CREB-binding protein transcription activation domain for enhanced transgene expression by a positive feedback system

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ABSTRACT

The positive feedback system using a fusion protein of the sequence-specific DNA binding domain of yeast GAL4 and the transcription activation domain of herpes simplex virus VP16 (GAL4-VP16), in which GAL4-VP16 promotes its own expression as well as that of a reporter gene product, is useful for efficient transgene expression from plasmid DNA. In this study, the transcription activation domains of endogenous proteins, instead of VP16, were fused to the GAL4 DNA binding domain, and the positive feedback systems employing the novel fusion proteins were examined. Plasmid DNAs encoding the transcription factors were introduced into mouse Hepa 1-6 cells by electroporation and lipofection. Among CREB-binding protein (226-460), sterol regulatory element-binding protein-1 (1-140), p53 (1-70), and Med15 (9-73), the CREB-binding protein functioned efficiently as an activator. These results indicated that the GAL4-CREB-binding protein is useful for enhanced transgene expression by the positive feedback system.

Keywords: artificial transcription factor; positive feedback system; CREB-binding protein

Abbreviations: CREB, cyclic AMP response element binding protein; TAD, transcription activation domain; G5, five tandem copies of the 17-bp GAL4 DNA binding site; qPCR, quantitative PCR; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-PCR.

1. Introduction

The development of techniques to express transgenes in living cells would benefit various biological, biotechnical, and biomedical applications. Plasmid DNA is often used as an expression vector, due to its simplicity and excellent safety profile (Demeneix et al., 2004; Gao et al., 2007; Itaka and Kataoka, 2009; Kamiya et al., 2003; Li and Huang, 2006; Yu and Wagner, 2009). However, transgene expression from plasmid DNA in mammalian cells is generally low. One of the reasons for this drawback is the poor delivery of plasmid DNA into the nuclei of the target cells. The other is the inefficient transcription from plasmid DNA. We and others reported that plasmid DNAs are silenced after nuclear entry (Ochiai et al., 2006, 2007; Herweijer et al., 2001), indicating that the avoidance of silencing is important for efficient expression.

We previously described a positive feedback system using GAL4-VP16, an artificial transcription factor, for efficient transgene expression from plasmid DNA (Ochiai et al., 2010). GAL4-VP16 is a fusion protein of the sequence-specific DNA binding domain of yeast GAL4 and the transcription activation domain (TAD) of herpes simplex virus VP16 (Sadowski et al., 1988). Five tandem copies of the 17-bp GAL4 DNA binding site (G5) were introduced into both the upstream and downstream regions of the expression cassette of the luciferase (reporter) gene. GAL4-VP16, produced from another plasmid DNA, acts as an activator to enhance reporter gene expression. The G5 sequences were also introduced into both the upstream and downstream regions of the GAL4-VP16 expression cassette to activate its own production, resulting in the positive feedback system. As a result, the reporter gene expression was strongly promoted in the positive feedback system (Ochiai et al., 2010). Similar positive feedback systems could be constructed, using a fusion protein of the sequence-specific DNA binding domain of a protein and the TAD of another protein.

Since many transcription factors are present in mammalian cells and the endogenous transcription factors would be preferable to viral VP16, from the viewpoint of safety, we attempted to find such transcription factors for the fusion proteins, as part of an ongoing effort to establish a positive feedback system comprising only endogenous proteins.

In this study, we focused on the following four endogenous proteins as transcription activators. The region encompassing amino acid residues 226-460 of the CREB-binding protein (Crebbp) binds to the TATA-binding protein (TBP) and activates transcription (Swope et al., 1996). We first selected the TAD of Crebbp (226-460), as an endogenous protein domain fused to GAL4 (Fig. 1). The second protein is sterol regulatory element-binding protein-1 (Srebp1). The TAD of Srebp1 is located in an acidic NH_2-terminal sequence (Sato et al., 1994; Ebmeier and Taatjes, 2010). We fused the NH_2-terminal 140 residues to the GAL4 DNA binding domain. Moreover, amino acid residues 1-70 of p53 were used, since its TAD is located within the N-terminal 63 residues (Zhu et al., 1998). Lastly, we tested the KIX domain of Med15, one of the components of Mediator that recruits RNA polymerase II, since the Med15 KIX domain is involved in the recruitment of Mediator to target genes (Jedidi et al., 2010). We introduced the GAL4-Crebbp/Srebp1/p53/Med15 activator plasmid DNAs, together with the reporter luciferase plasmid DNA, into mouse Hepa 1-6 cells by electroporation and lipofection. The GAL4-Crebbp plasmid DNA functioned efficiently as an activator, indicating its utility in the positive feedback system.

2. Materials and methods

2.1 Materials

The plasmid pBIND was purchased from Promega (Madison, WI, USA), and pBluescript II SK(+) was obtained from Stratagene (La Jolla, CA, USA). Oligodeoxyribonucleotides were obtained from Life Technologies Japan (Tokyo, Japan) in purified forms.

