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Establishment of a newly improved detection system for NF-κB activity

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Abstract

The transcription factor nuclear factor-κB (NF-κB) plays roles in apoptosis, inflammation and oncogenesis. It is important for biological and medical research to understand when proteins of interest are activated in cells, leading to the establishment of a luciferase/EGFP assay to monitor the activation of transcription factors. Here, we describe an improved reporter system for NF-κB, the NF-κB-activated transgene (NAT) system, that can detect NF-κB signalling with high sensitivity and specificity. The NAT system consists of large copy numbers of NF-κB consensus sequence and a minimal promoter derived from the mouse *interleukin-2* (*IL-2*) gene. Furthermore, we generated NAT systems with stable or unstable luciferase/EGFP proteins. Stable and unstable types of luciferase/EGFP are suitable for analyzing the accumulation of and the real-time activity of NF-κB signal, respectively. Our findings suggest that the NAT system is effective for *in vivo* imaging of NF-κB signalling using cells or animals.

Keywords: NF-κB; transcription; reporter system; luciferase; EGFP

1. Introduction

NF-κB is a ubiquitously expressed transcription factor that induces the expression of genes that regulate diverse cellular functions, such as inflammation, immunity, cell growth and apoptosis, in response to various intra- and extra-cellular stimuli [1,2]. NF-κB forms a homo- or hetero-dimeric complex of subunits that belong to the Rel family, including p105/50 (NF-κB1), p100/52 (NF-κB2), p65 (RelA), RelB and c-Rel, all of which have the Rel homology region (RHR), a highly conserved sequence of 300 amino acids. In a resting state, NF-κB is retained in the cytoplasm by binding to IκB. In the canonical and non-canonical NF-κB signalling pathways, IκB phosphorylation and ubiquitination followed by proteasomal degradation lead to the activation of NF-κB. Upon stimulation such as cytokines, chemokines or pathogens, the β subunit of IκB kinase (IKK) complex is phosphorylated. The activated IKK2 phosphorylates IκBα serine residues. Next, phosphorylated IκBα is ubiquitinated by the SCF^{Fbw1} (Skp1-Cul1-F-box ligase containing the F-box protein) E3 ligase and is then subsequently degraded through a proteasome-dependent pathway [3-6]. The activated NF-κB dimers translocate into the nucleus and bind to the κB sequences in the promoter or enhancer of the target genes [7]. Consequently, NF-κB signalling affects many biological functions such as inflammation and oncogenesis.

Various reporter systems have been established and are available for research to evaluate the activity of NF-κB signalling at *in vitro* (cell) or *in vivo* (mouse) levels. However, previous reporter systems are problematic with regard to sensitivity and

specificity. Conventional *in vitro* NF-κB reporter systems consist of a few NF-κB binding sequences and a TATA box to transcribe the firefly luciferase reporter gene. However, the sensitivity and specificity of conventional *in vitro* NF-κB reporter systems are insufficient, and it is therefore necessary to obtain large copy numbers of the NF-κB binding sequence and to select optimum conditions for a minimal promoter. Furthermore, the conventional luciferase protein *per se* is quite stable for a long period after translation and can be used for observing the accumulation of NF-κB signals. However, this application seems to be inappropriate for observation of the real-time activity of NF-κB. Recently though, luciferase with a PEST sequence at the C-terminus (Luc2CP) has become available for quantifying the real-time activity of transcription factors.

There has been a need recently for *in vivo* imaging systems that can detect luminescent or fluorescent signals from inside the living body [8-11]. To date, NF-κB reporter transgenic mice as p105*lacZ* or *cis*-NF-κB^{EGFP} mice have been reported [12-14]. However, these reporter mice still pose several problems regarding regulatory cassettes and reporter genes. Regulatory cassettes in these reporter mice contain an endogenous promoter that is likely to induce non-specific transactivation of reporter genes, due to cross-talking with other signalling pathways. The *LacZ* reporter gene used in previous studies has an advantage for the mapping of NF-κB activity in the embryonic stage, but non-specific LacZ activity interferes with its specificity in adult mice, leading to the necessity of probes such as luciferase and/or EGFP.

