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Positive Feedback System Provides Efficient and Persistent Transgene Expression

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Abstract: The two-step transcriptional amplification (TSTA) system, using artificial transcription factors, effectively enhances transgene expression. In this study, a TSTA system-based positive feedback system was developed to achieve efficient and persistent transgene expression. A fusion protein of the sequence-specific DNA binding domain of yeast GAL4 and the transcriptional activation domain of herpes simplex virus VP16 (GAL4-VP16) was used as an "activator" to amplify the expression of the luciferase "reporter" gene. It was found that the introduction of five tandem copies of the GAL4 recognition sequence (G5) into both the upstream and downstream regions of the expression cassette synergistically enhanced the transgene expression. The upstream and downstream G5 sequences were introduced into the expression cassette of the activator itself, and into that of the reporter, to form the positive feedback loop that enabled continuous activator expression. This positive feedback system maintained the expression levels of the reporter for 4 days in HeLa cells and for a week in mouse liver, while those from the usual plasmids decreased by 30- and 50-fold, respectively. These results constitute the first evidence that the positive feedback system is a useful method for long-term transgene expression in cultured cells and *in vivo*. This system would be applicable to gene therapy, *in vivo* imaging, and biotechnology.

Keywords: Long-term transgene expression; artificial transcription factor; positive feedback system

Introduction

Transgene expression from plasmid DNA (pDNA) in mammalian cells is occasionally used in biotechnology and is essential for gene therapy. However, the expression from pDNA is generally transient. Control of the intranuclear disposition of pDNA is necessary for achieving efficient and prolonged transgene expression.[1] Recently, we and others reported that the short duration of transgene expression was due to the silencing of pDNA,[2−4] indicating that the avoidance of silencing is important for long-term expression. To alleviate the silencing, depletion of the CpG motif and removal of the bacterial backbone were performed.[5−8] In some cases, the transgene expression was maintained by using the full genomic DNA sequence with its natural promoter.[9] These strategies have been recognized as useful ways to obtain prolonged transgene expression.

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Previously, we showed that the silenced transgene in mouse liver could be reactivated.[3] Thus, transgene silencing is a reversible process, and continuous activation of pDNA might be another way to achieve long-term transgene expression. In addition, the expression efficiency from pDNA was 3 orders of magnitude lower than that from an adenoviral vector.[10] Thus, the pDNA-specific transcriptional activation and its maintenance, in addition to the prevention of silencing, are necessary for sustained gene expression.

Artificial transcription factors are a very attractive tool for the activation of specific genes. GAL4-VP16, one of the most common artificial transcription factors, is a fusion protein of the DNA binding domain of yeast GAL4 and the transcriptional activation domain of herpes simplex virus VP16, and in mammalian cells, it induces robust expression of genes bearing the 17-bp GAL4 binding sequence near their promoters.[11] The genes of interest can be activated in a tissue-specific manner, when GAL4-VP16 is expressed under the control of tissue-specific promoters. This system, called two-step transcriptional amplification (TSTA), is a promising tool for many biotechnical fields, including noninvasive *in vivo* imaging and gene therapy.[12−14] Furthermore, the positive feedback system, in which an artificial transcription factor enhances its own expression, has been expected to increase the strength and the specificity of TSTA.[15] Nettelbeck et al. developed a bicistronic pDNA carrying both the reporter and activator (LexA-VP16) genes under the control of an activator-responsive promoter, and demonstrated that the expression driven by the very weak, but highly specific, von Willebrand factor (vWF) promoter was enhanced by the positive feedback loop.[16] In addition, Woraratanadharm et al. generated an inducible positive feedback system by using the Tet-off (TetR-VP16) system.[17] These systems, however, were used mainly for increasing transient transgene expression efficiency, but not for long-term expression.

In this study, we examined the positive feedback system based on TSTA for persistent expression. GAL4-VP16 was used as an activator to enhance the expression of the luciferase reporter gene. Tandem copies of the GAL4 biding sites were introduced into both the reporter and activator genes. The reporter and activator pDNAs were cointroduced into HeLa cells and mouse liver, and the luciferase expression was monitored for 4 and 7 days, respectively. We found that the introduction of the GAL4 binding sites downstream of the polyA signal, as well as upstream of the promoter, maintained the highest luciferase expression level driven by the human cytomegalovirus (CMV) promoter in HeLa cells. Moreover, this system efficiently suppressed the silencing of the luciferase gene, driven by the mouse albumin (ALB) promoter, in mouse liver. These results suggest that the maintenance of an activated state by the positive feedback loop is a useful method for long-term expression.