The pG5-ALB-luc-G5 plasmid (Ochiai et al., 2010) containing the luciferase gene driven by the ALB promoter and two G5 sequences was used as the reporter plasmid (Fig. 1).

2.2 Cell Culture

Hepa 1-6 cells were maintained in high glucose Dulbecco's modified Eagle medium (Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, penicillin, and streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C.

2.3 Construction of the activator plasmid DNAs

cDNA was prepared from total RNA isolated from Balb/c mouse brain. The DNA fragment corresponding to mouse Crebbp (226-460) was amplified with high fidelity KOD FX DNA polymerase (Toyobo, Japan). The primers used were X-mCBP226 (5'-dTCATCTAGACCTGCTCCAGCCATGCAGG) and mCBP460-N(R) (5'-dTGAGCGGCCGCTTAACCAACAGAACCAATTGTGTTTT). The amplified PCR fragment was ligated into the EcoRV site of the pBluescript II SK(+) plasmid, to yield the pBlue-mCBP plasmid. The XbaI-NotI fragment of pBlue-mCBP, including the mouse *Crebbp* (226-460) gene, was inserted into pBind-λ, which is a derivative of pBIND containing the λ repressor linker (corresponding to amino acid residues 92-132) (Ochiai et al., 2010), to yield the pCMV-mCBP plasmid. The BamHI-NotI fragment of pCMV-mCBP,

including the λ repressor linker and the *Crebbp* (226-460) genes, was inserted into pG5-ALB-GAL4-MCS-G5 SB, a derivative of pG5-ALB-GAL4-VP16-G5, to yield the pG5-ALB-GAL4-mCBP-G5 SB plasmid DNA (GAL4-Crebbp). The pG5-ALB-GAL4-Sreb(s)-G5, pG5-ALB-GAL4-p53-G5, and pG5-ALB-GAL4-Med15(KIX)-G5 plasmids, containing the Srebp1 (1-140), p53 (1-70), and Med15 (9-73) genes, respectively, were constructed in similar manners.

2.4 Reporter assay (electroporation)

The reporter and activator plasmid DNAs (200 fmol each) were mixed with an appropriate amount of pBluescript II SK(+) to keep the total amount of DNA constant (2 μg). The DNA mixture was electroporated into Hepa 1-6 cells (2 X 10^5 cells) with the Neon transfection system (Life Technologies) (850 mV, 30 msec pulse, 2 pulses), according to the supplier's instructions. The luciferase activity was measured with a Luciferase Assay System (Promega) at 48 h after transfection.

2.5 Reporter assay (lipofection)

Hepa 1-6 cells were plated in a 24-well plate (4 X 10^4 cells/well) one day before transfection. The reporter and activator plasmid DNAs (30 fmol each) were mixed with an appropriate amount of pBluescript II SK(+) to keep the total amount of DNA constant (400 ng). Transfection into Hepa 1-6 cells was conducted with the Lipofectamine Reagent (Invitrogen), according to the supplier's instructions. The luciferase activity was measured with a Luciferase Assay System at 48 h after transfection.

2.6 Quantification of Intranuclear plasmid DNA

Transfected cells were homogenized in phosphate-buffered saline (PBS). After centrifugation at 800 X *g* for 5 min at 4 °C, the pellet was washed with PBS. The pellet was resuspended in DNA Lysis Buffer (10 mM Tris–HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (w/v) IGEPAL-CA630, pH 7.4). After centrifugation at 1,400 X *g* for 5 min at 4 °C, the pellet was washed three times with DNA Lysis Buffer. The intranuclear DNA was extracted with SepaGene (Sanko Jun-yaku, Tokyo, Japan). Quantitative PCR (qPCR) was performed using an ABI 7500 real time PCR system, and SYBR-Green chemistry. Approximately 100 ng of the recovered DNA were analyzed by qPCR. The *luciferase* gene in the Hepa1-6 cells was detected using the following primers: Luc (+), 5'-dGGTCCTATGATTATGTCCGGTTATG; Luc (-), 5'-dATGTAGCCATCCATCCTTGTCAAT. Data were calculated as the ratio to the amount of the *Gapdh* gene, which was determined using the following primers: Gapdh (+), 5'-dTTGTGATGGGTGTGAACCAC; Gapdh (-), 5'-dGTTGTCATGGATGACCTTGG.

2.7 RT-PCR analysis in activator plasmid DNA

Total RNA was extracted from transfected cells using the TRIzol reagent (Life Technologies) and DNase I (Takara). First-strand cDNA synthesis was performed on 300 ng of total RNA, using a High Capacity RNA-to-cDNA kit (Life Technologies) according to the manufacturer's instructions. Each of the mRNA transcripts was amplified by PCR using the following primers: GAL4 (+), 5'-dTGCCGTCACAGATAGATTGG; GAL4 (-), 5'-dATTCCGGCGATACAGTCAAC. The *Gapdh* mRNA was amplified as a loading control, using the Gapdh (+) and Gapdh (-) primers described above.

