Insulators prevent transcriptional interference between two promoters in a double gene construct for transgenesis

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Abstract In transgenesis, the expression of two transgenes is often subject to mutual interference by each of the two expression cassettes when they are driven by different transcriptional regulatory elements in a single construct. To study this problem, we constructed vectors consisting of two expression units, one contains a strong ubiquitous promoter and the other contains a tissue-specific transcriptional element. The expression pattern of each transgene was examined in transfected cell lines and also in transgenic mice. In both cases, two expression units in a single construct were expressed in an independent manner and were controlled by their respective regulatory element only if we placed insulators at both ends of one expression unit. These results indicate that usage of insulators is a valuable tool for transfection of double gene constructs in transgenesis.

Key words: Double gene transfection; Enhancer blocking; Insulator; Fluorescent protein; Transcriptional interference; Transgenic mouse

1. Introduction

Double gene transfection is a useful approach for various purposes such as selection of transgenic offspring with a marker gene or examination of the relationship between two gene products. It also enables analysis of gene function in conditional transgenic mice by using a transgene and another modifier gene, such as Cre recombinase, driven by a tissue-specific promoter without mating of two different transgenic lines. For such double gene transfection, an internal ribosomal entry site (IRES) has sometimes been used to make single constructs where the same promoter controls both two transgenes [1]. Frequently, when two transgenes have different transcriptional regulatory elements, two constructs are co-transfected at the same time, but this results in a mixture of double-transfected and single-transfected cells or animals [2,3]. On the other hand, interference between two promoters often affects the expression of two transgenes contained in a single construct [4,5]. Such interference is known as 'transcriptional interference' or 'promoter suppression' [4–9] and is probably caused by competitive binding of transcription factors and/or modification of DNA structure at one site that affects the other site. In endogenous genes, however, most transcription occurs by gene-specific regulatory elements and is independent of adjacent genomic domains. It is believed that the extent of each domain is defined and maintained by special elements called 'insulators' [10–12]. Insulators have been identified by their functional ability to segregate an enhancer and an adjacent promoter into independent domains [13,14]. This ability is called 'enhancer blocking' [10–12,15].

Insulators have been reported in various animals, such as the *gypsy* retrotransposon of *Drosophila* [16], the 5′-flanking region of sea urchin arylsulfatase [17], the repeat organizer (RO) of *Xenopus* 40S ribosomal RNA gene [18], the 5′-DNaseI hypersensitive site 4 (HS4) of chicken β-globin [19] and the blocking element α/δ (BEAD) of human T-cell receptor α/δ [20]. Among these insulators, the chicken HS4 has been the best characterized. When it is inserted at both ends of a transgene, the insulators protect the expression of the transgene against transcriptional silencing, called 'position effect' in stable transfection [19,21–23]. In other reports, the HS4 insulator shows enhancer-blocking ability in transiently transfected cells [24]. Therefore, the HS4 insulator may prevent transcriptional interference in transfection using double gene constructs. A very recent report showed such effects of the HS4 insulator in the stable double-transfection of cultured cells, in which insertion of the insulator between two strong ubiquitous transcriptional regulatory elements, the herpes simplex virus thymidine kinase (HSV-tk) gene promoter and RNA polymerase II (Pol2) gene promoter, prevented blocking in a double gene construct [9]. However, there has been no report addressing the usage of insulators to block the interference of tissue-specific expression in double gene constructs in cultured cells and also in the production of transgenic animals.

In this study, we examined if the HS4 insulator can prevent transcriptional interference between ubiquitous and tissue-specific transcriptional regulatory elements in double gene constructs in both transient and stable gene expression in cultured cells and transgenic mice. We showed that inclusion of the HS4 insulator indeed alleviated transcriptional interference. We constructed double gene vectors consisting of a transgene for the ubiquitous expression of a mitochondria-localized fluorescent protein and another transgene that was expected to result in tissue-specific expression of another fluorescent protein. To examine their expression patterns, such double gene constructs were used to transfect cultured cells and also to produce transgenic mice. We obtained independent expression patterns of the two cassettes, where each was con-

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Abbreviations: CAG, cytomegalovirus enhancer and chicken β-actin promoter; GFP, green fluorescent protein; RFP, red fluorescent protein; YFP, yellow fluorescent protein

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trolled by the individual transcriptional regulatory elements, and specificity of the tissue-specific transcriptional element was protected only if the insulator was placed at both ends of one expression unit.