Materials and Methods

Materials. The plasmids pG5-luc, pACT, and pBIND were purchased from Promega (Madison, WI), and pBluescript II SK(+) was from Stratagene (La Jolla, CA). Oligodeoxyribonucleotides were obtained from Invitrogen Japan (Tokyo, Japan) and Sigma Genosys Japan (Ishikari, Japan) in purified forms.

Animals. 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.

Cell Culture. HeLa cells were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supple-

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mented with 10% fetal bovine serum (FBS), penicillin, and streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Construction of the Reporter pDNAs. Five tandem copies of the 17-bp GAL4 DNA binding site (G5) were amplified from pG5-luc, and were inserted into the *Kpn*I site of pG5-luc after blunting with KOD polymerase (pol) (Takara, Kusatsu, Japan), yielding the plasmid pG10-luc. To create the plasmid pΔG-luc, which lacked GAL4 DNA binding sites, pG5-luc was digested with *Kpn*I and *Nhe*I, blunted with KOD pol, and self-ligated. The plasmids pluc-G5 and pluc-G10 were constructed by inserting one and two G5 fragments, respectively, into the *Bam*HI site of pΔG-luc. The plasmid pG5-CMV-luc, containing the G5 sequence upstream of the CMV enhancer/promoter, was generated by replacement of the minimal TATA box (*Nhe*I−*Bgl*II) of pG5-luc with the CMV enhancer/promoter (*Bgl*II−*Nhe*I) of pBIND, after blunting with KOD pol. Similarly, the minimal TATA box of pG5-luc was replaced with the ALB promoter (−281/+39), containing the essential elements for liver-specific expression,[18−20] amplified from BALB/c mouse genomic DNA to yield pG5-ALB-luc. The G5 fragment was amplified by using the following primer set: (*Bgl*II-5G4951 (Δ*Kpn*I), 5′-ACAAGATCTCGATAAGTACCGAGTTTCTA-GA-3′, and 5G139(R)-*Bam*HI, 5′-TCAGGATCCAGC-CCCCCGCTAGCGTCTT-3′), and was inserted into the *Bam*HI sites of pG5-luc and pG5-CMV-luc to yield the plasmids pG5-luc-G5 and pG5-CMV-luc-G5, respectively. The plasmid pG5-ALB-luc-G5 was obtained by the replacement of the CMV promoter (*Kpn*I−*Hin*dIII) of pG5-CMV-luc-G5 with the ALB promoter (*Kpn*I−*Hin*dIII) of pG5-ALB-luc.

Construction of the Activator pDNAs. The λ repressor linker (corresponding to amino acid residues 92−132) was initially amplified from λ phage DNA and introduced into pBIND pretreated with *Bam*HI and *Xba*I. The transcription activation domain of the herpes simplex virus VP16 (corresponding to amino acid residues 411−454) was then amplified from pACT, and was introduced into the region between the *Xba*I and *Kpn*I sites to yield the plasmid pCMV-VP16. To create pG5-VP16, the CMV enhancer/promoter (*Bgl*II−*Pst*I) of pCMV-VP16 was replaced with the DNA fragment including the minimal TATA box and the upstream G5 sequence of pG5-luc. The plasmid pG5-CMV-VP16 was obtained by inserting the G5 fragment into the *Bgl*II site of pCMV-VP16. The plasmid pG5-ALB-VP16 was constructed by replacing the minimal TATA box (*Nhe*I−*Nhe*I) of pG5-VP16 with the mouse ALB promoter. One of the two *Cla*I sites in pG5-CMV-VP16, containing no overlapping GATC sequence, was initially deleted by blunt-end ligation. The resulting plasmid, containing a unique *Cla*I site overlapping the GATC sequence, was amplified in *Escherichia coli* strain INV110 (*dam*⁻). The G5 fragment was then amplified by using the following primers: *Cla*I-5G4951, 5′-ACAATC-GATCGATAGGTACCGAGTTTCTAGA-3′, and 5G139(R)-*Sal*I−*Cla*I, 5′-TCAATCGATAGTCGACAGCCCCCCG-CTAGCGTCTT-3′. The fragment was inserted into the *Cla*I site of the plasmid, to yield the plasmid pG5-CMV-VP16-G5. The plasmid pG5-ALB-VP16-G5 was obtained by substituting the expression cassette (*Bgl*II−*Mfe*I) of pG5-CMV-VP16-G5 with the expression cassette (*Bgl*I−*Mfe*I) of pG5-ALB-VP16.