2.8 Statistical analysis

Statistical significance was examined by a two-way ANOVA and a SNK post-hoc test. Levels of $P<0.05$ were considered to be significant.

3. Results and Discussion

3.1 Cloning of endogenous transcription factors

We used the fusion protein of yeast GAL4 and herpes simplex virus VP16 as an activator in a previous study (Ochiai et al., 2010). In this study, we examined the possibility of employing endogenous transcription factors, instead of the VP16 protein, fused to the GAL4 DNA binding domain. Four kinds of mouse transcriptional factors, Crebbp (226-460), Srebp1 (1-140), p53 (1-70), and Med15 (9-73), were selected. The cDNA was prepared from the total RNA of Balb/c mouse brain, and the PCR fragments corresponding to these proteins were amplified by high-fidelity DNA polymerase and cloned into the activator plasmid DNAs (Fig. 1).

3.2 Enhanced luciferase expression by activator plasmid DNAs including endogenous transcription factors

We examined the positive feedback system using endogenous transcription factors in cultured Hepa 1-6 cells. First, the activator plasmid DNAs were introduced into the cells, together with the luciferase reporter plasmid DNA, by electroporation. To confirm the expression of the activators, their mRNAs were extracted and analyzed by reverse transcription-PCR (RT-PCR), in which the region corresponding to GAL4 was amplified. As shown in Fig. 2, all activators were detected, indicating their successful expression.

When we measured the luciferase activity in the transfected Hepa 1-6 cells, the

GAL4-VP16 activator plasmid DNA increased the expression, as expected (Fig. 3A). The GAL4-Crebbp plasmid DNA activated the expression by two orders of magnitude, as compared to the reporter plasmid alone (none), and its activation ability was similar to that of GAL4-VP16 (statistically insignificant). Although the activations by the GAL4-Srebp1 and GAL4-p53 plasmids were detectable, they were weaker than that of GAL4-Crebbp. In contrast, the co-introduction of the GAL4-Med15 plasmid seemed to affect the luciferase expression only slightly.

We next measured the amounts of the reporter plasmid DNAs in the cells by quantitative PCR (qPCR), after isolation of the nuclei. As shown in Fig. 3B, the amounts were almost the same for all plasmid constructs. The expression efficiency from a single molecule of plasmid DNA, the luciferase activity divided by the amount of the reporter plasmid DNA, showed a tendency similar to that of the luciferase activity (Fig. 3C). These results suggested that GAL4-Crebbp is a useful activator.

3.3 Effects of endogenous transcription factors upon lipofection

We then examined the efficacy of the positive feedback system using the endogenous transcription factors, upon introduction with cationic lipids. As shown in Fig. 4, similar expression enhancement by the activator plasmid DNAs was observed. The GAL4-Crebbp plasmid enhanced luciferase expression by two orders of magnitude, albeit less efficiently than GAL4-VP16, followed by GAL4-Srebp1 and GAL4-p53. Slight activation by GAL4-Med15 was also observed. We concluded that GAL4-Crebbp is useful for the positive feedback system for efficient transgene expression, independent of the introduction method.

3.4 GAL4-Crebbp as a useful activator

In the present study, we examined the enhancement of transgene expression by endogenous transcription activators fused to GAL4 in the positive feedback system. We found that the GAL4-Crebbp plasmid DNA strongly activated the expression, and its activation ability was comparable to that of GAL4-VP16 (Figs. 3A and 4). Crebbp is a huge protein with a molecular mass of 265 kDa and contains multifunctional domains, including the TAD (226-460) used in this study. Crebbp interacts with many proteins including CREB. Various transcription factors, such as HIF-1α, p53, and Stat2, bind to the CH1 domain (323-423) encompassed in the TAD (226-460), one of the three cysteine-histidine-rich domains (Arany et al., 1996; Bhattacharya et al., 1996; Lill et al., 1997). The KIX domain (586-666) is bound with CREB, Myb, and Jun, and the region containing the bromodomain (1085-1196 (human Crebbp)) interacted with JMY (Bannister et al., 1995; Dai et al., 1996; Mujtaba et al., 2004; Radhakrishnan et al., 1997; Shikama et al., 1999). The CH3 (ZZ-TAZ2) domain (1700-1855), another cysteine-histidine-rich domain, is also bound with various transcription factors as NAP, p53, and Fos (Bannister and Kouzarides, 1995; De Guzman et al., 2000; Legge et al., 2004; Shikama et al., 2000). RNA helicase A interacts with the CH3 domain of Crebbp and recruits RNA polymerase II (Nakajima et al., 1997). Furthermore, over 300 viral and mammalian proteins interact with Crebbp (Kasper et al., 2006). The above-described proteins interacted with the TAD (226-460) (HIF-1α et al.), in addition to TBP (Swope et al., 1996), could contribute to the activation of luciferase expression observed in this study.

