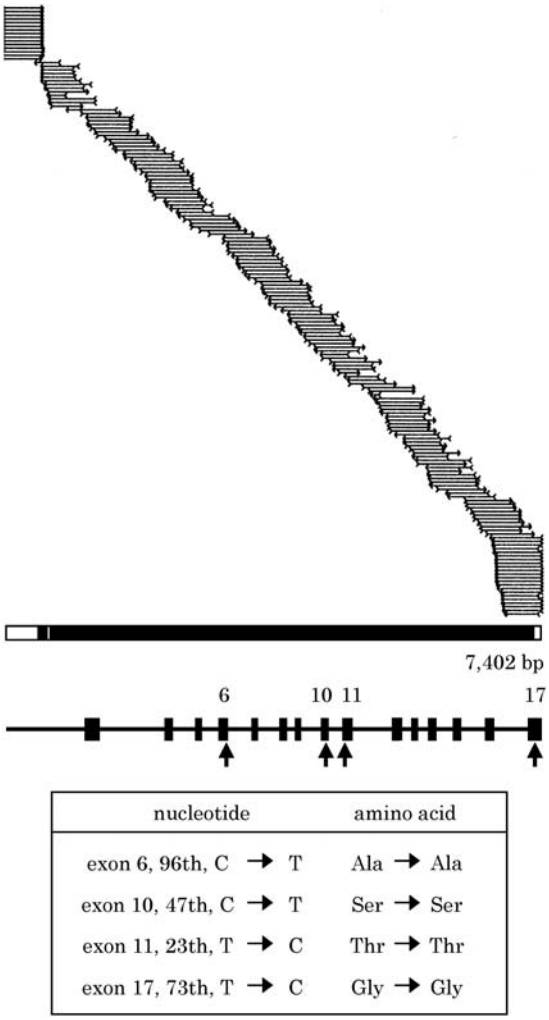

Fig. 2. **Contig view of d1cry-homologous DNA and comparison with a known sequence (accession number: M10806) (in exons).** Each horizontal arrow indicates sequence size and orientation of each subclone. Contig composed of multiple clones (a bold bar), while three regions contained unidirectional clones only (open bars). Corresponding d1 cry region shown under the bar; solid boxes and numbers indicate exons. Sites of nucleotide differences in exons shown by vertical arrows and summarized in panel.

Results and Discussion

The homologous DNA of d1cry from intron 2 to the stop codon in exon 17 was amplified by genomic PCR and then cloned. Sequence of the one clone was determined by