We have established an improved NF-κB reporter system, the NF-κB activated

transgene (NAT) system, that contains large copy numbers of the NF-κB binding sequence and an optimized *IL-2* minimal promoter. The *IL-2* promoter contains two NF-κB sites to which RelA homodimers, c-Rel-containing heterodimers, p50 homodimers, p50/p65 or p50/c-Rel heterodimers bind [15]. A previous study has shown that RelA homodimers and c-Rel-containing heterodimers activate IL-2 transcription but that p50 homodimers, p50/p65 or p50/c-Rel heterodimers negatively regulate IL-2 transcription [16]. The NAT system has diverse possibilities using a combination of different probes, such as stable or unstable luciferase or EGFP, and would be widely applicable for analysis of the activity of NF-κB signalling at the *in vivo* level for screening of drug for inflammation or oncogenesis.

2. Materials and Methods

2.1. Cell culture

The human embryonic kidney cell line HEK293 and the human cervical cancer cell line HeLa were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA).

2.2. Plasmid construction

The NF-κB binding sequences were generated from oligonucleotides (Fw and Rv) as indicated in Fig. 1A. These oligonucleotides were mixed in equimolar amounts and then annealed at 95˚C for 5 min, 68˚C for 1 min, and finally at 30˚C. Annealed oligonucleotides were ligated using a DNA ligation Kit Ver. 2.1 (Takara, Shiga, Japan) and separated according to the size of the polymerized oligonucleotide fragments by agarose gel electrophoresis. Purified fragments were subcloned into the *Hind*III site of pBluescript II SK^+(pBS-NF-κB). *IL-2* minimal promoter fragments were amplified from genomic DNA derived from a mouse E14 ES cell line by PCR with blend Taq (TOYOBO, Osaka, Japan) using the following primers (Fig. 1C): 5’-AGGAATTCAACAAAGGTAATGCTTTCTGCC–3’ (IL-2pFw1), 5’-AGGAATTCCATCGTGACACCCCCATATTAT-3’ (IL-2pFw2), 5’-AGGGAT

CCAGGCAGCTCTTCAGCATGGGAG-3' (IL-2pRv1) and 5'-AGGGATCCGCCTGCAGGACTTGAGGTCACT-3' (IL-2pRv2). Amplified PCR products of *IL-2* minimal promoters were subcloned into the *Eco*RI/*Bam*HI site of pBS-NF-κB (pBS-NF-κB-IL2p: pNAT1-12 in Fig. 1D). The IRES-nEGFP fragment isolated from pCIG vector with the *Eco*RI/*Not*I was subcloned into the *Eco*RI/*Not*I site of pBS (pBS-IRES-nEGFP), the polyA fragment from pCIG was subcloned into the *Not*I/*Sal*I site of pBK-CMV, and then the polyA fragment was subcloned into the *Not*I/*Sac*I site of pBS-IRES-nEGFP (pBS-IRES-nEGFP-polyA). Luc2CP cDNA was amplified from pGL4.22 (Promega, Madison, WI) by PCR using the primers 5'-AGAGGATCCCCACCATGGAAGATGCCAAAAACATTAAG–3' (Luc2CP-Fw) and 5'-AGAAGATCTTTAGACGTTGATCCTGGCGCTGGC–3' (Luc2CP-Rv). The Luc2CP PCR product was digested by *Bam*HI/*Bgl*II and subcloned into the *Bam*HI site of pBS-IRES-nEGFP-polyA (pBS-Luc2CP-IRES-nEGFP-polyA). For construction with EGFP:Luc2 and EGFP:Luc2CP cassettes, the EGFP fragment isolated from pEGFP-C1 (Clontech, Mountain View, CA) with *Nhe*I/*Bgl*II was subcloned into the *Nhe*I/*Bgl*II sites of pGL4.20 or pGL4.22 (pGL4.20/22-EGFP). NAT fragments isolated from pBS-NAT1-12 vector were subcloned into the the *Xho*I/*Bam*HI site of pBS-Luc2CP-IRES-nEGFP-polyA (pNAT1-12-Luc2CP-IRES-nEGFP-polyA reporters), and NAT fragments isolated from NAT11 vector were subcloned into the *Kpn*I/*Sac*I site of pGL4.20-EGFP and pGL4.22-EGFP (NAT11-EGFP:Luc2, NAT11-EGFP:Luc2CP).