The results shown in Figs. 3 and 4 raised the question of why the TAD of Crebbp was more effective than the TADs of other endogenous proteins. We confirmed that the amounts of the activator plasmid DNAs were similar (data not shown). Thus, the differences

in (i) the amount of protein, (ii) the nuclear entry efficiency, and (iii) the recruiting ability of RNA polymerase II would determine the luciferase expression. Regarding the amounts of the proteins, only slight differences were observed for the mRNAs of the TADs fused to GAL4 (Fig. 2), although we could not detect the GAL4-TADs by western blotting using an anti-GAL4 antibody (data not shown). If their amounts in the nuclei were similar, then the observed luciferase activities would depend on the strength of transcription enhancement by each TAD.

In the present study, we demonstrated that enhanced transgene expression was achieved by the positive feedback system using GAL4-Crebbp. This system would be useful in many biological, biotechnical, and biomedical (gene therapy) applications.

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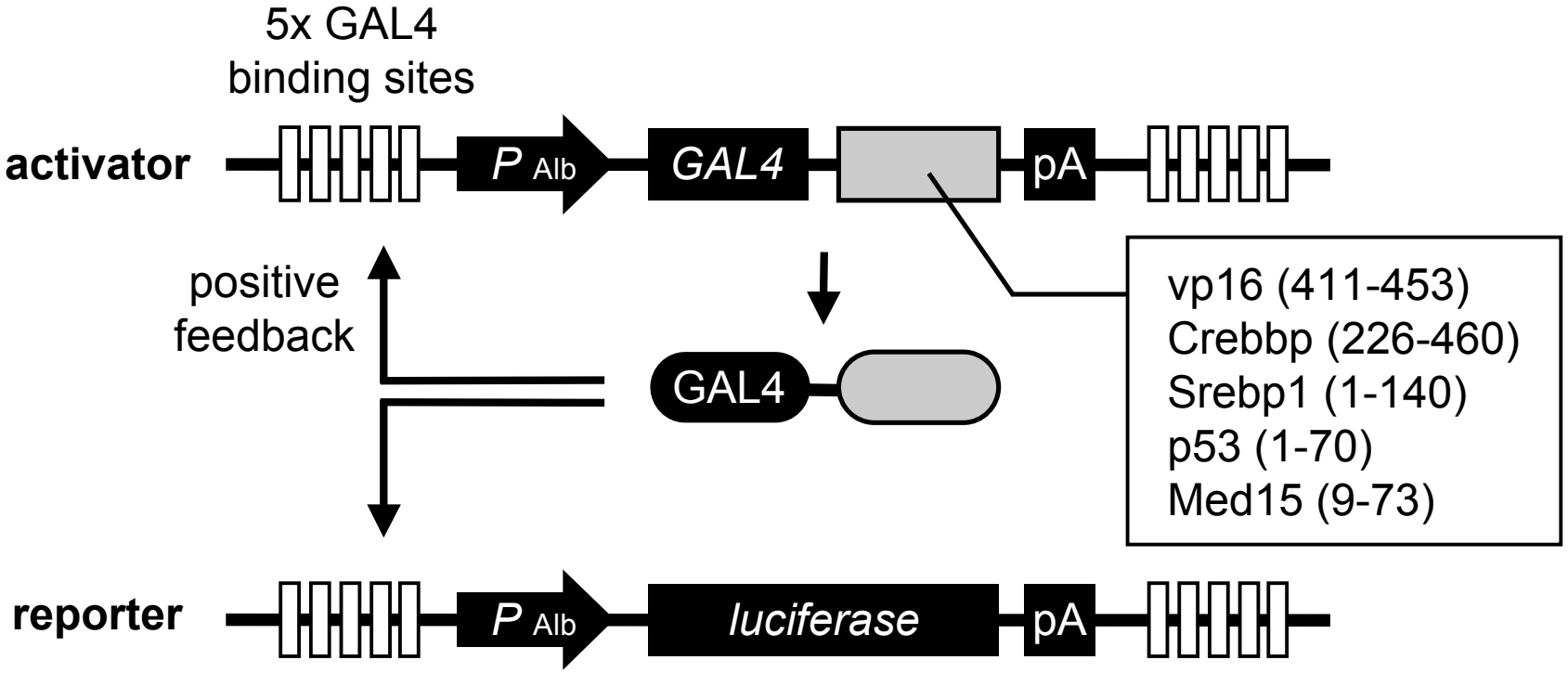
Figure Legends

Figure 1. The positive feedback system used in this study. The white box indicates the 17-bp GAL4 binding site. P_{Alb} and pA refer to the mouse albumin promoter and the SV40 polyA signal, respectively.

