The chicken hypersensitive site-4 insulator cannot fully shield the murine phosphoglycerate kinase-1 promoter from integration site effects in transgenic mice

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Abstract

Differential expression of transgenes in transgenic animals is one of the main drawbacks of pronuclear injection. To overwhelm this issue, the genetic constructs are equipped with insulators. In this study, the consensus of exerting chicken hypersensitive site-4 (*cHS4*) insulator was examined on the shield of phosphoglycerate kinase-1 (*Pgk-1*) promoter from the surrounding chromatin in transgenic mice. The PGK-EGFP cassette was flanked by insertion of three copies of the *cHS4* insulators. Mouse zygotes' microinjection by the constructed cassette was resulted in the birth of nine transgenic founders (F0). Copy-number-dependent expression of the *EGFP* was investigated in the transgenic F1 offspring by fluorometry and real-time PCR. They showed no correlation between the expression level of transgene and gene copy number among the transgenic lines. Moreover, dissection of the *EGFP*-expressing mice revealed heterogeneous expression of the *EGFP* in the different organs. In conclusion, for the first time, these findings indicated that the *cHS4* sequence is not a perfect insulator to fully protect the *Pgk-1* promoter from the side effects of integration site in transgenic mice and it needs probably to some additional elements of the *cHS4* locus to reach this goal.

Keywords *Pgk-1* promoter · *cHS4* insulator · Chromosomal position effect · Transgenic mouse

Introduction

Genetically modified mice are extensively used for the purpose of studying gene function and generating disease models (Hickman-Davis and Davis 2006). Pronuclear injection (PI) of fertilized oocytes is the most common used protocol for generation of transgenic mice, which works through random insertion of a transgene into the host genome. Designing genetic constructs is a critical step determining successful expression of transgene in transgenic animals. Expression cassettes typically consist of three crucial components including promoter, gene of interest, and

transcription terminator/polyadenylation sequence (Haruyama et al. 2009; Whitelaw et al. 1993). Transgene expression can be result of chromosomal position effect (CPE), in which the chromatin structure adjacent to the integration site of a transgene can influence on its expression through variegated, ectopic, uncontrolled, and even silenced expression of the transgene. Such undesired side effects result in irreproducible and unpredictable expression patterns of transgenes in transgenic animals (Wilson et al. 1990). To counteract the CPE, encompassing the expression cassettes with cis-regulatory elements, such as insulators, locus control regions, and matrix attachment regions, is suggested to achieve copy-number-dependent, promoter-dependent and position-independent expression of transgenes (Emery et al. 2003).

Chromatin insulators are regulatory DNA elements forming functional boundaries between adjacent chromatin domains by their enhancer blocking and barrier insulating activities (Emery et al. 2003). Chicken hypersensitive site-4 (*cHS4*) is the first identified insulator in vertebrate genome located at the 5' end of chicken β -globin locus with both

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enhancer blocking and barrier insulating activities (Chung et al. 1993). To overcome CPE, several studies showed that encompassing the expression cassettes with *cHS4* insulators leads to increase proportion of high-expressing lines and to reduce variation of expression and somewhat copy-number-dependent expression in transgenic mice (Giraldo et al. 2003; Moltó et al. 2011; Recillas-Targa et al. 2004).

The phosphoglycerate kinase-1 (*Pgk-1*) is a housekeeping gene-encoding X-linked 3-phosphoglycerate kinase, which is essential for glycolysis in all cell types with the exception of sperms (McBurney et al. 1994). The *Pgk-1* promoter could drive long-term expression of transgene in cells that were at the risk of promoter silencing due to epigenetic alterations (Hamaguchi et al. 2000). To best our knowledge, the ability of the *cHS4* insulator to shield *Pgk-1* promoter from undesirable effects of integration site in transgenic mouse is unknown. To achieve this goal, hence, the present study was performed for in vivo assessment of the PGK-EGFP cassette equipped with *cHS4*.

Methods

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from chicken and mouse using the commercial DNAfast solution (NIGEB) as the manufacture's protocol. The chicken *cHS4* insulator and mouse *Pgk-1* promoter were amplified by PCR using sets of specific primers (Table 1). All primers were designed to have appropriate restriction enzyme sites at 5'-termini for cloning. The PCR was carried out in a 20 µL volume using iNtRON Maxime PCR PreMix (iMAXII) kit under the following thermal cycling condition: an initial denaturation at 94 °C for 10 min, 30 cycles of (94 °C for 1 min, 60–63 °C for 30 s, 72 °C for 1 min), and a final extension for 10 min at 72 °C. The amplicons were separately cloned into the pTG19-T vector (Vivantis) and sequenced for verification.

