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Effects of insulator cHS4 on transgene expression from plasmid DNA in a positive feedback system

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## **ABSTRACT**

The effects of a genetic insulator (chicken cHS4) on transgene expression in a positive feedback system using the GAL4-VP16 activator and luciferase reporter plasmids were examined. The introduction of cHS4 enhanced luciferase expression in mouse liver, indicating that this system would be useful for efficient transgene expression.

**Keywords:** genetic insulator; cHS4; plasmid; positive feedback system; transgene expression

Nonviral gene delivery systems are recognized as an appealing alternative to viral vectors, due to their potential avoidance of the immunogenicity and toxicity problems associated with the use of viral systems. However, transgene expression from plasmid DNA delivered with nonviral vectors is generally low and transient. One possible reason for the problem is suppressed transgene expression in the nucleus. We and others have reported the silencing of plasmid DNA (1-3), indicating that the avoidance of silencing is required for high, long-term expression. To alleviate the silencing, strategies including the decrease/depletion of CpG motifs and the removal of the bacterial backbone from the plasmid DNA, as well as the use of the full genomic DNA sequence with its natural promoter, have been reported (4-8). These results suggested that the transgene silencing is induced by the bacterial DNA-like characteristics of the plasmid DNA.

A genetic insulator is a boundary element in chromosomal DNA, and it reportedly prevents the silencing of transgenes integrated into chromosomal DNA (9). To examine whether it could prevent the silencing caused by the bacterial backbone of plasmid DNA, Chen *et al.* inserted two tandem units of the 5'-end of the chicken  $\beta$ -globin locus cHS4, a

well-known genetic insulator, both upstream and downstream of the human  $\alpha$ 1-antitrypsin expression cassette containing a viral promoter, the Rous sarcoma virus (RSV) long terminal repeat (LTR) (10). They observed that the insertion of cHS4 partially suppressed the silencing, suggesting the usefulness of the genetic insulator for enhanced transgene expression.

Recently, we constructed a positive feedback system, using an artificial transcription factor, GAL4-VP16 (11). In this system, GAL4-VP16, expressed from plasmid DNA (activator), binds to the GAL4 binding sequences located both upstream and downstream of the luciferase expression cassette in another plasmid DNA (reporter), resulting in enhanced luciferase expression. In addition, the GAL4 binding sequence was introduced both upstream and downstream of the GAL4-VP16 expression cassette, to enhance its own expression. When the reporter and activator plasmid DNAs were co-introduced into mouse liver, the luciferase expression was increased, indicating the potential of this positive feedback system.

In this study, we examined the effects of cHS4 on luciferase expression driven by the mouse albumin (ALB) promoter in mouse liver. The introduction of the genetic insulator increased luciferase expression in the positive feedback system, indicating that the positive feedback system including the insulator and the ALB promoter would be useful for efficient transgene expression.

The plasmid pJC13-1 (12) was kindly provided by Drs. Gary Felsenfeld and Noriko Saitoh. Oligodeoxyribonucleotides were obtained from Life Technologies Japan (Tokyo, Japan) in purified forms. Six-week-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). The mice were kept under specific pathogen-free conditions and were maintained in a temperature-controlled room with a 12 h light/dark illumination cycle.

All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee.

The locus control region was deleted from the pJC13-1 plasmid by EcoRI digestion, followed by self ligation to yield pJC13-1( $\Delta$ LCR). Five tandem copies of the 17-bp GAL4 DNA binding site (G5) were amplified from pG5-luc (Promega, Madison, WI, USA), and the expression cassette containing the upstream G5, the ALB promoter, the luciferase gene, and the downstream G5 was constructed. The expression cassette was inserted into the backbone of pJC13-1( $\Delta$ LCR) to yield p2-G5-AL-G5-2, containing two tandem units of cHS4 both upstream and downstream of the expression cassette (Fig. 1). The p2-G5-AV-G5-2 plasmid DNA was constructed in a similar way. The four copies of cHS4 were deleted from the pJC13-1( $\Delta$ LCR) plasmid by stepwise restriction enzyme plus self ligation reactions. The expression cassettes containing G5, the ALB promoter, and the luciferase/GAL4-VP16 gene were inserted into the cHS4-deleted vector, to yield plasmid DNAs without cHS4 (Fig. 1).

The plasmid DNA (1 pmol) was administered to BALB/c mice by the hydrodynamics-based procedure (13,14). In the positive feedback system experiments, the reporter (luciferase) plasmid DNA and the activator (GAL4-VP16) plasmid DNA (1 pmol each) were co-administered by the hydrodynamics-based procedure. The luciferase activities in the livers were measured at 8 h, and on days 2 and 7 after injection, as described previously (1,2). Statistical significance was examined by the Student's *t*-test. Levels of  $P < 0.05$  were considered to be significant.