Construction of Single-Plasmid System. The expression cassette of pG5-CMV-VP16-G5, including the two G5 sequences, was purified by agarose gel electrophoresis after digestion with *Bgl*II and *Sal*I, and then inserted into pG5-CMV-luc-G5 pretreated with *Bam*HI and *Sal*I, to yield p5CL5-5CV5. The p5AL5-5AV5 plasmid was constructed by similar procedures from pG5-ALB-VP16-G5 and pG5-ALB-luc-G5.

Reporter Assay (HeLa Cells). For transient expression, HeLa cells were plated in a 24-well plate (4×10^4 cells/well) one day before transfection. The reporter pDNA (200 ng) and the activator plasmid pCMV-VP16 (200 ng) were cotransfected with the LipofectAMINE Reagent (Invitrogen) according to the manufacturer's instructions. The luciferase activity was measured with a luciferase assay system (Promega) at 48 h after transfection.

For time-course experiments, the reporter (200 ng) and the activator pDNAs (200 ng each) were cotransfected into HeLa cells (1×10^4 cells/well). In the case of the single-plasmid system, the p5CL5-5CV5 plasmid (200 ng) was cotransfected with the same amount of pBluescript II SK(+). Cells were recovered on 1, 2, 3, and 4 days after transfection, and the luciferase activity was examined.

***In Vivo* Reporter Assay.** The reporter plasmid pG5-ALB-luc-G5 (1 pmol, 3.40 μg) was coadministered to BALB/c mice by the hydrodynamics-based procedure[21,22] with the same molar amount of the activator plasmid pG5-ALB-VP16-G5 (3.85 μg), pΔG-ALB-VP16 (3.68 μg), or pBluescript II SK(+) (1.87 μg). In the case of the single-plasmid system, 1 pmol (4.43 μg) of the p5AL5-5AV5 plasmid was injected. The luciferase activities and the amounts of the intranuclear luciferase DNA in the livers were measured at 8 h, and on 2 and 7 days after injection, as described previously.[2,3]

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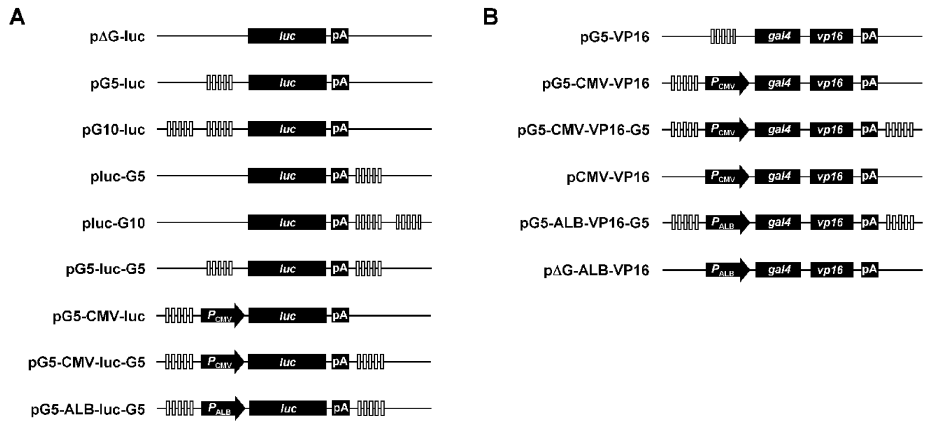

Figure 1. Schematic diagrams of the reporter (A) and activator (B) pDNAs used in this study. The white box indicates the 17-bp GAL4 binding site, and pA refers to the polyA signal.

Statistical Analysis. Statistical significance was examined by Student's t test. Levels of $P < 0.05$ were considered to be significant.

Results

The Effect of a GAL4 DNA Binding Site Downstream of the PolyA Signal. We first examined the efficiency of the TSTA system in cultured cells. The firefly luciferase gene, bearing five tandem copies of the 17-bp GAL4 DNA binding site (G5) located 19-bp upstream of the minimal TATA box, was used as the reporter gene (Figure 1A). The reporter plasmid pG10-luc, containing two G5 sequences, was also constructed (Figure 1A). The activator was constructed by fusing the GAL4 DNA binding domain (amino acid residues 1−147) to the VP16 transcription activation domain (amino acid residues 411−454). The λ repressor linker (amino acid residues 92−132) was introduced between the two domains, to increase the spacing and flexibility.[23] This activator (GAL4-VP16) was expressed under the control of the CMV promoter (pCMV-VP16, Figure 1B), and was cotransfected into HeLa cells with the same amount of either the pG5-luc or pG10-luc reporter.