2. Materials and methods

2.1. Construction of transgenes

pCAGYm has been previously described as pCAG-EYFP-mito [25], which was constructed from pEYFP-mito (Clontech, USA) and pCAGGS [26]. pCAGGS contains the CAG promoter which contains a cytomegalovirus enhancer and the chicken β-actin promoter. pAlbR was constructed by inserting a *Bam*HI–*Ssp*I fragment from pDsRed1-N1 (Clontech, USA) into the *Bam*HI and *Eco*RV sites of p2335A-I (a gift from Dr. Richard Palmiter) [27], which contains the albumin enhancer and promoter. pCAGYm-AlbR and pCAGYm-AlbR(R) were constructed by inserting a 3.2 kb *Sac*I–*Kpn*I fragment of pAlbR into the *Hin*dIII site of pCAGYm. The chicken β-globin HS4 insulator was obtained from pJC5-4 (a gift from Dr. Gary Felsenfeld) [19]. pCAGYm-I-AlbR was constructed by inserting a 1.2 kb *Sac*I fragment carrying the insulator of pJC4-5 into the *Sac*I site of pCAGYm-AlbR. pCAGYm-I-AlbR-I and pCAGYm-I-AlbR(R)-I were constructed by inserting DNA fragments carrying the 1.2 kb insulator of pJC5-4 into the *Sac*I site and *Cla*I site of pCAGYm-AlbR or pCAGYm-AlbR(R). pI-CAGYm-I-AlbR was constructed from pCAGYm-I-AlbR-I by digesting at the *Cla*I and *Spe*I sites followed by self-ligation. pCAGYm-0.7-AlbR-0.7 and pCAGYm-2.1-AlbR-2.1 were constructed by replacing DNA fragments carrying the insulators of pCAGYm-I-AlbR-I with a 0.7 kb *Pvu*I fragment or 2.1 kb *Sac*I fragments of the LacZ gene from pCMV-SPORT β-gal (Gibco, USA). pCAGRm was constructed by replacing a DNA fragment encoding the EYFP protein of pCAGYm with a *Bam*HI–*Not*I fragment encoding the DsRed1 protein of pDsRed1-N1. pInsG was constructed by inserting a insulin enhancer/promoter (−363 to +15) [28], which was obtained by polymerase chain reaction from a healthy human volunteer using the forward primer: 5′-GACAGCAGCGCAAA-GAGCCCCGCCCTGC-3′ and the reverse primer: 5′-CTGT-CCTGGAGGGCTGAGGGCTGCTGGG-3′, into the *Ase*I and *Sac*II sites of pEGFP-N1 (Clontech, USA). pCAGRm-InsG and pCAGRm-InsG(R) were constructed by inserting a 1.3 kb *Spe*I–*Afl*II fragment of pInsG into the *Hin*dIII site of pCAGRm. pCAGRm-I-InsG-I and pCAGRm-I-InsG(R)-I were constructed by inserting DNA fragments carrying the insulator of pJC5-4 into the *Sac*I site and *Cla*I site of pCAGRm-InsG or pCAGRm-InsG(R). For transfection, all plasmids were linearized and purified from the vector backbone.

2.2. Cell culture and transfection

Mouse fibroblasts, SL10 (a STO subline, gift from Dr. Hirofumi Suemori in our laboratory), and mouse hepatoma cells 1ME (ATCC, USA) [29] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). The rat pancreatic β cells, HIT-T15 (ATCC, USA) [30], which has been widely used for analysis of the human insulin transcriptional regulatory element [31–33], were cultured in Ham's F12K medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10% dialyzed horse serum and 2.5% FBS. All cells were incubated at 37°C with 5% CO_2 in a humidified incubator. 10^5 cells were cultured in a chamber slide overnight, and transfected with 2 μg linearized DNA by using Effectene (Qiagen, Germany) according to the manufacturer's procedure. 6 h after transfection, the cells were washed once with phosphate-buffered saline, cultured with fresh medium for 48 h and fixed with 4% formaldehyde. Expression of fluorescent proteins was examined with a fluorescence microscope BX60 (Olympus, Japan). To count the cell number, nuclei were stained with Hoechst 33342 (Sigma, USA) after fixation and fluorescence positive cells was calculated by counting more than 500 Hoechst stained nuclei as the total cell number in each experiment.

2.3. Production of transgenic mice

For production of transgenic mice, CAGYm-AlbR or CAGYm-I-AlbR-I were cut out from the plasmid, purified and microinjected into the pronucleus of fertilized mouse eggs obtained from mating between male and female mice of B6C3F1 (purchased from CLEA Japan, Japan), as previously described [34]. After pronuclear injection, two-cell stage embryos were transferred to oviducts of recipient ICR female mice (purchased from CLEA Japan, Japan).

2.4. Examination of expression patterns in transgenic mice

Transgenic embryos and mice were identified by yellow fluorescent protein (YFP) fluorescence using a fluorescence stereomicroscope MZFLIII (Leica, Germany) at 11.5 days post coitum (dpc), 14.5 dpc or on the day of birth. Fluorescence positive embryos were fixed with 4% formaldehyde, embedded in OCT (Tissue-Tek, Sakura, Japan) and sectioned at 10 μm thickness. Such sections were permeabilized in acetone, incubated in a blocking solution (Block Ace, Dainippon-pharm, Japan) and incubated with primary antibodies, which were rabbit anti-red fluorescent protein (RFP) antibodies (Living Colors D.s. Peptide Antibody, Clontech, USA) or goat anti-albumin antibodies (Bethyl Lab, USA). The sections were then washed and incubated in same blocking solution with secondary antibodies, which were rhodamine-labeled anti-rabbit IgG antibodies (TAGO, USA) or AlexaFluor350-labeled anti-goat IgG antibodies (Molecular Probes, USA). Thus, YFP expression was examined directly with a fluorescence microscope, but RFP expression was detected as rhodamine signals by immunostaining because of weak fluorescence of the RFP itself. Albumin expression was detected as Alexa Fluor 350 signal.