Figure 2. Expression of activators, examined by an RT-PCR analysis. Activator and reporter plasmid DNAs (200 fmol each) were co-transfected into Hepa 1-6 cells by electroporation. At 48 h after transfection, total RNA was extracted and reverse-transcribed. The region corresponding to the GAL4 moiety was amplified by PCR. The *Gapdh* mRNA was also amplified. The gel was stained with ethidium bromide, and is shown as a reverse image. Lane 1, reporter plasmid DNA alone; lane 2, vp16; lane 3, Crebbp; lane 4, Srebp1; lane 5, p53; lane 6, Med15.

Figure 3. Effects of activators on luciferase expression. Activator and reporter plasmid DNAs (200 fmol each) were co-transfected into Hepa 1-6 cells by electroporation. (A) The luciferase activity and (B) the amount of intranuclear luciferase DNA were measured at 48 h after transfection. (C) The expression efficiency was calculated by dividing the luciferase activity (panel A) by the amount of luciferase DNA (panel B). The values represent the averages of four to five independent experiments. Error bars indicate SD (standard deviation). RLU, relative light units. **Statistically significant versus none, Srebp1, p53, and Med15 ($P < 0.01$). †Statistically significant versus p53 and Med15 ($P < 0.01$), and none and Srebp1 ($P < 0.05$). §Statistically significant versus none, p53, and Med15 ($P < 0.05$). N.S., no statistical significance.

Figure 4. Effects of activators on luciferase expression, examined by lipofection. Activator and reporter plasmid DNAs (30 fmol each) were co-transfected into Hepa 1-6 cells with the Lipofectamine Reagent. The luciferase activity was measured at 48 h after transfection. The values represent the averages of three independent experiments. Error bars indicate SD (standard deviation). RLU, relative light units. **Statistically significant versus none, Srebp1, p53, and Med15 ($P < 0.01$). ††Statistically significant ($P < 0.01$).


5x GAL4
binding sites
activator
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GAL4
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positive
feedback
GAL4
vp16 (411-453)
Crebbp (226-460)
Srebp1 (1-140)
p53 (1-70)
Med15 (9-73)
reporter
P Alb
luciferase
pA

Figure 1

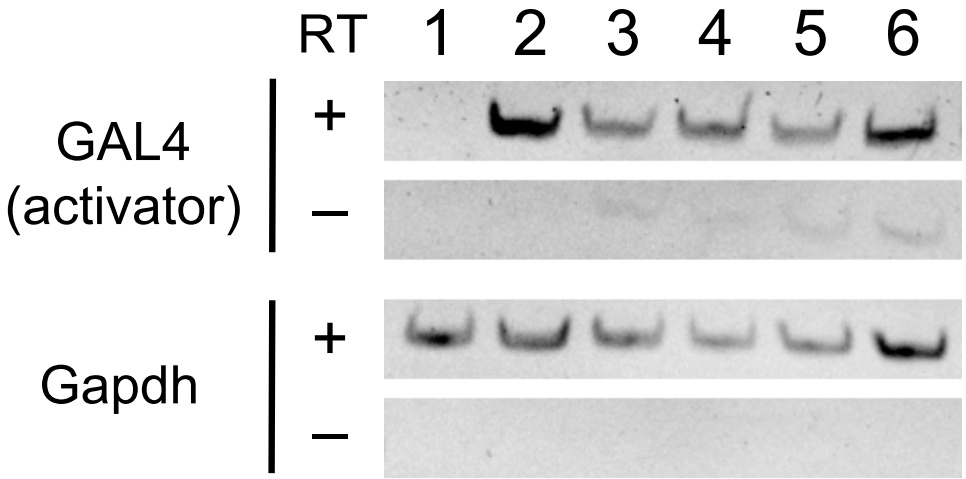
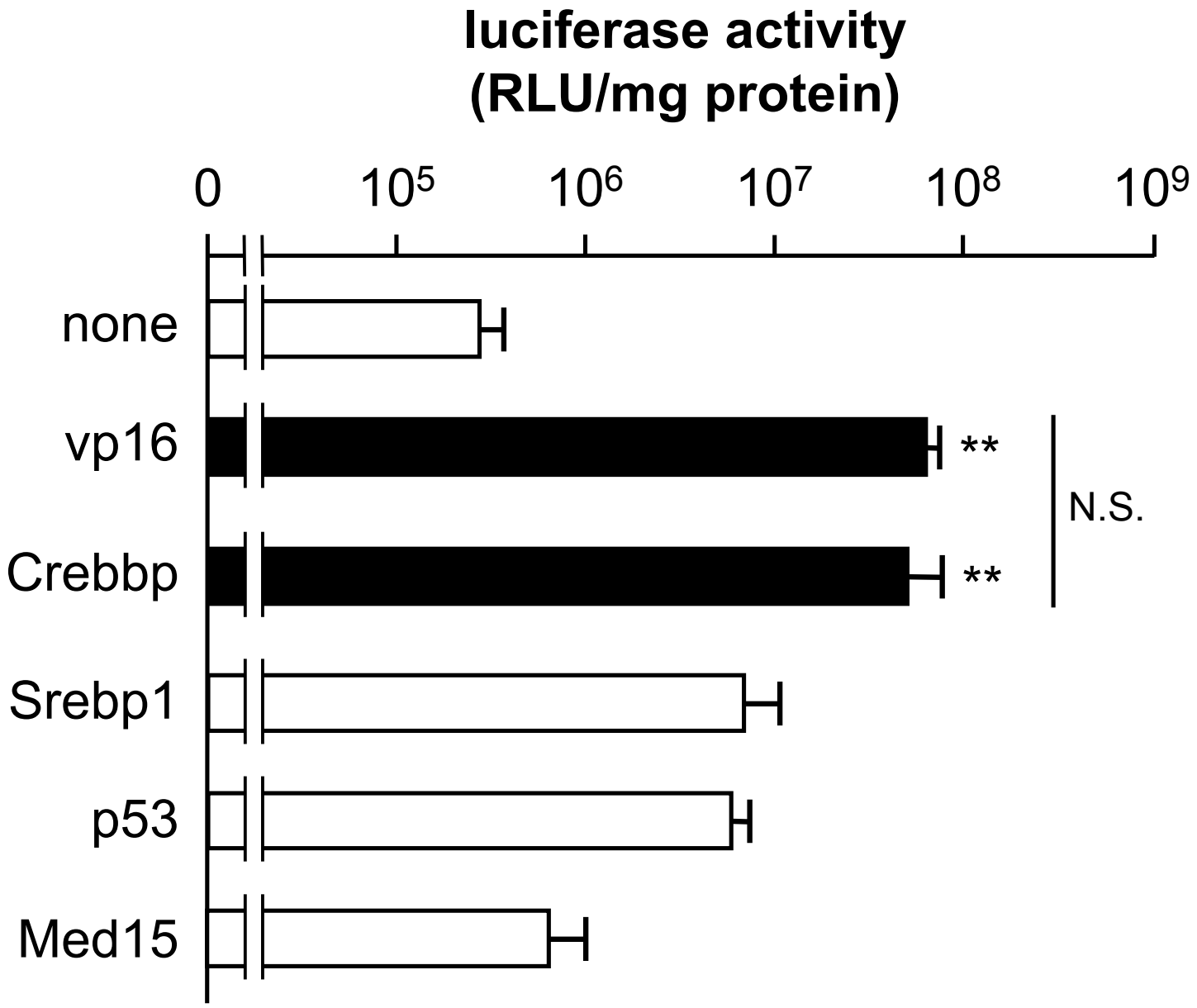