Figure 2 shows the luciferase activity at 48 h after transfection. The activity was quite low (3.5×10^5 RLU/mg protein) when the reporter pDNA lacked a GAL4 DNA binding site (pΔG-luc). By attaching the G5 sequence(s), the expression was activated by ~600- and 3600-fold for pG5-luc and pG10-luc, respectively, as compared to pΔG-luc. The effect of GAL4-VP16 seemed to be synergistic, rather than additive, since the two tandem G5 sequences activated the expression by ~6-fold over that of the single G5 sequence.

Next, we evaluated the effect of the location of the GAL4 DNA binding site on the reporter pDNA. Ptashne and his colleagues showed that the GAL4-VP16 protein could also stimulate transcription when the GAL4 binding site was present downstream of the expression cassette.[11,24] Thus, the reporter pDNAs containing one and two G5 sequences located 38-bp downstream of the polyA signal were constructed (pluc-G5 and pluc-G10, respectively, Figure 1A). In addition, a reporter pDNA bearing G5 sequences located both upstream and downstream of the expression cassette (pG5-luc-G5) was constructed. These reporter pDNAs were cotransfected into HeLa cells with the activator plasmid pCMV-VP16. As shown in Figure 2, the luciferase expression levels from pluc-G5 and pluc-G10 were ~70- and 220-fold, respectively, higher than that from pΔG-luc, suggesting a synergistic effect of the downstream GAL4 binding sites. Moreover, the pG5-luc-G5 plasmid exhibited the highest activity among all of the reporter pDNAs shown in Figure 2 (~6400-fold higher than pΔG-luc) (statistically significant,

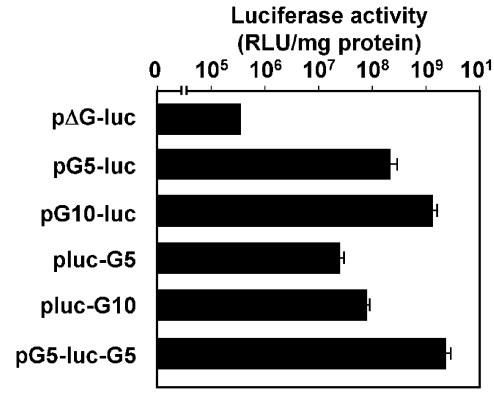

Figure 2. The effects of the number and the location of the GAL4 binding site on the reporter pDNAs. Each reporter plasmid (pΔG-luc, pG5-luc, pG10-luc, pluc-G5, pluc-G10, or pG5-luc-G5, 200 ng) was cotransfected into HeLa cells with the same amount of the activator plasmid pCMV-VP16. The luciferase activity was measured at 48 h after transfection. The values represent the averages of at least three independent experiments, except for pΔG-luc ($n = 1$). Bars indicate SD (standard deviation). RLU, relative light units.

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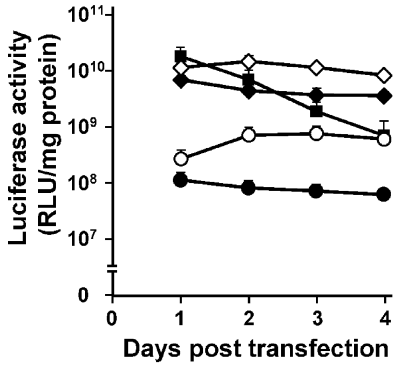

Figure 3. Prolonged luciferase expression by the positive feedback system in HeLa cells. The reporter pG5-luc (closed circles), pG5-luc-G5 (open circles), pG5-CMV-luc (closed diamonds), or pG5-CMV-luc-G5 (open diamonds) was cotransfected into HeLa cells with the activator pG5-VP16. The plasmid pG5-CMV-luc-G5 was also transfected with pBluescript II SK(+) (closed squares) as a control. Note that pBluescript II SK(+) contains no mammalian expression cassette and was used to adjust total amount of DNA. The luciferase activity was measured on 1, 2, 3, and 4 days after transfection. The values represent the averages of at least three independent experiments. Bars indicate SD (standard deviation). RLU, relative light units.