The liver of adult transgenic mice was fixed by perfusion with 4% paraformaldehyde, embedded in OCT, sectioned at 10 μm and examined with a fluorescence microscope. Alternatively, liver tissues were dissociated by perfusion with collagenase solution to obtain and culture hepatocytes as previously described [35]. Such hepatocytes were collected by decantation, cultured in dishes coated with collagen I for 24 h, fixed with 4% paraformaldehyde, stained with Hoechst 33342, and examined with a fluorescence microscope.

3. Results

3.1. Insulators prevent transcriptional silencing in double gene transfection

The chicken HS4 insulator has been reported to have the ability to protect against positional effects in studies of various cells using a variety of genes. To investigate whether the HS4 insulator could protect against transcriptional interference between ubiquitous and tissue-specific transcriptional regulatory elements in a double gene construct, we constructed the vectors consisting of two expression units with or without the insulator. Such constructs are illustrated in Fig. 1 and contain an expression unit CAGYm that consisted of a strong ubiquitous transcriptional element with the CAG promoter and mitochondria-localized YFP cDNA (YFPmito) and another unit, AlbR, that consisted of a hepatocyte-specific albumin enhancer/promoter and RFP cDNA.

When the two expression units were co-transfected as separate constructs, both were expressed in albumin-expressing hepatoma cells (1ME line). On the other hand, only YFPmito was expressed in fibroblasts (SL10 line). However, double-expressing hepatoma cells were not abundant in such co-transfection experiments (data not shown). Next, we examined gene expression in transfection using double gene constructs, CAGYm-AlbR or CAGYm-AlbR(R), where two units were arranged in a head-to-tail manner. As shown in Fig. 2, cells transfected with CAGYm-AlbR or CAGYm-AlbR(R) expressed YFPmito alone. These results indicated that transcriptional silencing of the AlbR unit was caused by transcriptional interference from the CAG promoter. To avoid such silencing, the HS4 insulator was interposed between the two expression units (CAGYm-I-AlbR in Fig. 1), but this construct could not protect against transcriptional silencing (Fig. 2). This result suggested that, in the transient expression assay, transcriptional interference might come from interaction of not only

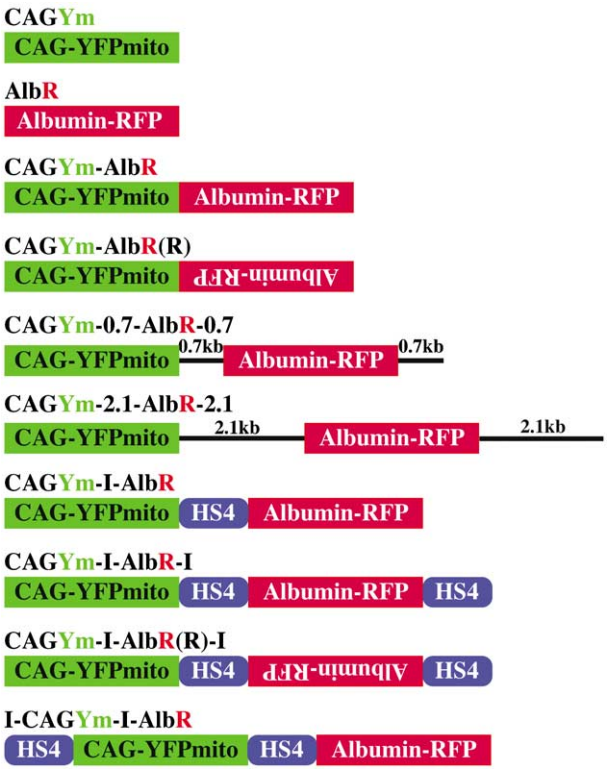

Fig. 1. Structure of double gene constructs containing CAGYm and AlbR for double gene transfection. Each construct consisted of two expression units with or without insulators. The CAG-YFPmito unit (green box), which contains the strong ubiquitous CAG promoter and mitochondria-localized YFP cDNA, was named CAGYm. The albumin-RFP unit (red box), comprising the mouse albumin enhancer/promoter and RFP cDNA, was named AlbR. The blue box indicates the 1.2 kb chicken HS4 insulator and was named I. Constructs in which the CAGYm and the AlbR units are connected in reverse orientation were named (R). Black lines under 0.7 or 2.1 kb indicate 0.7 or 2.1 kb LacZ fragment.

the adjacent units, but also the exterior end of the two units in the double gene constructs.