We inserted the insulator into luciferase plasmid DNA containing the liver-specific ALB promoter (p2-G5-AL-G5-2, Fig. 1). We chose the 5'-end of the chicken  $\beta$ -globin locus cHS4, a well-known insulator, and inserted two tandem units of cHS4 both upstream and

downstream of the expression cassette. We also inserted cHS4 at each end of the expression cassette for an activator, GAL4-VP16, driven by the ALB promoter (Fig. 1).

The activator GAL4-VP16 and reporter luciferase plasmid DNAs without cHS4 (p0-G5-AV-G5-0 plus p0-G5-AL-G5-0, 1 pmol each) were co-administered into BALB/c mice by the hydrodynamics-based administration method, which enables the efficient introduction of naked DNA into the liver (13,14), and the luciferase activity in the liver was measured for 1 week. As shown in Fig. 2A, the luciferase activity seemed to be enhanced by the co-administration (compare p0-G5-AL-G5-0 alone and p0-G5-AL-G5-0 plus p0-G5-AV-G5-0). These results were similar to those in our previous study (also shown in Fig. 2) (11).

When the activator GAL4-VP16 and reporter luciferase plasmid DNAs containing cHS4 (p2-G5-AV-G5-2 plus p2-G5-AL-G5-2) were co-administered into BALB/c mice, the luciferase expression was strongly increased (Fig. 2). On day 7 after injection, the luciferase expression from the positive feedback system (p2-G5-AV-G5-2 plus p2-G5-AL-G5-2) was 3,000-fold higher than that from p2-G5-AL-G5-2 alone and 10-fold higher than that from the positive feedback system without cHS4.

A genetic insulator is a boundary element in chromosomal DNA. It plays two distinct roles in gene expression, as an enhancer-blocking element and as a barrier against condensed chromatin proteins spreading onto active chromatin. Felsenfeld and his collaborators demonstrated that the cHS4 insulator element protected the transgene of an integrated plasmid from chromosomal silencing, and that the transcription factor USF1 was required for the barrier activity (9). This insulator has also been shown to impair the silencing of retroviral and lentiviral vectors (15,16). Kay and his collaborators reported that the introduction of cHS4 into plasmid DNA partially suppressed the silencing, in the case of

the human  $\alpha$ 1-antitrypsin gene driven by the RSV-LTR (10). The finding that the cHS4 insulator enhanced the expression in the positive feedback system (Fig. 2) suggested contribution of its barrier activity.

Control of the intranuclear disposition of plasmid DNA is necessary for achieving efficient and prolonged transgene expression (17). Previously, we showed that a transgene introduced on plasmid DNA was silenced in mouse liver, and that the silenced transgene could be reactivated (1,2). Thus, transgene silencing might be overcome in various ways. The combination of the insulator and the positive feedback system are strategies for the prevention of transgene suppression. The successful enhancement of transgene expression by the combination confirms the usefulness of this system in many biotechnical fields, including the generation of transgenic models, *in vivo* imaging, and gene therapy.

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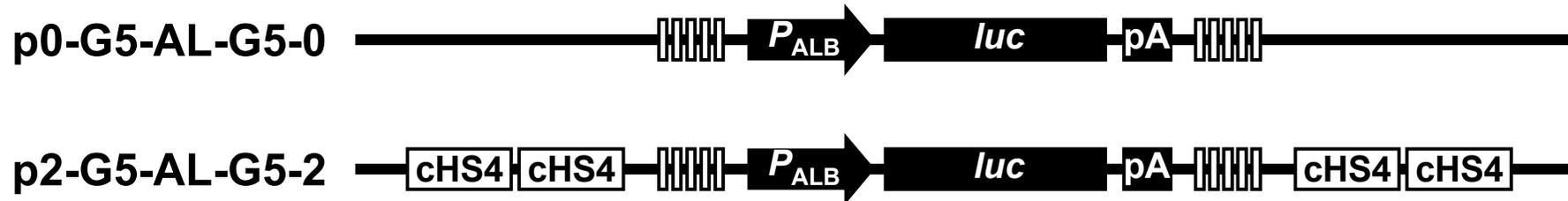
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**Figure 1.** Schematic diagrams of the reporter and activator plasmid DNAs used in this study. The white box indicates the 17-bp GAL4 binding site.  $P_{ALB}$ , *luc*, and pA refer to the ALB promoter, the luciferase gene, and the polyA signal, respectively.