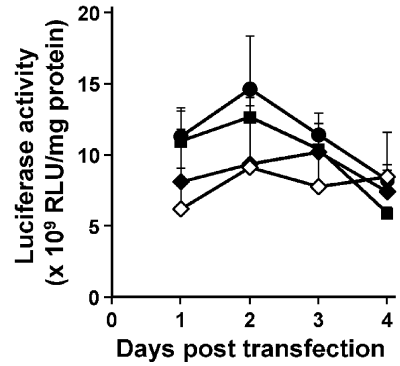

Figure 4. The effects of the number and the location of the GAL4 binding site on the activator pDNAs. The reporter pG5-CMV-luc-G5 was cotransfected into HeLa cells with the activator pG5-VP16 (closed circles), pCMV-VP16 (closed squares), pG5-CMV-VP16 (closed diamonds), or pG5-CMV-VP16-G5 (open diamonds). The luciferase activity was measured on 1, 2, 3, and 4 days after transfection. The data for pG5-CMV-luc-G5 plus pG5-VP16 (closed circles) are the same as those in Figure 3. The values represent the averages of at least three independent experiments. Bars indicate SD (standard deviation). RLU, relative light units.

pG5-luc-G5 vs pG5-luc, pG10-luc, pluc-G5, and pluc-G10, $P < 0.05$). Most importantly, the introduction of the two G5 sequences to both ends of the expression cassette worked synergistically and was more effective than that of the two tandem G5 sequences in the upstream region (pG5-luc-G5 vs pG10-luc).

Maintenance of Reporter Gene Expression by the Positive Feedback Loop. We then constructed the positive feedback system. To do so, the G5 sequence was introduced upstream of the minimal TATA box of the activator gene (pG5-VP16, Figure 1B). This activator pDNA was cotransfected into HeLa cells with either pG5-luc or pG5-luc-G5. The luciferase activity was measured on 1, 2, 3, and 4 days after transfection. As shown in Figure 3, the luciferase activity by pG5-luc transfection was maintained during 4 days (closed circles). In contrast, in the absence of the activator, the activity was quite low (6.1×10^5 RLU/mg protein on day 1) and declined more than 1 order of magnitude within 3 days (3.6×10^4 RLU/mg protein on day 3). Moreover, the additional attachment of the G5 sequence downstream of the polyA signal (pG5-luc-G5) maintained a higher expression level (~10-fold) than pG5-luc on day 4 (open circles). However, these luciferase activities were low, as compared with that driven by the CMV promoter alone (closed squares) at the earlier time points. Next, we constructed reporter pDNAs containing the G5 sequence upstream of the CMV promoter (pG5-CMV-luc, Figure 1A) and the two G5 sequences both upstream and downstream of the expression cassette (pG5-CMV-luc-G5, Figure 1A). These reporter pDNAs achieved great increases in the luciferase activity (closed and open diamonds, Figure 3). In particular, the pG5-CMV-luc-G5 plasmid maintained a high expression level in HeLa cells for at least 4 days. Statistical significance was observed in the luciferase activity on day 4 (pG5-CMV-luc-G5 plus pG5-VP16 vs other plasmid combinations, $P < 0.001$).

The Effect of the Downstream GAL4 Binding Site on the Activators. We next tested the effects of both the number and location of the GAL4 binding sites on the activator pDNAs. The activator pDNAs bearing the G5 sequence(s) upstream of the CMV promoter (pG5-CMV-VP16) and both upstream and downstream of the CMV promoter (pG5-CMV-VP16-G5) were constructed (Figure 1B). The activator plasmids, pG5-VP16, pCMV-VP16, pG5-CMV-VP16, and pG5-CMV-VP16-G5, were each cotransfected into HeLa cells with the reporter plasmid pG5-CMV-luc-G5. In contrast to the case of the reporter pDNAs, the insertion of the GAL4 binding sites into the activator pDNAs only slightly affected the luciferase expression (Figure 4). The expression elicited by pG5-VP16 (closed circles) and pCMV-VP16 (closed squares) was high on day 2, but declined gradually. The expression induced by pG5-CMV-VP16 was also lower after day 3 (closed diamonds). Conversely, the luciferase activity was maintained for 4 days when pG5-CMV-VP16-G5 was used as an activator (open diamonds). Therefore, the introduction of the G5 sequences into the upstream and downstream regions of the expression cassettes for both the activator and reporter pDNAs was most effective and facilitated prolonged expression in cultured cells. These results are in striking contrast to the rapid decrease in expression in the case of a typical transfection with pDNA containing the CMV promoter: the luciferase activity on day 4 was only ~1/30th of that on day 1 (Figure 3, closed squares).