Therefore, we constructed and transfected CAGYm-I-AlbR-I, CAGYm-I-AlbR(R)-I or I-CAGYm-I-AlbR, in which HS4 insulators were placed between the two units and also at the end of the transgene (Fig. 1). When such constructs were transfected, RFP expression was detected only in hepatoma cells, and all RFP positive cells also expressed YFPmito (Fig. 2 and data not shown). When control constructs in which the 1.2 kb HS4 insulators were replaced by 0.7 or 2.1 kb LacZ fragments (CAGYm-0.7-AlbR-0.7 or CAGYm-2.1-AlbR-2.1 in Fig. 1) were transfected, double positive cells were not detected (Fig. 2). These results showed that, in the transient expression assay using cultured cells, the HS4 insulator could prevent transcriptional silencing of the tissue-specific transcriptional regulatory element in double gene constructs.

3.2. *Insulators prevent leaky transcription in double gene transfection*

Transcriptional interference causes not only transcriptional silencing but also leaky transcription from promoters that should not be expressed. We transfected double gene constructs consisting of the CAGRm unit consisting of the CAG promoter and mitochondria-localized RFP cDNA (RFPmito) and the InsG unit consisting of the pancreatic β cell-specific insulin enhancer/promoter and green fluorescent protein (GFP) cDNA (Fig. 3). Both insulin-expressing pancreatic β cells (HIT-T15 line) and fibroblasts (SL10 line) were transfected. When the InsG unit was transfected alone, GFP expression was detected only in pancreatic β cells (Fig. 4). However, when double gene constructs connecting InsG and CAGRm in a head-to tail manner (CAGRm-InsG or CAGRm-InsG(R) in Fig. 3) were transfected, GFP expression was detected in both pancreatic β cells and fibroblasts (Fig. 4). These results indicated that leaky transcription of the InsG unit was caused by transcriptional interference from the ubiquitous promoter.

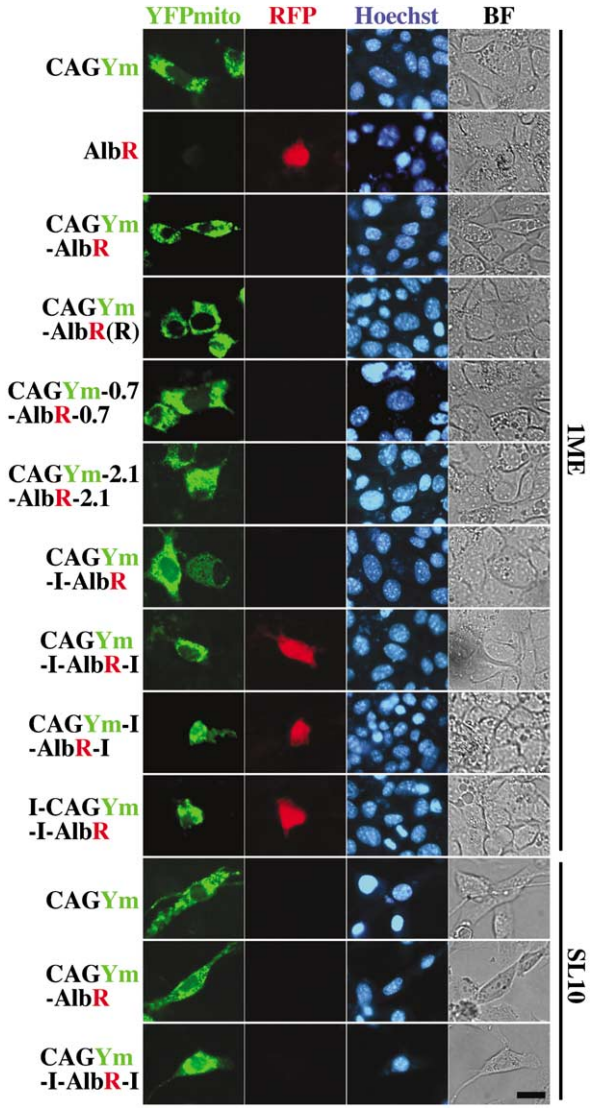

Fig. 2. Placing of insulators prevents transcriptional silencing of AlbR in double gene constructs. The albumin-expressing mouse hepatoma cell line (1ME) or fibroblast cell line (SL10) was transfected by lipofection. Names of transfected constructs and cell lines were described on the left and right of photographs, respectively. Four photographs in a row show the same field. YFPmito and RFP were detected by fluorescence microscopy (YFPmito and RFP columns). Nuclei were shown by Hoechst 33342 staining (Hoechst column). Cell shapes were examined with differential interference contrast (BF column). A bar indicates 20 μm.

To avoid such leakage, HS4 insulators were placed at both ends of the InsG unit in the double gene constructs (CAGRm-I-InsG-I or CAGRm-I-InsG(R)-I in Fig. 3). As shown in Fig. 4, these double gene constructs resulted in expression of both RFPmito and GFP in pancreatic β cells but only that of RFPmito in fibroblasts. These results showed that the HS4 insulator could protect against not only the transcriptional silencing but also the leakage of the tissue-specific transcriptional regulatory element in the double gene constructs in transient expression assays in cultured cells.