2.3. Transfection and reporter assay

HEK293 cells (7.5 x 10^4 cells) were seeded into 24-well plates and transfected with NF-κB reporter plasmids (100 ng/well), Renilla reporter gene plasmids (2 ng/well) and IKK2 expression vector (pCR-Flag-IKK2) at the indicated combinations by FuGENE 6 Transfection Reagent (Roche, Branchburg, NJ). HeLa cells (5 x 10^4 cells) were seeded into 24-well plates and transfected with NF-κB reporter plasmids (490 ng/well) or TopFlash Wnt reporter (250 ng/well) and with Renilla reporter gene plasmids (10 ng/well). Twelve h after transfection, the indicated concentration of TNFα (R&D Systems, Minneapolis, MN), LTα1/β2 (R&D Systems) or LPS (Sigma) was added to the culture medium. For expression of Wnt3a, pCIG-Wnt3a (80 ng/well) was transfected. The cells were harvested and lysed in 100 μl of cell culture lysis reagent, and then luciferase activities were measured using 20 μl of lysate and 100 μl of luciferase assay substrates (Promega). The luminescence was quantified with a luminometer (Tuner Designs, Sunnyvale, CA).

2.4. Flow cytometric analysis

All flow cytometric analyses were performed with a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA).

3. Results

3.1. Generation of NF-κB-activated transgene (NAT) reporter system

To establish a sensitive NF-κB reporter system, we referred to a β-catenin-activated transgene (BAT) system for detection of Wnt/β-catenin signalling that has multiple Tcf/Lef binding sequences and a minimal promoter of *Xenopus laevis Siamois*, which is known to be a direct target gene of Wnt/β-catenin signalling, and that enhances the expression and eliminates non-specific activation of the reporter gene [17,18]. We designed three different copy numbers (4, 6 and 8 copies) of NF-κB binding sequences with two different types of NF-κB binding sequence derived from the mouse *IL-2* gene (Fig. 1A) and combined them with a minimal promoter sequence of the mouse *IL-2* gene, which is known to be a direct target gene of NF-κB signalling (Fig. 1B). Since the *IL-2* promoter has been well characterized with regard to binding sequences of diverse transcription factors and regulation by diverse signalling cascades among direct target genes of NF-κB signalling, we chose the *IL-2* promoter region as a minimal promoter. Four candidates of *IL-2* promoter fragments (a: 134 bp, b: 181 bp, c: 71 bp, d: 119 bp) were amplified (Fig. 1C). We generated twelve series of NAT cassettes (NAT1-12) from the combination of three different copy numbers of NF-κB binding sequences with four different regions of the *IL-2* minimal promoter (Fig. 1D).

Previous reporter systems for NF-κB, which contain normal firefly luciferase or EGFP genes, are likely to detect the accumulation of signals after stimulation because

luciferase and EGFP proteins are more stable in cells. However, such systems are not suitable for the detection of real-time activity of signals. To improve the system in this respect, we designed three different types of a detection system, Luc2CP-IRES-nEGFP, EGFP:Luc2 and EGFP:Luc2CP fusion proteins (Fig. 1E). Luc2 with a PEST sequence at the C-terminus (Luc2CP) is more unstable than Luc2 in cells. Luc2CP-IRES-nEGFP encodes Luc2CP and EGFP with a nuclear localizing signal (NLS) (nEGFP) and produces two independent proteins by insertion of an internal ribosome entry site (IRES).

3.2. Evaluation of NAT cassettes

To survey candidates for an efficient regulatory cassette, we examined twelve generated NAT cassettes by luciferase assays in HEK293 cells with or without overexpression of IKK2, which phosphorylates IκB and promotes sequential degradation of IκB to activate NF-κB as a transcription factor. NAT11 showed the highest NF-κB activity among NAT3, 7 and 11 cassettes which contain different copy numbers (4, 6, and 8) of the NF-κB binding sequence with the same minimal promoter region c, indicating that larger copy numbers of the NF-κB binding sequence contribute to sensitive NF-κB-dependent transactivation (Fig. 2). Small copy numbers (4 or 6 copies) of the NF-κB binding sequence caused weak transcriptional activities. Next, a comparison of the luciferase activities of NAT9-12, which contain the same copy numbers (8 copies) of the NF-κB binding sequence and four different regions of the *IL-2* promoter (a-d),

showed that luciferase activity levels of NAT11 and 12 were significantly higher than those of NAT9 and 10, suggesting that the common sequence in NAT9 and 10 contains an Oct binding sequence and that it negatively regulates NF-κB signalling. These findings suggest that larger copy numbers of the NF-κB binding sequence and the *IL-2* promoter region c are indispensable for sensitive and specific reporter systems for NF-κB. The specific activity by NAT11 (18.8 fold) is significantly higher than that of NAT12 (12.8 fold) when compared between with and without overexpression of IKK2. Eventually, we used the NAT11 cassette, which has a low level of background signal without stimulation and high intensity of signal with stimulation, for the following experiments.