Transgenic cassette construction

The transgenic cassette was generated by subcloning of the PCR fragments into the pEGFP-C1 plasmid (Fig. 1). To obtain the PGK-EGFP cassette equipped with insulator (hereafter INPGK-EGFP), two copies of the *cHS4* insulators were inserted into the upstream of the *EGFP* gene using *AseI-SnaBI* and *SnaBI-NheI* restriction sites. The third insulator was inserted at the downstream of termination sequence using *MluI-DraIII* sites. Finally, the *Pgk-1* promoter was cloned into the upstream of the *EGFP* gene using the *NheI* site.

Table 1 Primer sequences used in this study (the underlines show restriction enzyme sites)

Primers	Sequences
cHS4-I	
InsF- <i>AseI</i>	5' <u>ATTAAT</u> GGGACAGCCCCCCCCCAAAG 3'
InsR- <i>SnaBI</i>	5' <u>TACGTAG</u> CCCCATCCTCACTGACTCCG 3'
cHS4-II	
InsF- <i>SnaBI</i>	5' <u>TACGTAG</u> GGGACAGCCCCCCCCCAAAG 3'
InsR- <i>NheI</i>	5' <u>GCTAGCG</u> CCCCATCCTCACTGACTCCG 3'
cHS4-III	
InsF- <i>MluI</i>	5' <u>ACGCGT</u> GGGACAGCCCCCCCCCAAAG 3'
InsR- <i>DraIII</i>	5' <u>CACTACG</u> TGGCCCCATCCTCACTGACTCCG 3'
PGK	
PGKF- <i>NheI</i>	5' <u>GCTAGCT</u> TCTACCGGGTAGGGGA 3'
PGKR- <i>NheI</i>	5' <u>GCTAGC</u> AGGTCGAAAGGCCCGGA 3'
PGKF- <i>AseI</i>	5' <u>ATTAAT</u> TTCTACCGGGTAGGGGA 3'
EGFP^a	
EGFP-F	5' CAAGGACGACGGCAACTACA 3'
EGFP-R	5' GTCACGAAGTCCAGCAGGACCAT 3'
Taf3	
Taf3-F	5' TGTAGCCATAGAAACCCCAAAGTC 3'
Taf3-R	5' ATAAGCAAGCCAACCTGAAATGAC 3'
RT-EGFP^b	
RT-EGFPF	5' GCAGAAGAACGGCATCAAGGTG 3'
RT-EGFPR	5' AGTGGTTGTCTGGGCAGCAG 3'

^aMouse genotyping primers

^bReal-time PCR primers

Mammalian cell transfection

HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% v/v fetal bovine serum (FBS, Gibco), 2 mM L-glutamine and 1% v/v penicillin–streptomycin (Gibco) in a CO₂ incubator at 37 °C. The cells were seeded at 2 × 10⁵ cells/well in six-well plates, grown to 60% confluence, and transfected with 1 µg of the plasmid DNA using a calcium-phosphate transfection protocol (Kingston et al. 2003). Briefly, the cells were plated at night before transfection and grown to 60–70% confluence by the day of transfection. For each well of a six-well plate, two micrograms DNA were mixed with ddH₂O (to make the total volume of 180 µL) and 20 µL 2.5 M CaCl₂. When the DNA mixture was ready, 200 µL of 2X HBS [50 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM dextrose, and 50 mM HEPES (pH 7.5)] was added dropwise while vortex. The mixture was added into the wells and incubated in 37 °C incubator overnight. The EGFP expression was visualized by a Nikon Eclipse fluorescence microscope 48 h post-transfection.

Fig. 1 Schematic representation of the cassettes and its in vitro assessment. **a** INPGK-EGFP cassette was designed by replacing the CMV promoter in the CMV-EGFP cassette with the *Pgk-1* promoter. After that, three copies of *chs4* insulators amplified from hen genome were inserted at the sides of the cassette. **b** Transient transfection of the two cassettes, including CMV-EGFP as a control and INPGK-EGFP, separately into HEK293T cells confirmed the capability of the engineered cassette in expression of the EGFP protein by a fluorescent microscope

Mouse transgenesis

All animals were conducted according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications no. 8023, revised 1978). The mice were kept on 12:12 light/dark cycle at 20–22 °C with unlimited access to food and water. Four-week-old females were subjected for superovulation by injection of PMSG and hCG and immediately caged with normal males. Next morning after successful mating, the oocytes were extracted and subjected to injections into the male pronucleus of the fertilized oocytes. For PI, the transgenic cassette was released from the rest of the vector by *Ase*I-*Dra*III restriction enzymes, gel purified and diluted in embryo injection buffer (Millipore) at final concentration of 1.2 ng/ μ L. The injected embryos were transferred into the oviduct of pseudo-pregnant females and the newborns were analyzed by PCR to amplify a 375 bp fragment of the EGFP from tail biopsy DNA and by visualizing EGFP emission using Dual Fluorescent Protein Flashlight (NightSea). For positive control, TATA box-binding protein (TBP)-associated factor (*Taf3*) gene was also amplified as a 127 bp endogenous fragment (Table 1). PCR was performed at a volume of 20 μ L using Taq DNA Polymerase Master Mix Red (Ampliqon) as the following thermal cycles: an initial denaturation of 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The transgenic founders with fluorescence emission were crossed with wild-type strain to obtain transgenic pups at the next generation.