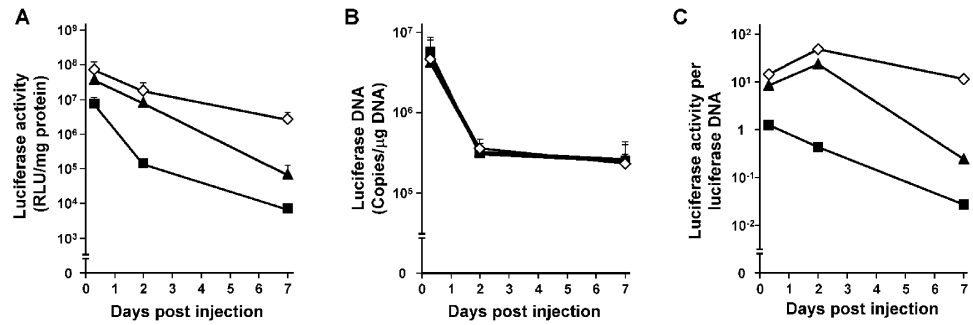

Figure 5. The effect of the positive feedback system in the mouse liver. The reporter plasmid pG5-ALB-luc-G5 (1 pmol) was coinjected into BALB/c mice with the same amount of the activator plasmid pG5-ALB-VP16-G5 (open diamonds), pΔG-ALB-VP16 (closed triangles), or pBluescript II SK(+) (closed squares). The luciferase activity (A) and the amount of the intranuclear luciferase DNA (B) were measured at 8 h, and on 2 and 7 days after injection. The values represent the averages of at least three independent experiments, except for pBluescript II SK(+) on day 2 ($n = 2$). Bars indicate SD (standard deviation). The expression efficiency (C) was calculated by dividing the average luciferase activities (panel A) by the average amounts of luciferase DNA (panel B) at the same time points. RLU, relative light units.

Prolonged Transgene Expression by a Positive Feedback Loop in Mouse Liver. The results obtained in HeLa cells prompted us to examine the effect of this positive feedback system *in vivo*. A hydrodynamics-based administration method, which enables the efficient introduction of naked DNA into mouse liver,[21,22] was chosen for this study. For this purpose, a liver-specific mouse albumin (ALB) promoter was used, instead of the CMV promoter (pG5-ALB-luc-G5 and pG5-ALB-VP16-G5, Figure 1). These pDNAs (1 pmol each) were coadministered into BALB/c mice, and the luciferase activity was measured for 1 week. As shown in Figure 5A, the luciferase activity was quite low, and declined rapidly within 2 days in the absence of the activator (closed squares). Conversely, in the presence of the activator pG5-ALB-VP16-G5, the luciferase activity was dramatically increased by the positive feedback loop (open diamonds), and was prolonged for 1 week. When the activator pDNA lacking GAL4 binding sites (pΔG-ALB-VP16) was used for comparison, the decrease in the luciferase activity was much more evident. At 7 days after injection, the luciferase expression from the positive feedback system was 390- and 40-fold higher than those from pG5-ALB-luc-G5 plus pBluescript II SK(+) (containing no mammalian expression cassette) and pG5-ALB-luc-G5 plus pΔG-ALB-VP16, respectively (statistically significant, pG5-ALB-luc-G5 plus pG5-ALB-VP16-G5 vs pG5-ALB-luc-G5 plus pΔG-ALB-VP16, $P < 0.05$).

We previously reported that the amount of exogenous DNA in the nucleus after delivery into the mouse liver decreased at the early time points.[2,3] Thus, the decrease in the luciferase activity reflects both the silencing and the elimination of exogenous DNA. To normalize the influence of the DNA elimination, the amounts of the reporter pDNAs in the liver were examined by quantitative polymerase chain reaction (Q-PCR) after isolation of the nuclei. As shown in Figure 5B, the amounts were almost the same for all pDNA constructs, and they declined quickly within 2 days and then gradually. Thus, the expression efficiency from a single molecule of pDNA, the luciferase activity divided by the amount of the reporter pDNA at the same time point, was maintained for 7 days by the positive feedback loop (Figure 5C, open diamonds). In contrast, the expression efficiency was low and decreased rapidly (by ~50-fold from 8 h to 7 days after injection) without the activator (Figure 5C, closed squares). These results indicate that the positive feedback loop worked *in vivo*, and increased the efficacy and the duration of the conventional TSTA system.