3.3. Characterization of different types of luciferase or EGFP for detection

We measured luciferase activities and EGFP signals from Luc2CP-IRES-nEGFP, EGFP:Luc2 and EGFP:Luc2CP fusion proteins under control of the CMV promoter in HEK293 cells. The lysate from EGFP:Luc2 showed a high level of luciferase activity, whereas that from Luc2CP-IRES-nEGFP and EGFP:Luc2CP showed low activity levels (Fig. 3A). The intensity of the EGFP signal from EGFP:Luc2CP was weaker than that from EGFP:Luc2, indicating that the stability of the fusion protein also depends on the PEST sequence (Fig. 3B). Moreover, the results using Luc2CP-IRES-nEGFP showed that unstable Luc2CP is probably better for detection of the real-time activity of signalling, whereas stable EGFP is useful for evaluation of the accumulation of total

signals, suggesting that the reporter system with Luc2CP-IRES-nEGFP is a dual functional reporter system both for short-term and long-term NF-κB signals (Fig. 3A and B).

3.4. Activation of reporter genes by several cytokines

To examine the reactivity to extracellular physiological stimulation, we performed luciferase assays with NAT11-EGFP:Luc2 vectors in HeLa cells by lipopolysaccharides (LPS), tumour necrosis factor α (TNFα) and lymphotoxin β (LTβ), the stimuli of which utilize the NF-κB signalling pathway. Luciferase activity with NAT11-EGFP:Luc2 by TNFα and LTβ showed reactivity in a dose-dependent manner, whereas stimulation by LPS showed little activity (Fig. 4A). Since HeLa cells are known to express only a low of or not express CD14 and Toll-like receptor 4 (TLR4), which act as LPS receptors on the cell surface, HeLa cells are not likely to react to LPS [19,20]. These findings indicated that NAT11-EGFP:Luc2 is also responsive to an alternative NF-κB pathway including p52 via LTβR, in addition to the classical NF-κB pathway via TNFα.

To further examine the specificity of NAT11, we performed luciferase assays with NAT11-EGFP:Luc2 vector or TopFlash as a positive control in 293 cells cotransfected with a Wnt3a expression vector (Fig. 4B and C). Luciferase activity with NAT11-EGFP:Luc2 by Wnt3a stimulation showed little reactivity, suggesting that NAT11-EGFP:Luc2 is specifically responsive to NF-κB signalling (Fig. 4C). These findings indicate that the regulatory cassette NAT11 can specifically detect NF-κB

signalling via TNF receptors (TNFR). Next, we compared the efficiency of this reporter system with that of a conventional reporter system by TNFα stimulation. The activated reporter construct, NAT11-EGFP:Luc2, displayed much higher sensitivity than that of the conventional system (pNF-κB-Luc) (Fig. 4D). Considering the ratio of presence to absence of TNFα, the specific activity of NAT11-EGFP:Luc2 (56 fold) was higher than that of pNF-κB-Luc (11 fold). Next, the efficiencies to detect NF-κB signalling were compared among NAT11 reporter vectors. The luciferase activities of NAT11-EGFP:Luc2 showed higher activity than that of NAT11-Luc2CP-IRES-nEGFP or NAT11-EGFP:Luc2CP by TNFα stimulation (Fig. 4E). We also examined the EGFP signal by flow cytometric analysis. Almost half (48%) of the reporter-transfected cells with NAT11-EGFP:Luc2 expressed EGFP, but weak EGFP signals were detected in the other two reporters (NAT11-Luc2CP-IRES-nEGFP: 6.4%, NAT11-EGFP:Luc2CP: 10.9%) (Fig. 4F). These results indicate that improved reporter systems are suitable for efficiently detecting the transcriptional activity of NF-κB under a biological signal.