Fluorometry and real-time PCR

The relationship between the *EGFP* gene copy number and the fluorescence intensity was investigated in the mice by fluorometry and real-time PCR. To do this, the EGFP-expressing fibroblasts from the F1 transgenic mice were cultured in DMEM medium containing 10% v/v FBS, 2 mM L-glutamine, and 1% v/v penicillin–streptomycin and placed in humidified 37 °C incubator with 5% CO₂. The fluorescence intensity was measured by a Cary Eclipse Fluorometer (Varian, Inc.) and excitation and emission filters were set at 488 and 509 nm, respectively. In continue, copy-number-dependent expression of the *EGFP* was studied by real-time PCR using a RealQ Plus 2x Master Mix Green kit (Ampliqon) in an ABI 7500 system (Applied Biosystems). A three-step run protocol was used according to: (1) an initial hold for 10 min at 95 °C to activate Taq polymerase; (2) an amplification step with 40 cycles of denaturation 20 s at 95 °C, annealing 20 s at 58 °C and extension 20 s at 72 °C; and (3) a melting curve analysis from 58 to 95 °C to check the specificity of the amplified product. The *Taf3* gene was used as an endogenous reference gene to normalize the data. Relative transgene copies were determined by calculating $2^{-\Delta C_t}$ formula.

Statistical analysis

Fluorometry and real-time PCR were conducted in three independent experiments. The results for each experiment were presented as mean $\pm$ SD.

Results

Construction of gene expression cassette

To investigate the capability of the *cHS4* insulator in protection of the *Pgk-1* promoter from CPE, a genetic construct expressing *EGFP* under the control of *Pgk-1* promoter was designed and equipped with the insulators. The fragments of chicken *cHS4* insulator and mouse *Pgk-1* promoter in length of 1211 and 511 bp, respectively, were amplified by PCR and inserted into the pEGFP-C1 plasmid to reach the PGK-EGFP cassette flanked by insulators (INPGK-EGFP) (Fig. 1a).

In vitro assessment of the engineered cassette

For in vitro assessment of the designed construct, it was transfected into the HEK293T cells by calcium-phosphate method and the *EGFP* expression was confirmed by visualization of the green fluorescence. The original CMV-EGFP cassette was also transfected separately as a control of transfection (Fig. 1b). This result demonstrated the capability of the engineered construct for in vitro expression of the transgene.

Generation of transgenic mice

The *EGFP*-expressing cassette was excised from the prokaryotic backbone of the designed plasmid by *AseI*-*DraIII* digestion and then injected into the pronucleus of fertilized oocytes. PCR from the tail biopsy DNA positively amplified a part of the *EGFP* sequence. As an endogenous control, the *Taf3* gene was simultaneously amplified from each mouse (Fig. 2a). Molecular analysis indicated that the microinjection resulted in the birth of nine transgenic founders. Meanwhile, phenotypic analysis of the transgenic founders was performed visually, which revealed unequal emissions of the EGFP fluorescence between the different mice (Fig. 2b).

In vivo assessment of transgene expression

For in vivo assessment, the copy-number-dependent expression of transgene in the transgenic mice was investigated by fluorometry and real-time PCR. The mice, which had visually different intensity of the EGFP signal, were subjected for fibroblast culture and fluorometry. The fluorescent quantification data confirmed that the generated transgenic lines expressed different levels of the EGFP (Fig. 3a). For the determination of the relation between fluorescent intensity and relative transgene copy number, the genomic DNA from the same samples of fluorometry was extracted and

Fig. 2 Transgenic mice generated by injection of the designed cassette. **a** Transgenic founders were identified by PCR on tail-biopsies extracted DNA. The *Taf3* endogenous sequence was amplified to examine the PCR compatibility of the extracted DNA. **b** Transgenic mice produced by the pronuclear injection showed the different intensities of fluorescent signal

subjected for real-time PCR. Comparison of the results did not reveal an absolute direct relationship between the fluorometry and PCR data (Fig. 3b). For instance, while the fibroblasts derived from the transgenic mice 1 and 2 had a similar fluorescent emission, the calculated copy number of transgene differed twofold between them. In addition, the transgene was silenced in line 4 of transgenic mouse. In continue, the fluorescent intensity of the internal organs was compared each other after dissection of transgenic mice, and interestingly, the heterogeneous expression of EGFP was observed between the different organs. As shown in Fig. 4, while the most fluorescent emission was observed in the heart, it was expressed at much lower levels in the liver, kidney, and spleen.