Development of Single-Plasmid Systems. The positive feedback loop functions only when the reporter and activator pDNAs are concurrently present in the same nucleus. Therefore, we constructed a single-plasmid system carrying both the reporter and activator genes. The pG5-CMV-VP16-G5 expression cassette was introduced downstream of the pG5-CMV-luc-G5 expression cassette, to yield p5CL5-5CV5. This pDNA (200 ng) was transfected into HeLa cells with the same amount of pBluescript II SK(+), and the luciferase activity was measured for 4 days. As shown in Figure 6A, the activity of the single-plasmid system was about twice as high as that of the two-plasmid system, although a lower molar amount of the reporter gene was used (37 fmol for the single-plasmid system vs 52 fmol for the two-plasmid system).

We further examined the effect of the single-vector system *in vivo*. The plasmid p5AL5-5AV5 containing two ALB promoters, instead of the CMV promoter, was constructed. One picomole of this pDNA was injected into BALB/c mice by the hydrodynamics-based method. In contrast to the results from HeLa cells, no substantial difference was observed between the single-plasmid and two-plasmid systems (Figure 6B). These results indicate that the single-plasmid system was at least as effective as the two-plasmid system.

Discussion

Control of the intranuclear disposition of exogenous DNA is extremely important for efficient and prolonged transgene expression. The TSTA system is an attractive strategy for controlling transcription. Moreover, the positive feedback system has been used to increase the efficiency and specificity

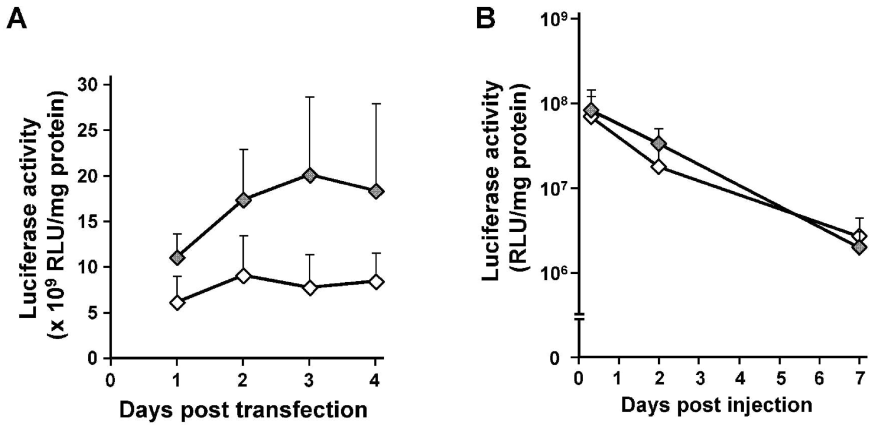

Figure 6. Comparison of the single-plasmid system with the two different plasmid system in HeLa cells (A) and in mouse liver (B). (A) 200 ng of p5CL5-5CV5 (gray diamonds) was cotransfected into HeLa cells with the same amount of pBluescript II SK(+). The luciferase activity was measured on 1, 2, 3, and 4 days after transfection. The data from the two different plasmids (open diamonds) are the same as the values of pG5-CMV-luc-G5 plus pG5-CMV-VP16-G5, shown in Figure 4. (B) 1 pmol of p5AL5-5AV5 (gray diamonds) was administered to BALB/c mice by the hydrodynamics-based procedure. The luciferase activity was measured at 8 h, and on 2 and 7 days after injection. The data from the two different plasmids (open diamonds) are the same as the values of pG5-ALB-luc-G5 plus pG5-ALB-VP16-G5, shown in Figure 5A. The values represent the averages of at least three independent experiments. Bars indicate SD (standard deviation). RLU, relative light units.

of TSTA for transient gene expression.[15−17] In the present study, we examined the effects of the positive feedback system on the duration of transgene expression in cultured cells and *in vivo*. We found that five tandem copies of the GAL4 binding sites, downstream of the polyA signal, acted synergistically with the upstream GAL4 binding sites, and dramatically increased the reporter gene expression (Figure 2). In addition, we successfully maintained a high expression level of the CMV promoter for 4 days in HeLa cells by the positive feedback system with the two G5 sequences (Figure 4). Furthermore, the expression driven by the ALB promoter was remarkably prolonged by the positive feedback loop in mouse liver (Figure 5).