Stability of the luciferase protein is an important factor when considering accumulated or real-time transcriptional activity, and it is useful to establish the NF-κB reporter system including luciferase with a PEST sequence for degradation. We assayed the luciferase activity of each reporter after the depletion of TNFα stimulation in HeLa cells (Fig. 5A). The luciferase activity of NAT11-EGFP:Luc2 was extremely stable even at 12 h after depletion of TNFα stimulation. However, vectors with a PEST sequence, NAT11-Luc2CP-IRES-nEGFP and NAT11-EGFP:Luc2CP, showed attenuation of luciferase activities after depletion of TNFα (Fig. 5B), indicating that

both NAT11-Luc2CP-IRES-nEGFP and NAT11-EGFP:Luc2CP are probably useful to detect real-time activity of the NF-κB signal. We also assayed the EGFP signal of each reporter after the depletion of TNFα stimulation in HeLa cells. Flow cytometric analysis indicated that EGFP signals derived from NAT11-EGFP:Luc2 and NAT11-Luc2CP-IRES-nEGFP were stable, whereas the EGFP signal of NAT11-EGFP:Luc2CP was attenuated after depletion of TNFα (Fig. 5C). Thus, these findings demonstrate that the reporter system with Luc2CP-IRES-nEGFP is useful as a dual functional reporter system both for real-time activity and for accumulation of NF-κB signal.

4. Discussion

We have described a sensitive reporter system for NF-κB, the NAT system, that has large copy numbers of NF-κB consensus sequence with a minimal promoter derived from the mouse *IL-2* gene. To improve a reporter system for the detection of NF-κB signalling activity, it is pivotal to maintain both high sensitivity and low background level. There are several candidates for suppressing background signals in the absence of stimulations. The first candidate is a split luciferase system, which has the advantage of being able to detect protein-protein interaction *in vivo* with a low background level [21]. However, we did not use this system because we do not detect protein-protein interaction and because it is difficult to generate transgenic mice with the system. The second candidate is a degraton probe system for NF-κB signalling with a luciferase:IκB fusion protein, which is degraded by NF-κB signalling from receptors [22]. This luciferase reporter gene is active in the absence of stimulation, and luciferase activity fades away together with IκB degradation by stimulation. Actually, it is more difficult to detect attenuation of a signal than to detect an increase from no signal. Consequently, we adopted the traditional transcription factor-activated system of BAT-*LacZ*, combined with a modified degron-tag probe.

In this report, we have presented results of evaluation of a new reporter system for the NF-κB signalling pathway. In two (Luc2CP-IRES-nEGFP and EGFP:Luc2CP) of the three generated probes, we introduced a degron-tag probe system by the addition of PEST at the C-terminus of a Luc2 reporter gene. As expected, luciferase activity levels

with PEST (Luc2CP) were significantly low without TNFα stimulation (Fig. 4E). The PEST degradation signal avoids the expression of luciferase protein at the static stage by its instability, resulting in lower activity levels of the Luc2CP reporter gene than that of Luc2 (Fig. 3A and 4E). However, considering the ratio of the presence to the absence of stimulation, the ratio of the activity level of Luc2CP was two-times higher than that of Luc2, suggesting that Luc2 with PEST provides us with high specificity despite its low sensitivity. These results suggest that NAT11-EGFP:Luc2 is effective for *in vivo* imaging due to its high sensitivity, while NAT11-Luc2CP-IRES-nEGFP and NAT11-EGFP:Luc2CP are presumably useful for *in vitro* experiments that require detection of real-time activity of its transcription.

Taken together, the results have shown that the new reporter system, NAT system, detects the activity of NF-κB signalling with high sensitivity, specificity and low background. Moreover, the NAT system can be linked to detection probes such as luciferase and EGFP. Establishment of an exquisite detection system for transcriptional activity such as NF-κB signalling is necessary to evaluate several important signalling pathways, and spatiotemporal information on transcriptional activity in living cells or animals is essential for biological and medical research. Hence, the NAT system can presumably be used as a strong tool for *in vitro* and *in vivo* imaging of NF-κB signalling activity and may be helpful in screening of drugs for inflammation or cancer with cell lines and transgenic mice.