3.3. Insulators prevent transcriptional interference in double transgenic mice

In the transient expression assays in cultured cells using the double gene construct, the HS4 insulator prevented transcriptional interference between the ubiquitous and tissue-specific transcriptional regulatory elements. To examine whether the same insulator can prevent transcriptional interference in stable transfection in transgenic animals, we used the double gene constructs with or without the insulators (CAGYm-I-AlbR-I or CAGYm-AlbR illustrated in Fig. 1) for production of double transgenic mice. The constructs were injected into pronuclei of fertilized eggs for transgenesis. Embryos at 14.5 dpc were sectioned and examined for the expression of the fluorescent proteins. In transgenic mice harboring the double gene constructs, YFPmito expression was detected in all tissues of transgenic mice for both constructs. However, RFP was detected specifically in liver of transgenic mice only when using the construct with insulators, CAGYm-I-AlbR-I (Fig. 5A,B and Table 1). Using immunohistochemistry of the liver, we confirmed that the RFP-expressing cells coincided with albumin-expressing cells (data not shown).

For further analysis, hepatocytes of transgenic mice were collected from the liver and the expression of the transgenes was examined in cell culture. As shown in Fig. 5C, RFP driven by the AlbR unit was expressed only in the hepatocytes of the CAGYm-I-AlbR-I transgenic mice but not of the CAGYm-AlbR transgenic mice. We detected no RFP expression in the fibroblasts of either type of transgenic mice. RFP expression was detected in all examined hepatocytes of the CAGYm-I-AlbR-I transgenic mice. From these results, we concluded that the HS4 insulator could protect the transcriptional specificity of the tissue-specific expression units in double gene constructs against transcriptional interference in transgenic mice.

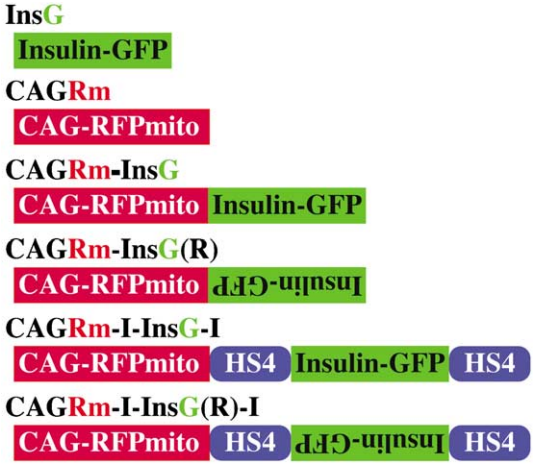

Fig. 3. Structure of double gene constructs containing CAGRm and InsG for double gene transfection. Each construct consisted of two expression units with or without insulators. The CAG-RFPmito unit (red box), which contains CAG promoter and mitochondria-localized RFP cDNA, was named CAGRm. The insulin-GFP unit (green box), comprising the human insulin enhancer/promoter and GFP cDNA, was named InsG. The HS4 insulator (blue box) was named I. Construct in which the CAGRm and the InsG units are connected in reverse orientation were named (R).

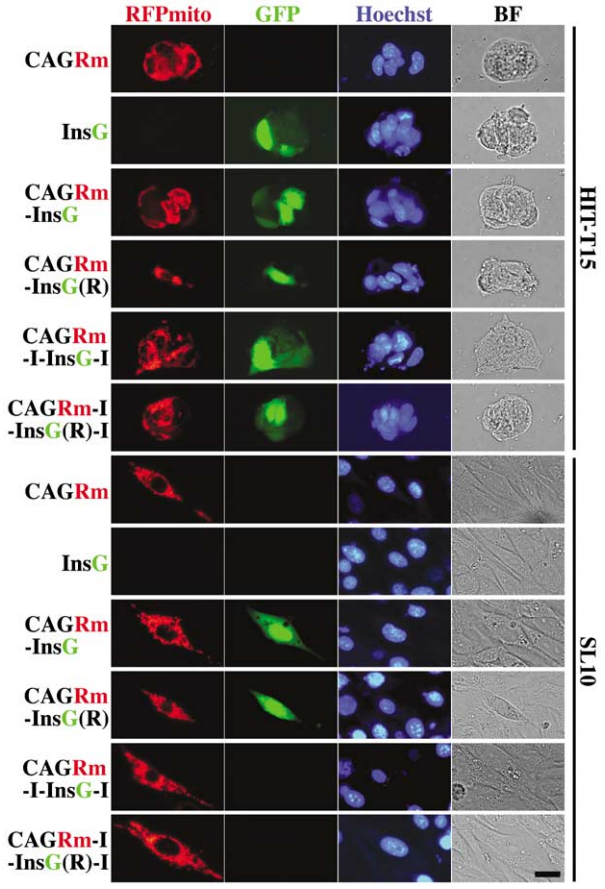

Fig. 4. Placing of insulators in double gene transfection prevents leaky transcription of InsG in double gene constructs. Lipofection was used for double gene transfection into the insulin-expressing rat pancreatic β cell line (HIT-T15) and mouse fibroblast cell line (SL10). Names of the transfected constructs and cell lines were described on the left and right of photographs, respectively. Four photographs in a row show the same field. RFPmito and GFP were detected by fluorescence microscopy (RFPmito and GFP columns). Nuclei were shown by Hoechst 33342 staining (Hoechst column). Cell shapes were examined with differential interference contrast (BF column). A bar indicates 20 μm.