The function of the GAL4-VP16 protein has been widely studied, and its activation mechanism has been well characterized. In mammalian cells, the VP16 domain interacts with many transcription factors, including the 92-kDa subunit of human activator-recruited cofactor/Mediator (ARC92), which facilitates the recruitment of RNA polymerase (pol) II.[25−27] In addition, the remodeling and modification of histones are also facilitated by VP16.[28,29] The activation is efficient when the GAL4 binding sites reside upstream of the TATA box, while GAL4-VP16 binding to the downstream region elicits relatively weak reporter gene expression.[11,24] These results are consistent with our observations shown in Figure 2. Unexpectedly, we found that the highest reporter gene expression was obtained when the G5 sequences were present both upstream and downstream of the expression cassette (Figure 2). The binding of GAL4-VP16 to both ends of the expression cassette might influence the chromatin structure and lead to a more favorable conformation for transcription within the entire coding region.

Although the positive feedback system was reported more than 10 years ago,[16] there has been no report on prolonged transgene expression by this system. One possible reason is that the transgene expression with the previous positive feedback systems was not sufficient to maintain a high expression level. Indeed, in our experiment, the combination of the reporter pG5-luc and the activator pG5-VP16, which both have the GAL4 binding sites only upstream of the TATA box, showed low expression (Figure 3). The inclusion of the GAL4 binding sites downstream of the reporter pDNA (pG5-luc-G5) greatly augmented the expression (Figure 3). In addition, the reporter pG5-CMV-luc-G5 successfully maintained a high expression level driven by the CMV promoter, while the reporter pG5-CMV-luc displayed a moderate expression level. Moreover, the use of the activator pG5-CMV-VP16-G5 elicited the highest expression on day 4, among all of the activator constructs (Figure 4). These results indicate that the introduction of the GAL4 binding sites into both ends of the expression cassettes facilitated efficient and continuous expression. Furthermore, the positive

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feedback system also worked in the mouse liver, and prolonged the expression, as compared to the conventional TSTA system (Figure 5A,C). This result suggests that our positive feedback system would be useful for *in vivo* applications, such as imaging and gene therapy.

Our system could also be used with other artificial transcription factors, besides GAL4-VP16. For example, chemical induction systems are one of the most interesting targets for positive feedback loops. The lactose repressor (LacR),[30,31] the tetracycline repressor (TetR),[32,33] and the fusion protein of the progesterone ligand binding domain with the GAL4 DNA binding domain (GAL4-PR-LBD),[34] instead of the GAL4 DNA binding domain, can induce reporter gene expression upon the addition of IPTG, tetracycline (doxycycline), and mifepristone, respectively. The combination of these systems with a positive feedback loop could achieve the spatial and temporal control of transgene expression, in addition to the persistent expression.

The single-plasmid system enhanced the reporter gene expression more efficiently than the two-plasmid system in HeLa cells, while no substantial difference was observed between the two systems in mouse liver (Figure 6). The reasons for this discrepancy are being investigated. The quiescent state of the liver cells and the transfection methods might be related to the discrepancy. Further investigations to elucidate the differences would improve the single-plasmid system.

We examined the luciferase activity in liver on day 28 after the hydrodynamics-based administration of pG5-ALB-luc-G5 plus pG5-ALB-VP16-G5, pG5-ALB-luc-G5 plus pΔG-ALB-VP16, and pG5-ALB-luc-G5 plus pBluescript II SK(+). The luciferase activity was 2.7×10^4 RLU/mg protein for pG5-ALB-luc-G5 plus pG5-ALB-VP16-G5, while those for pG5-ALB-luc-G5 plus pΔG-ALB-VP16 and pG5-ALB-luc-G5 plus pBluescript II SK(+) were 5.2×10^3 and 3.5×10^3 RLU/mg protein, respectively. This reduction in luciferase expression could be due to (i) immune response to the foreign protein and/or (ii) increased binding of histones to pDNA.[3,35] Thus, long-term transgene expression for clinical gene therapy requires further improvements of the current positive feedback system. However, the results obtained in this study indicate that the positive feedback system has potential ability to maintain transgene expression.

Many efforts have been made toward prolonging transgene expression. The expression of a transgene was reportedly maintained when the full genomic DNA sequence was used with its natural promoter.[9] The introns and 3′-untranslated regions contributed to long-term cDNA expression.[20,36] Moreover, the nonmammalian DNA sequences within the plasmid backbone and the high density of CpG dinucleotides are undesirable for long-term expression.[5−8] Thus, mimicking the structure of genomic DNA may be important for prolonged transgene expression. In the present study, we demonstrated that prolonged transgene expression was also achieved by a positive feedback loop. Our results shed light on the importance of the maintenance of the activated state of pDNAs for long-term expression. This system would be useful in many biotechnical fields, including the generation of transgenic models, *in vivo* imaging, and gene therapy.

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