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Legends for figures

Fig. 1. Construction of NAT reporter vectors. (A) Oligonucleotides of the NF-κB binding sequence. Oligonucleotides containing two different NF-κB binding sequences were designed for polymerization by the addition of a complementary sequence at the 5'-end of oligonucleotides. (B) Schematic representation of conventional (upper) and improved (lower) NF-κB reporter constructs. Luciferase and EGFP genes are linked to polymerized NF-κB binding sequences and *IL-2* minimal promoters for visualization in an improved reporter system. (C) Schematic representation of mouse *IL-2* minimal promoter region. Binding sites of known transcription factors are indicated as open boxes, and four different fragments that were amplified by indicated primers are displayed as bold lines (a-d). (D) Variation of NF-κB-activated elements. Twelve different combinations of NAT elements were constructed from three different copy numbers of NF-κB sites and four different *IL-2* minimal promoters. (E) Schematic representation of reporter gene cassettes. Three types of reporter genes including luciferase and EGFP in the presence of IRES or as fusion proteins were tested using HEK293 cells.

Fig. 2. Evaluation of NAT cassettes. Twelve different cassettes (NAT1-12) that have three different copy numbers of the NF-κB binding sequence with four different regions of the *IL-2* minimal promoter (a-d) were examined for IKK2-derived activation, and then a dual-luciferase assay was performed at 48 h after transfection. The luciferase

activity of pcDNA3 transfection as a negative control was defined as 1.

Fig. 3. Characterization for combinations of reporter genes. (A) Comparison of luciferase reporter activities among different types of probe proteins. Three combinations of reporter genes, including Luc2CP-IRES-nEGFP, EGFP:Luc2 and EGFP:Luc2CP fusion proteins, were driven under control of the CMV promoter. Luciferase activity of pcDNA3 transfection was defined as 1. (B) Expression of different EGFP probes. Three combinations of reporter genes were driven under control of the CMV promoter and then compared by flow cytometric analysis 48 h after transfection. The percentage of EGFP-positive cells by pEGFP-N1 transfection was defined as 100%.

Fig. 4. Activation of reporter genes by physiological stimulations. (A) Luciferase reporter activities by LPS, TNFα or LTβ stimulation. The reporter vector, NAT11-EGFP:Luc2, was transfected in HeLa cells and the cells were incubated with several concentrations of extra-cellular stimulations such as LPS, TNFα or LTβ, and a dual-luciferase assay was performed 6 h after treatment. The luciferase activity of pcDNA3 transfection was defined as 1. (B) Luciferase assay with TopFlash Wnt reporter by Wnt3a. The reporter vector, TopFlash, was transfected in HEK293 cells with or without a Wnt3a expression vector, and then a dual-luciferase assay was performed. (C) Specificity of the NAT system. The luciferase assays were performed using NAT11-EGFP:Luc2-transfected HEK293 cells stimulated by TNFα (4 ng/ml) or

Wnt3a. (D) Comparison of the NAT system with a conventional system. Luciferase activities in NAT11-EGFP:Luc2-transfected HeLa cells were compared with those in pNF-κB–Luc-transfected cells in the absence and presence of TNFα stimulation (4 ng/ml). (E) Comparison of three different types of luciferase probes. NAT11-Luc2CP-IRES-nEGFP, NAT11-EGFP:Luc2 and NAT11-EGFP:Luc2CP were examined for luciferase activities in the absence or presence of TNFα stimulation (4 ng/ml). (F) Comparison of EGFP expression using different reporter systems. EGFP signals were examined under the same conditions as those in (E). EGFP signals were detected by flow cytometry 24 h after stimulation. The percentage of EGFP-positive cells by pEGFP-N1 transfection was defined as 100.

Fig. 5. Stability of luciferase and EGFP proteins. (A) Schematic representation of the time course after depletion of TNFα stimulation. Transfected HeLa cells with three types of reporters were stimulated by TNFα (4 ng/ml), and the medium was changed 6 h after stimulation to remove TNFα. Luciferase activities in cells at the indicated times (0, 3, 6 and 12 h after TNFα depletion) were measured by dual-luciferase assays. (B) Changes in luciferase activities after depletion of TNFα stimulation. Luciferase activities of three different vectors, NAT11-Luc2CP-IRES-nEGFP, NAT11-EGFP:Luc2 and NAT11-EGFP:Luc2CP, were examined. Luciferase activities at the time point when TNFα was depleted were defined as 1. Circles, boxes and triangles indicate relative luciferase activities of NAT11-Luc2CP-IRES-nEGFP, NAT11-EGFP:Luc2 and NAT11-EGFP:Luc2CP, respectively. (C) Changes in EGFP signal after depletion of

TNFα stimulation. The ratio of EGFP-positive cells at the time point when TNFα was depleted was defined as 1. Circles, boxes and triangles indicate the ratios of EGFP-positive cells of NAT11-Luc2CP-IRES-nEGFP, NAT11-EGFP:Luc2 and NAT11-EGFP:Luc2CP, respectively.