Discussion

In this study, we tested the ability of the *cHS4* insulator to shield the murine *Pgk-1* promoter from CPE. To reach this goal, a PGK-EGFP cassette flanked with three copies of the *cHS4* insulator was designed and used to generate transgenic mouse by PI. Our results showed that the *cHS4* insulator is not perfect enough to shield the transgenic cassette from CPE to obtain a copy-number-dependent expression of transgene and also a uniform expression of transgene in whole body. All quantitative analyses were performed on

Fig. 3 Investigation of the transgene copy-number-dependent intensity of the fluorescent signal. **a** Measurement of the EGFP fluorescence in the culture of fibroblast cells derived from the transgenic F1 offspring (TG) and wild-type (WT) mice. **b** Determination of relative gene copy number in the fibroblasts by real-time PCR. The data showed no correlation between the EGFP fluorescent signal and gene copy number. All experiments were performed in three independent experiments and the results were presented as Mean $\pm$ SD

the F1 offspring, because the potential of mosaicism in the founders, which is raised from delaying in integration of the transgene in genome during pronuclear microinjection.

Chromatin remodeling system is responsible for different distribution of euchromatin and heterochromatin regions of genome during development and can epigenetically influence on gene expression. Transgene expression in genetically engineered animals is affected by several factors such as integration site, gene copy number, cis-acting elements, cell lineage, and genetic background (Martin and Whitelaw 1996). To avoid these issues, it is highly recommended to incorporate genomic insulators in transgenic construct to achieve a stable, uniform and copy-number-related expression of transgenes. The previous study using DNA fragments containing *cHS4* displayed that the insulator protected hybrid constructs from the position effect in *Drosophila* (Chung et al. 1993). Insulators also induced similar effects on transgene expression in genetically engineered cell lines. For example, Rincón-Arango and colleagues reported that the *cHS4* β -globin insulator protects transgene expression against telomeric position effects in chicken HD3 cell line (Rincón-Arango et al. 2007). Several other studies also

characterized *cHS4* insulator effect on transgene expression in stable transfected cell lines (Emery et al. 2000; Pikaart et al. 1998; Aker et al. 2007; Rivella et al. 2000; Yannaki et al. 2002). Studies in transgenic mammals also showed reduction of variable expression and increase tissue-specific expression of transgenes using the *cHS4* fragments (Potts et al. 2000; Taboit-Dameron et al. 1999; Wang et al. 1997). Frazar et al. reported achieving a copy-number-dependent expression of a transgene derived from murine band 3 (AE1) promoter by adding insulators to a transgenic cassette (Frazar et al. 2003).

The *Pgk-1* gene located on X-chromosome permanently encodes an essential enzyme of glycolysis pathway. A previous study conducted by McBurney and coworkers showed that injection of a DNA fragment containing *Pgk-1* promoter caused long term, but not uniform expression of β -galactosidase in transgenic mice (McBurney et al. 1994). However, they did not apply genetic insulators in their cassette. To best our knowledge, this is the first report that investigates the potential of *cHS4* insulator on protection of *Pgk-1* promoter from side effects of integration site in transgenic animals. Although we equipped the PGK-EGFP cassette with three copies of the full-length *cHS4* insulators, they failed to shield the promoter from CPE. Our transgenic mice had different expression levels of the EGFP in internal tissues. This is in line with the Grajevskaja et al. report showing that chicken β -globin *cHS4* insulator failed to shield the *Nkx2.5* promoter from integration site effects in zebrafish (Grajevskaja et al. 2013). Based on these studies, it seems that applying *cHS4* elements can moderately protect integrated transgenes from CPE; however, additional elements of the *cHS4* locus are required to achieve the complete insulating activity.

Conclusions

The results of this study support that the universal insulators are often formed by long genomic fragments, such as the locus control regions (LCRs), containing elements with different and complementary functions. Synergic effects of these elements may result in perfect insulating activity and *cHS4* may be just one of these elements present in the LCR of β -globin locus and it is not lonely a perfect insulator. The outcomes of the present study declare that employing the *cHS4* insulator in transgenic cassettes is insufficient to shield the *Pgk-1* promoter and may be several other promoters from CPE to reach a uniform and also copy-number-dependent expression of transgenes in transgenic mice.

Fig. 4 Visual observation of the fluorescent signal heterogeneity in the F1 transgenic mouse organs. **a** Heart, **b** liver, **c** kidney, and **d** spleen

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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