4. Discussion

There have been a few reports that transfection of a double gene construct, in which two expression units were connected in a head-to-tail manner, resulted in independent expression of each unit [36,37]. However, in other reports, transcription of one expression unit is interfered by the other in such double gene constructs [9]. Transcriptional interference in double gene constructs probably shows variation between different transcriptional regulatory elements, and it is unpredictable. To avoid this problem, we constructed double gene vectors consisting of two expression units and chicken β-globin HS4

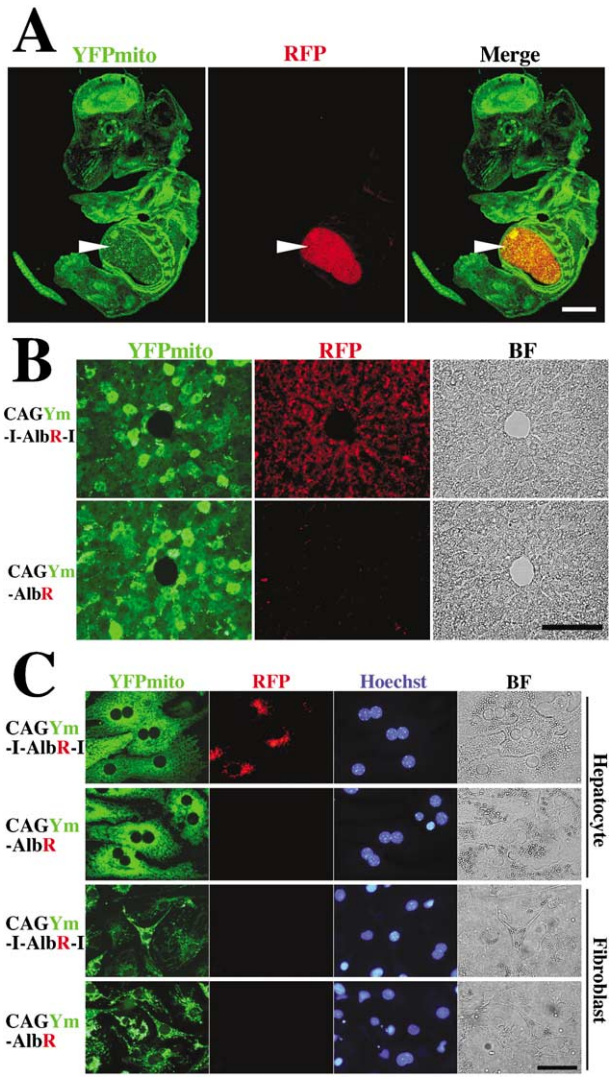

Fig. 5. Placing of insulators prevented transcriptional silencing in double transgenic mice. Expression of YFPmito and RFP was examined in CAGYm-I-AlbR-I or CAGYm-AlbR transgenic mice. (A) A sagittal section of a CAGYm-I-AlbR-I transgenic mouse embryo at 14.5 dpc. To enhance the RFP signal, embryo sections were immunostained by rabbit anti-RFP antibodies and rhodamine-labeled goat anti-rabbit IgG antibodies. Expression of YFPmito was detected directly but that of RFP was detected as rhodamine signals by fluorescence microscopy of the same field (YFPmito and RFP). The right photograph is a merged image of the YFPmito and RFP signals. Arrowheads indicate the liver, and a bar indicates 1 mm. (B) Sections of the liver of adult transgenic mice. The liver of 3-month-old transgenic mice were sectioned and examined for the YFPmito and RFP expression directly by fluorescence microscopy (YFPmito and RFP columns). The construct used for producing the transgenic mouse is described on the left of photographs. Cell shapes were examined with differential interference contrast (BF column) and a bar indicates 400 μm. (C) Hepatocyte-specific expression of the AlbR in double transgenic mice. Hepatocytes of 3-month-old transgenic mice were dissociated by perfusion and cultured on collagen coated chamber slide. After 24 h of culture, the hepatocytes were identified as large binucleated cells with Hoechst 33342 staining of nuclei (Hoechst column) and observation with differential interference contrast (BF column). The YFPmito and RFP expression was observed by fluorescence microscopy (YFPmito and RFP columns). Fibroblasts from the lung, skeletal muscle and skin were mixed, cultured and used as controls of the albumin enhancer/promoter specificity. Four photographs in a row show the same field. Names of the transgenes and cells are described on the left and right of photographs, respectively. A bar indicates 50 μm. →

insulators. We demonstrated that placing of the insulators prevented the transcriptional interference between the ubiquitous and tissue-specific regulatory elements in double gene constructs.