**Figure 2.** The effect of the positive feedback system involving the insulator in the mouse liver. The reporter plasmid DNA (1 pmol) was co-injected into BALB/c mice with the same amount of the activator plasmid DNA. The luciferase activity was measured at 8 h, and on days 2 and 7 after injection. The values represent the averages of two to five independent experiments. Bars indicate SD (standard deviation). Open squares, p0-G5-AL-G5-0 alone; open circles, p0-G5-AL-G5-0 plus p0-G5-AV-G5-0, closed squares, p2-G5-AL-G5-2 alone; closed circles, p2-G5-AL-G5-2 plus p2-G5-AV-G5-2. \* $P < 0.05$  versus p0-G5-AL-G5-0. The dashed line represents the data reported in the previous study, obtained with the positive feedback system without cHS4 (11).

## Reporter plasmid DNAs



## Activator plasmid DNAs

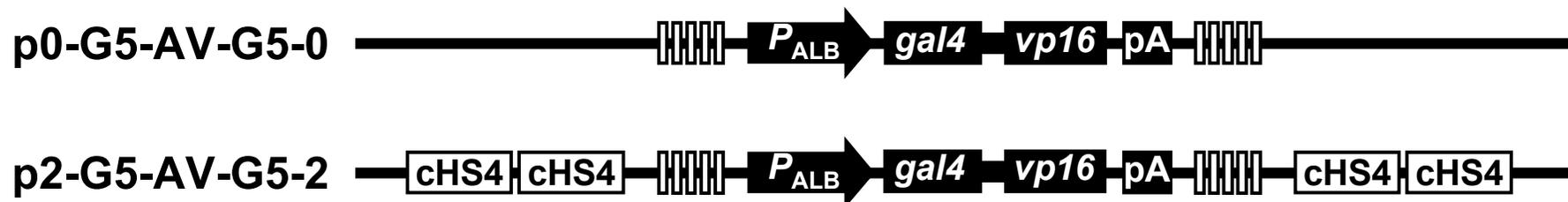


Fig. 1

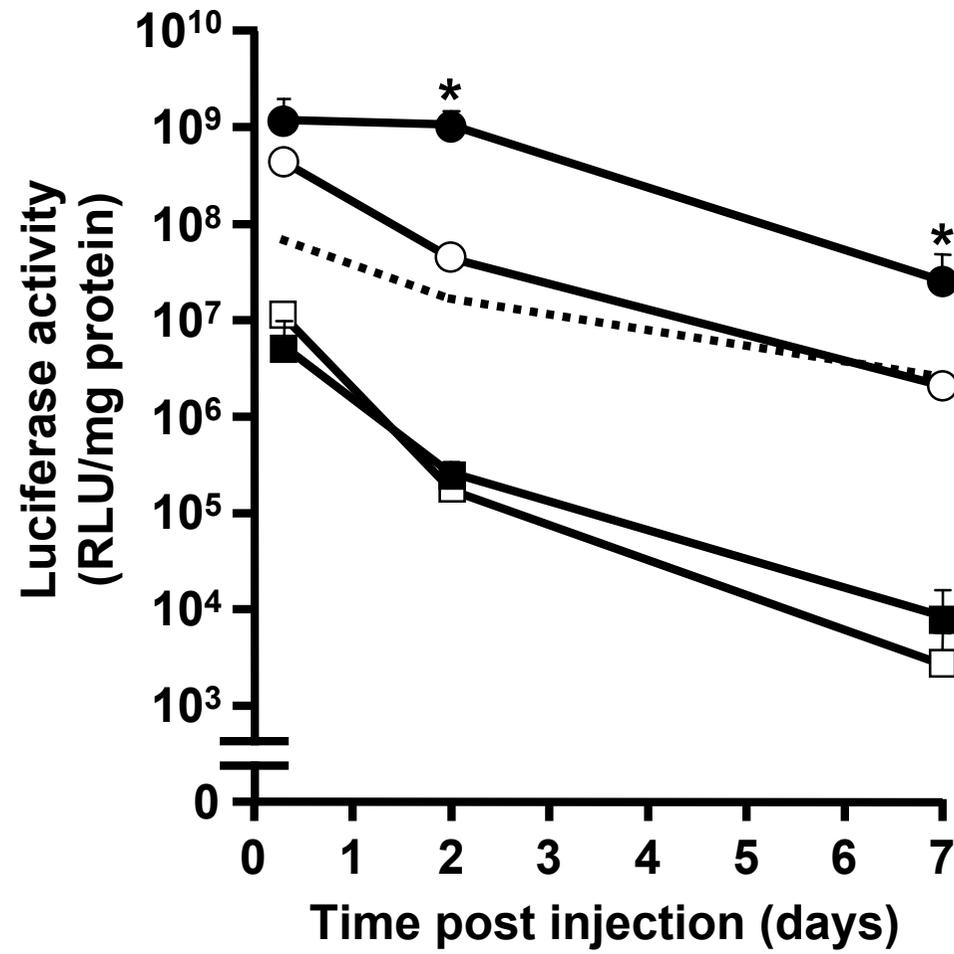


Fig. 2