Figure 2

A)


luciferase activity
(RLU/mg protein)
0
10^5
10^6
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none
vp16
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Crebbp
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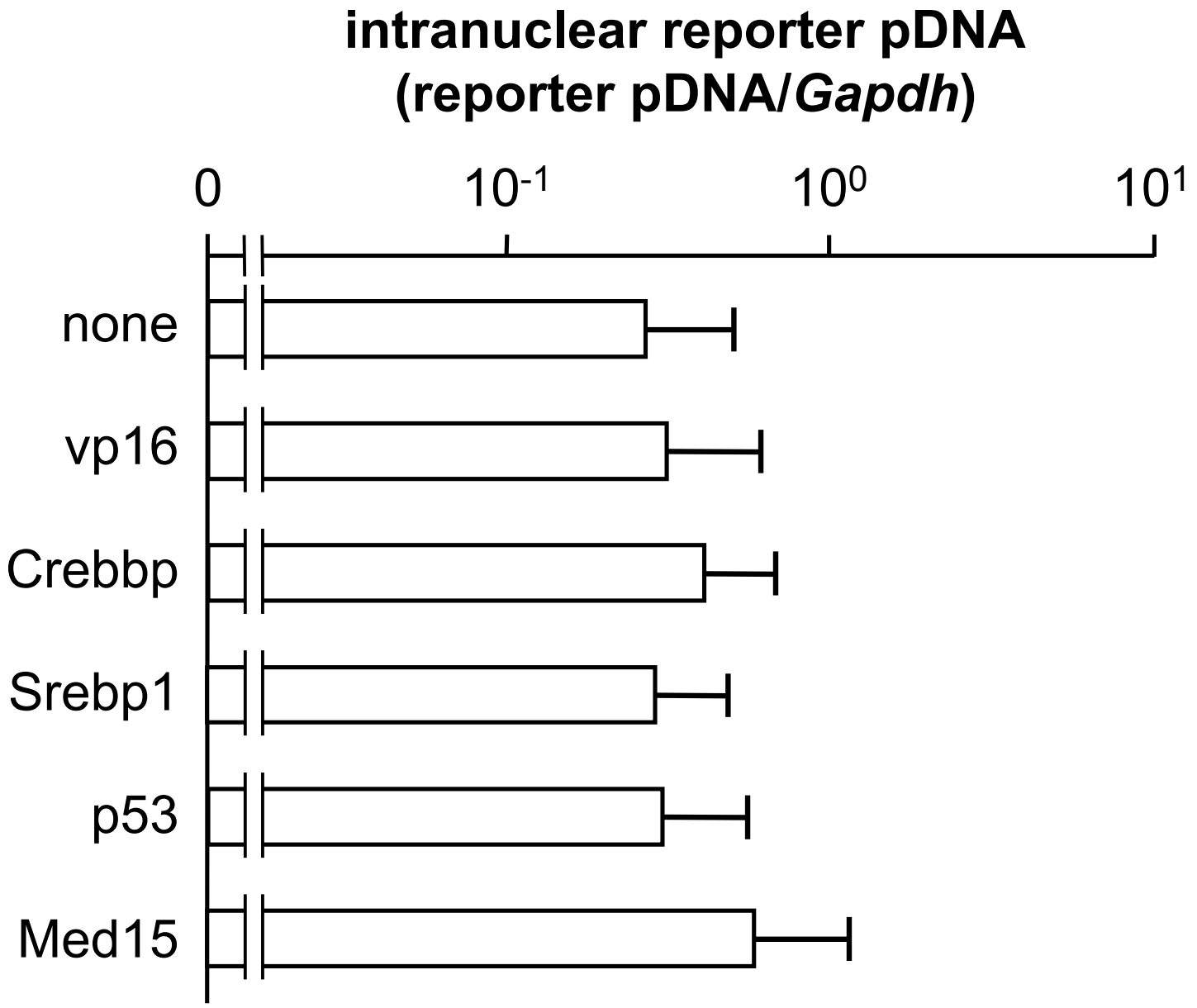
N.S.
Srebp1
p53
Med15