The HS4 insulator is widely used to prevent position effects, that is, variability in expression levels of transgenes depending on the integration site in transgenesis of *Drosophila*, mice and rabbits or transfection of human cell lines [19,21–23]. Such position effects may result from the proximity of an endogenous enhancer or silencer. Protection of transgenes against position effects is probably caused by the enhancer blocking ability of the HS4 insulator, which may be produced by bind-

Table 1
Expression of fluorescence marker genes in transgenic mice

Transgenes	YFPmito positive mice	Stage	Fluorescence (RFP/YFPmito)						
			brain	skin	skeletal muscle	lung	heart	liver	intestine
CAGYm-I-AlbR-I	A	147 dpp	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	B	92 dpp	ND	−/+	−/+	−/+	−/+	+/+	−/+
	C	89 dpp	ND	−/+	−/+	−/+	−/+	+/+	−/+
	D	56 dpp	ND	−/+	−/+	−/+	−/+	+/+	−/+
	E	17 dpp	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	F	17 dpp	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	G	6 dpp	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	H	1 dpp	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	I	17.5 dpc	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	J	14.5 dpc	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	K	14.5 dpc	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	L	14.5 dpc	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	M	14.5 dpc	−/+	−/+	−/+	−/+	−/+	+/+	−/+
	N	11.5 dpc	−/+	−/+	−/+	−/+	−/+	+/+	−/+
CAGYm-AlbR	a	115 dpp	−/+	−/+	−/+	−/+	−/+	−/+	−/+
	b	66 dpp	−/+	−/+	−/+	−/+	−/+	−/+	−/+
	c	14.5 dpc	−/+	−/+	−/+	−/+	−/+	−/+	−/+
	d	14.5 dpc	−/+	−/+	−/+	−/+	−/+	−/+	−/+
	e	14.5 dpc	−/+	−/+	−/+	−/+	−/+	−/+	−/+

YFPmito and RFP were detected in dissected organs and their sections of independent founders. dpp, days post partum; dpc, embryonic days post coitum. ND, not determined; −/+, YFPmito only was detected; +/+, both RFP and YFPmito were detected.

ing of the CTCF protein (CCCTC binding factor) [38] and blocking of the methylation of histone H3 [39]. Although the mechanism of insulators is still unclear, it has been well established that the enhancer blocking ability of the HS4 insulator can protect independent gene expression from transcriptional interference. A very recent report showed that insertion of single copy of the HS4 insulator between two strong ubiquitous transcriptional regulatory elements in a double gene construct prevented the interference in the stable double-transfection of cultured cells [9]. However, there has been no report of the usage of insulators to block the interference to the tissue-specific expression in double gene constructs in cultured cells or transgenic animals.

In this study, specificity of the tissue-specific transcriptional regulatory element was protected from inhibitory or leaky transcriptional interference from the strong ubiquitous expression unit only when the insulators were placed at both ends of the tissue-specific expression unit in double gene constructs. Using such constructs, we observed that two expression units successfully expressed marker proteins according to their individual transcriptional regulatory elements in transient expression assays using cultured cells. This indicated that the inclusion of HS4 insulators is valuable for double gene transfection using the double gene constructs. Also, in our results, expression of the two expression units was controlled independently in all of the CAGYm-I-AlbR-I transgenic mice, where both units were included in a single construct. Therefore, it is a more optimal method for producing double transgenic mice than simultaneous injection of two constructs or mating between two transgenic lines.

This double gene transfection method is a valuable approach for various purposes such as selection of transfected cells with an antibiotic resistance gene or identification of transgenic animals with a marker gene. Furthermore, it can be used in production and selection of functional cells from stem cells for cell transplantation therapy involving transfection of both tracer and differentiation marker genes. Finally, such constructs are valuable to introduce useful genes into spermatogenic cells in testis and select the transfected spermatozoa in the testis-mediated transgenesis we reported previously [25].