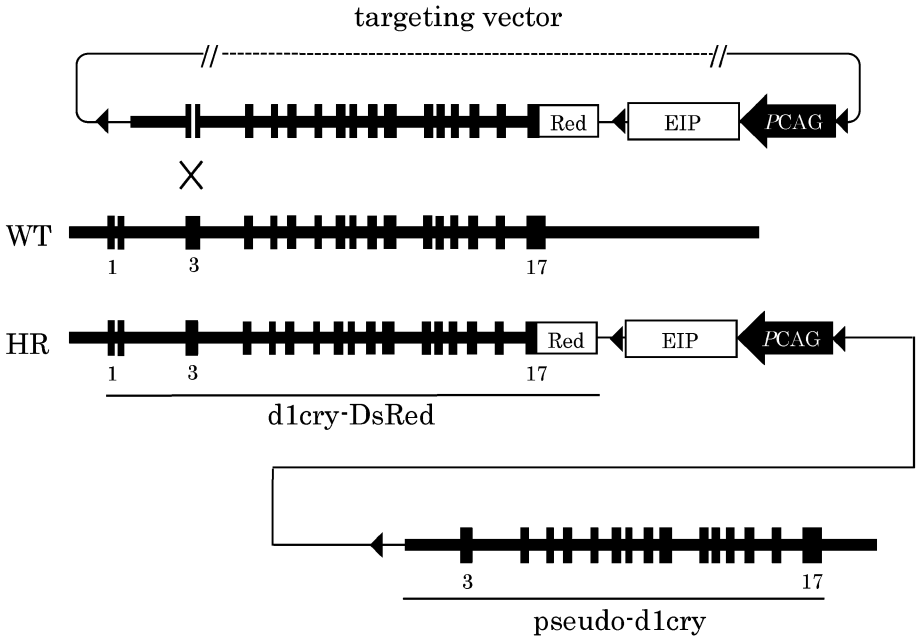

Fig. 3. **d1cry locus HR.** Insertion targeting vector, wild-type (WT), HR d1cry loci with numbered exons shown.

shotgun sequencing method. One hundred sixty-seven subclones were assembled with a total size of 7,402 bp (Fig. 2). Three regions contained unidirectional clones only but the contig was composed of multiple ones throughout (Fig. 2). The chicken delta-crystallin locus consists of two tandemly-arranged genes, delta1 and delta2. There are the strong sequence identities especially in the exons: it ranges from 70% (exon 2) to 100% (exon 7, 12, and 15), with the remaining exons having 89–98% (Nickerson *et al.,* 1986). However, the sequences of some introns were greatly different as follows: 47% (intron 2 and 5), 56% (intron 9). Our cloned DNA was d1cry because the decided sequences were identical to the d1cry in introns. Moreover, the enhancer sequence (Kamachi and Kondoh, 1993) in the intron 3 was conserved. Next, the exon regions were closely compared to the known d1cry sequence (accession number: M10806). There was one nucleotide substitution in each of exon 6, 10, 11 and 17, but these were silent mutations. The cloned DNA was used in following construction because no amber and frameshift mutations were found. On the other hand, A 1.0 kb linkerDsRed fragment was amplified from pCMV-DsRed-Express and then cloned. One of five clones had accurate sequence without artificial errors throughout but two clones obviously included them at linker regions. Consequently, the accurate clone was used in following construction.

PCC1BAC was adopted as a fundamental DNA of the d1cry-targeting vector because such an F-factor-based plasmid enables stable maintenance of a large DNA fragment (Shizuya *et al.,* 1992). EGFP gene, Pac and the homologous DNA were added for visualization, positive selection and HR of transfected cells, respectively. In addition, linkerDsRed was inserted into the downstream of the homologous DNA to evaluate HR in chickens. The accuracy of construction was confirmed by sequencing of ligated portions. In the result, the d1cry-targeting vector was accomplished; the total size was approximately 20.7 kb including 7,402-bp homologous DNA (Fig. 3). This vector belongs to insertion types on the basis of its structure and recombination strategy. They are known to cause HR efficiently (Hasty *et al.,* 1991; Hastings *et al.,* 1993; Hasty *et al.,* 1994; Leung *et al.,* 1997; Adams *et al.,* 2004).

When gene targeting of pluripotent stem cells is conducted using the d1cry-vector, a fusion gene d1cry-DsRed regulated by the endogenous promoter forms at the target locus (Fig. 3). By transplantation of the recombinant cells into recipient embryos, chicken transformation would be observed rapidly and easily by the mutant gene-derived red fluorescence in the lens at an early stage of embryogenesis. Another d1cry mutant simultaneously occurring downstream would be pseudo due to a lack of a proper promoter, exon 1 and 2 (termed pseudo-d1cry) (Fig. 3). Moreover, EGFP regulated by the ubiquitous CAG promoter would be useful to identify transformed cells *in vitro* and *in vivo.*

Recently, several researchers have established chicken ES cell lines in culture (Pain *et al.,* 1996; van de Lavoir *et al.,* 2006). Chicken leukemia inhibitory factor (LIF) was effective in maintaining chicken ES cells in an undifferentiated state (Horiuchi *et al.,* 2004). In the near future, it seems probable that several chicken ES cell lines will be capable of germinal transmission after the genetic modification. The evaluation of HR is necessary for those cells and the insertion d1cry-targeting vector will be useful

in that study.

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