Figure 3

B)


intranuclear reporter pDNA
(reporter pDNA/*Gapdh*)
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10^{-1}
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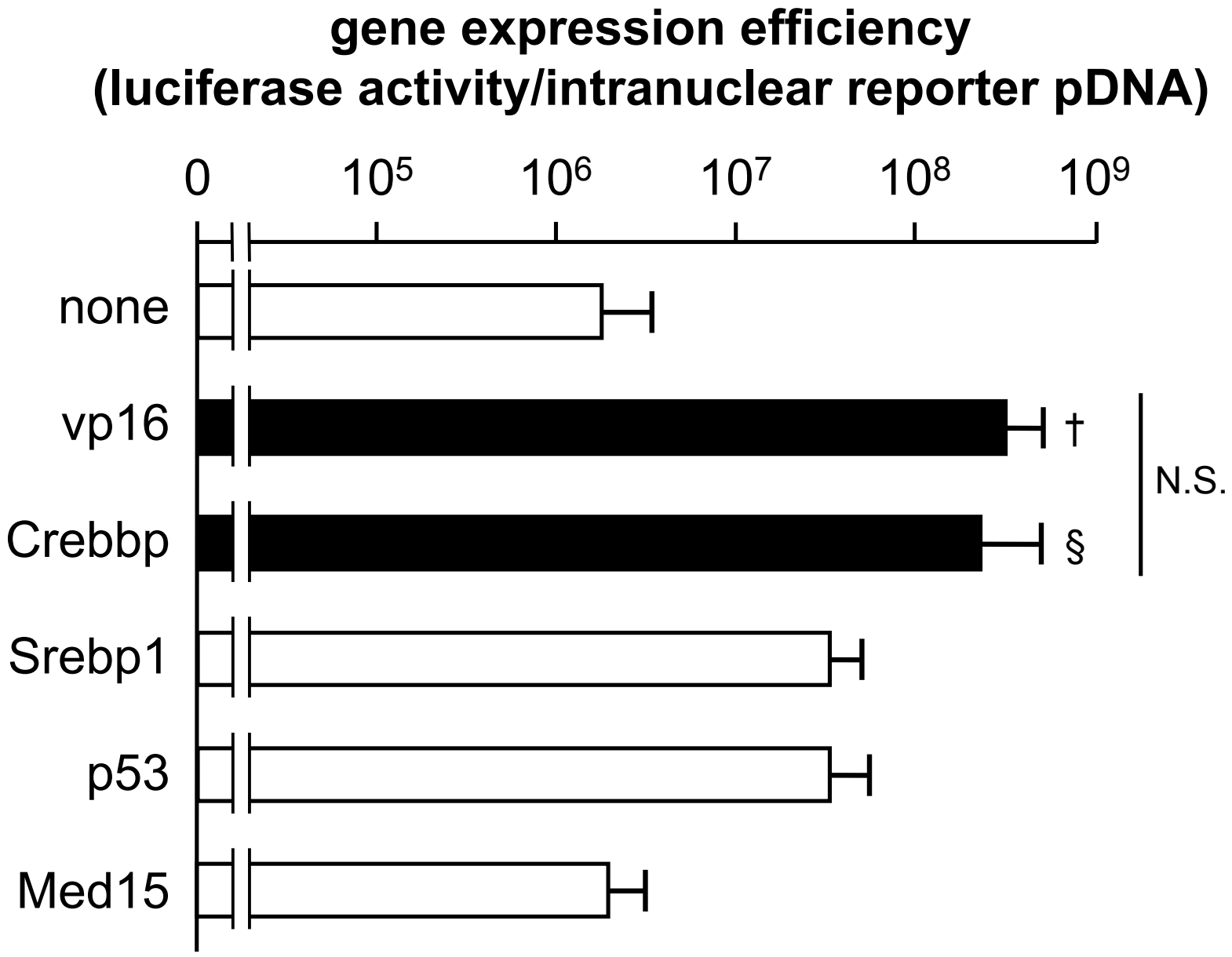
none
vp16
Crebbp
Srebp1
p53
Med15