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References

[1] Martinez-Salas, E. (1999) Curr. Opin. Biol. 10, 458–464.
[2] Overbeek, P.A., Aguilar-Cordova, E., Hanten, G., Schaffner, D.L., Patel, P., Lebovitz, R.M. and Lieberman, M.W. (1991) Transgenic Res. 1, 31–37.
[3] Clark, A.J., Cowper, A., Wallace, R., Wright, G. and Simons, J.P. (1992) Biotechnology 10, 1450–1454.
[4] Kadesch, T. and Berg, P. (1986) Mol. Cell. Biol. 6, 2593–2601.
[5] Proudfoot, N.J. (1986) Nature 322, 562–565.
[6] Cullen, B.R., Lomedico, P.T. and Ju, G. (1984) Cell 39, 459–467.
[7] Corbin, V. and Maniatis, T. (1989) Nature 337, 279–282.
[8] Emerman, M. and Temin, H.M. (1986) Nucleic Acids Res. 14, 9381–9396.
[9] Villemure, J.-F., Savard, N. and Belmaaza, A. (2001) J. Mol. Biol. 312, 963–974.
[10] Bell, A.C., West, A.G. and Felsenfeld, G. (2001) Science 291, 447–450.
[11] Sun, F.-L. and Elgin, S.C.R. (1999) Cell 99, 459–462.
[12] Udvardy, A. (1999) EMBO J. 18, 1–8.
[13] Udvardy, A., Maine, E. and Schedl, P. (1985) J. Mol. Biol. 185, 341–358.
[14] Holdridge, C. and Dorsett, D. (1991) Mol. Cell. Biol. 11, 1894–1900.
[15] Kellum, R. and Schedl, P. (1992) Mol. Cell. Biol. 12, 2424–2431.
[16] Geyer, P.K. and Corces, V.G. (1992) Genes Dev. 6, 1865–1873.
[17] Akasaka, K., Nishimura, A., Takata, K., Mitsunaga, K., Mibuka, F., Ueda, H., Hirose, S., Tsutsui, K. and Shimada, H. (1999) Cell. Mol. Biol. 45, 555–565.
[18] Robinett, C.C., O'Connor, A. and Dunaway, M. (1997) Mol. Cell. Biol. 17, 2866–2875.
[19] Chung, J.H., Whiteley, M. and Felsenfeld, G. (1993) Cell 74, 505–514.
[20] Zhong, X.P. and Krangel, M.S. (1997) Proc. Natl. Acad. Sci. USA 94, 5219–5224.
[21] Pikaart, M.J., Recillas-Targa, F. and Felsenfeld, G. (1998) Genes Dev. 12, 2852–2862.
[22] Wang, Y., DeMayo, F.J., Tsai, S.Y. and O'Malley, B.W. (1997) Nat. Biotech. 15, 239–243.
[23] Taboit-Dameron, F., Malassagne, B., Viglietta, C., Puissant, C., Leroux-Coyau, M., Chereau, C., Attal, J., Weill, B. and Houdebine, L.M. (1999) Transgenic Res. 8, 223–235.
[24] Recillas-Targa, F., Bell, A.C. and Felsenfeld, G. (1999) Proc. Natl. Acad. Sci. USA 96, 14354–14359.
[25] Huang, Z., Tamura, M., Sakurai, T., Chuma, S., Saito, T. and Nakatsuji, N. (2000) FEBS Lett. 487, 248–251.
[26] Niwa, H., Yamamura, K. and Miyazaki, J. (1991) Gene 108, 193–199.
[27] Pinkert, C.A., Ornitz, D.M., Brinster, R.L. and Palmiter, R.D. (1987) Genes Dev. 1, 268–276.
[28] Itier, J.-M., Douhet, P., Desbois, P., Joshi, R.L., Dandoy-Dron, F., Jami, J. and Bucchini, D. (1996) Differentiation 60, 309–316.
[29] Patek, P.Q., Collins, J.L. and Cohn, M. (1978) Nature 30, 510–511.
[30] Santerre, R.F., Cook, R.A., Crisel, R.M., Sharp, J.D., Schmidt, R.J., Williams, D.C. and Wilson, C.P. (1991) Proc. Natl. Acad. Sci. USA 78, 4339–4343.
[31] Walker, M.D., Edlund, T., Boulet, A.M. and Rutter, W.J. (1983) Nature 306, 557–561.
[32] Clark, A.R., Wilson, M.E., London, N.J., James, R.F. and Docherty, K. (1995) Biochem. J. 309, 863–870.
[33] Olson, L.K., Qian, J. and Poitout, V. (1998) Mol. Endocrinol. 12, 207–219.
[34] Hogan, B., Costantini, F. and Lacy, E. (1986) Manipulating the Mouse Embryo: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[35] Hirose, T., Terajima, H., Yamauchi, A., Kinoshita, K., Furuke, K., Gomi, T., Kawai, Y., Tsuyuki, S., Nakamura, Y., Ikai, I., Taniguchi, T., Inamoto, T. and Yamaoka, Y. (1997) J. Hepatol. 27, 1081–1088.
[36] Eiant, P., Bergham, Y., Yaffe, D. and Shani, M. (1987) Genes Dev. 1, 1075–1084.
[37] Sato, M., Watanabe, T., Oshida, A., Nagashima, A., Miyazaki, J. and Kimura, M. (2001) Mol. Reprod. Dev. 60, 446–456.
[38] Bell, A.C., West, A.G. and Felsenfeld, G. (1999) Cell 98, 387–396.
[39] Litt, M.D., Simpson, M., Gaszner, M., Allis, C.D. and Felsenfeld, G. (2001) Science 293, 2453–2455.