Figure 3

C)


gene expression efficiency
(luciferase activity/intranuclear reporter pDNA)
0
10^5
10^6
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vp16
Crebbp
Srebp1
p53
Med15
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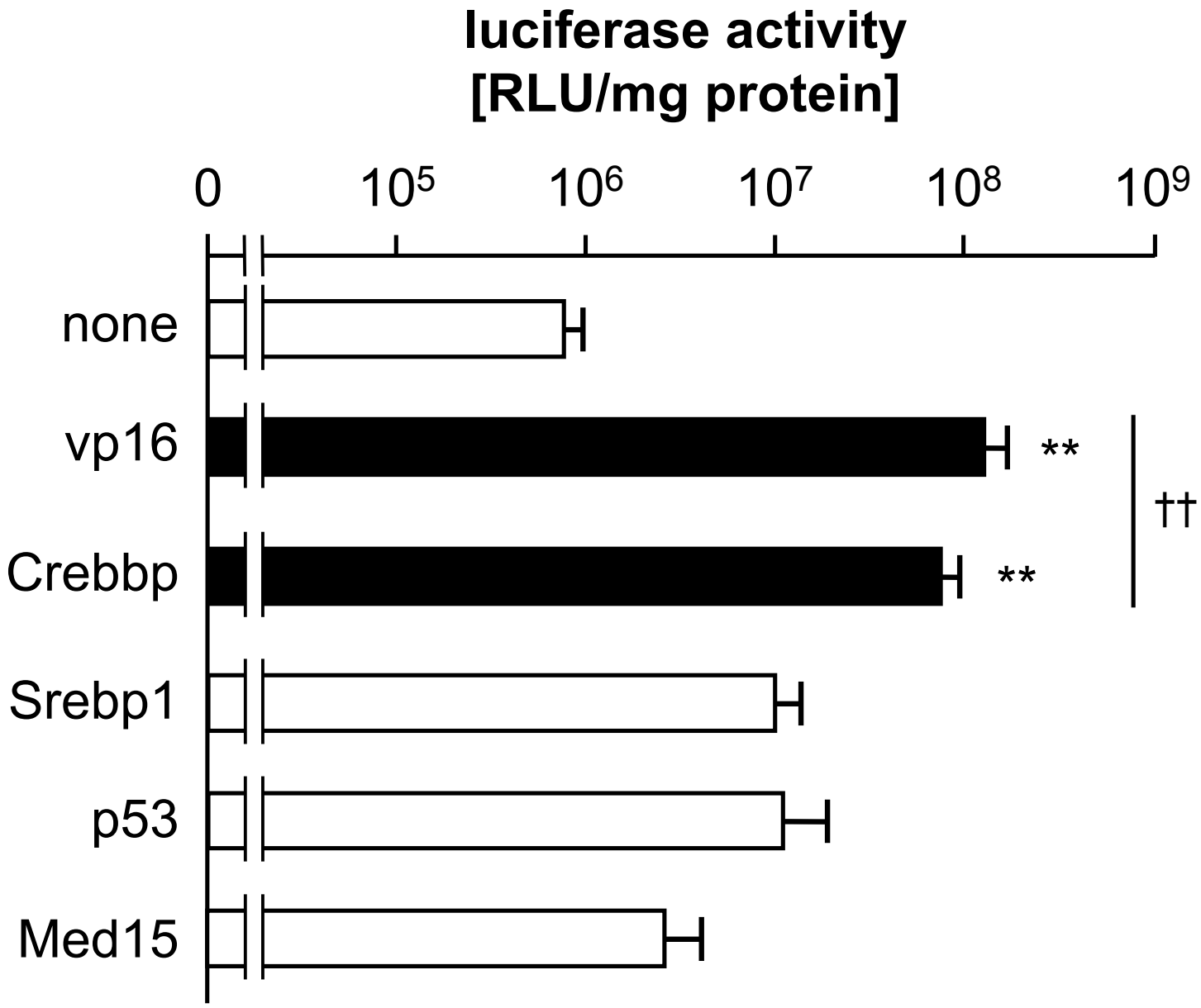
N.S.

Figure 3


luciferase activity
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0
10^5
10^6
10^7
10^8
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none
vp16
Crebbp
Srebp1
p53
Med15
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Figure 4