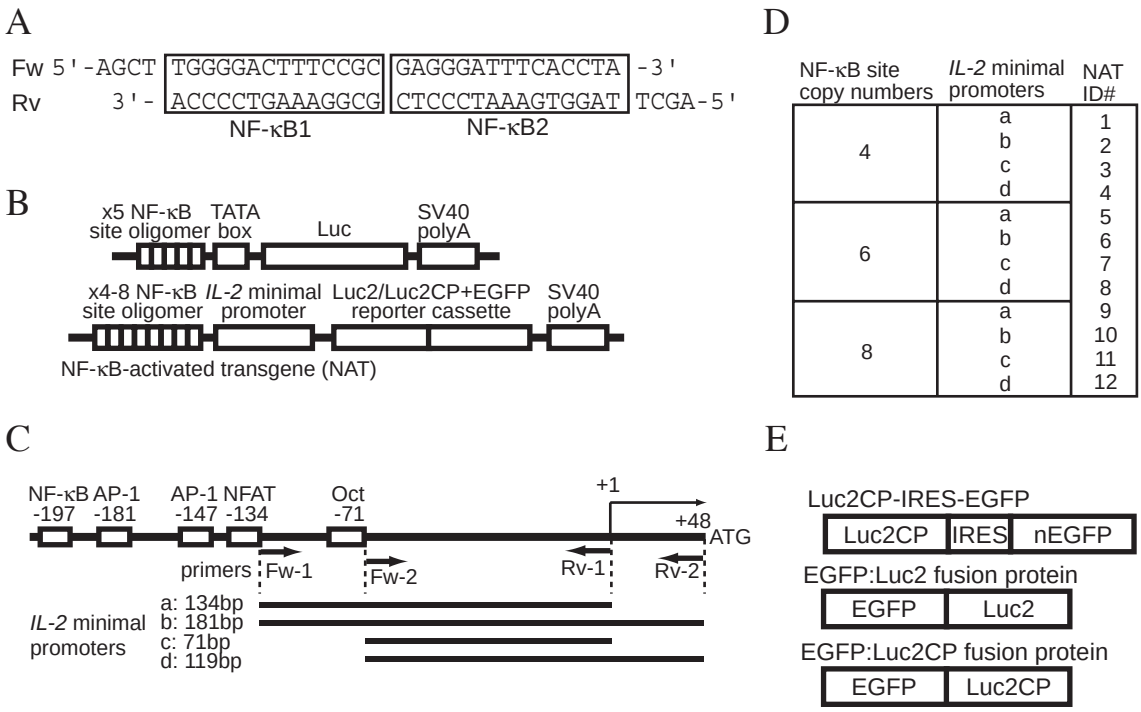

Figure 1, Matsuda

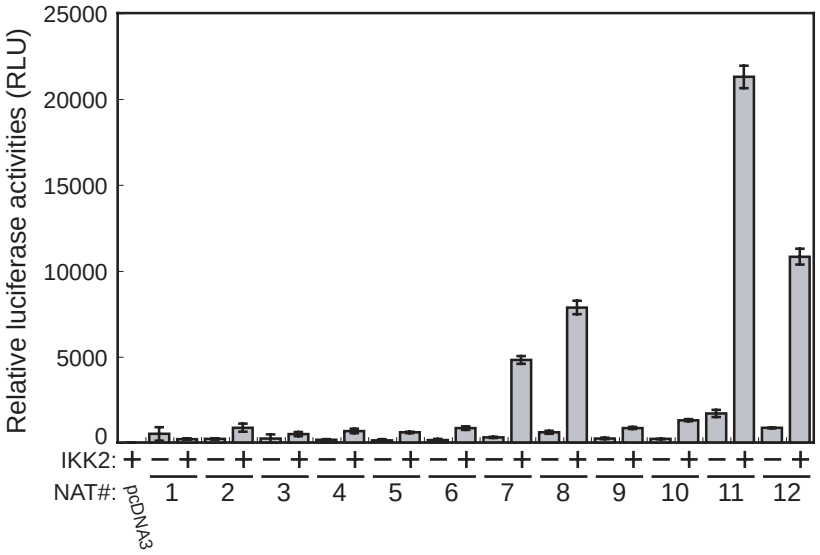

Figure 2, Matsuda

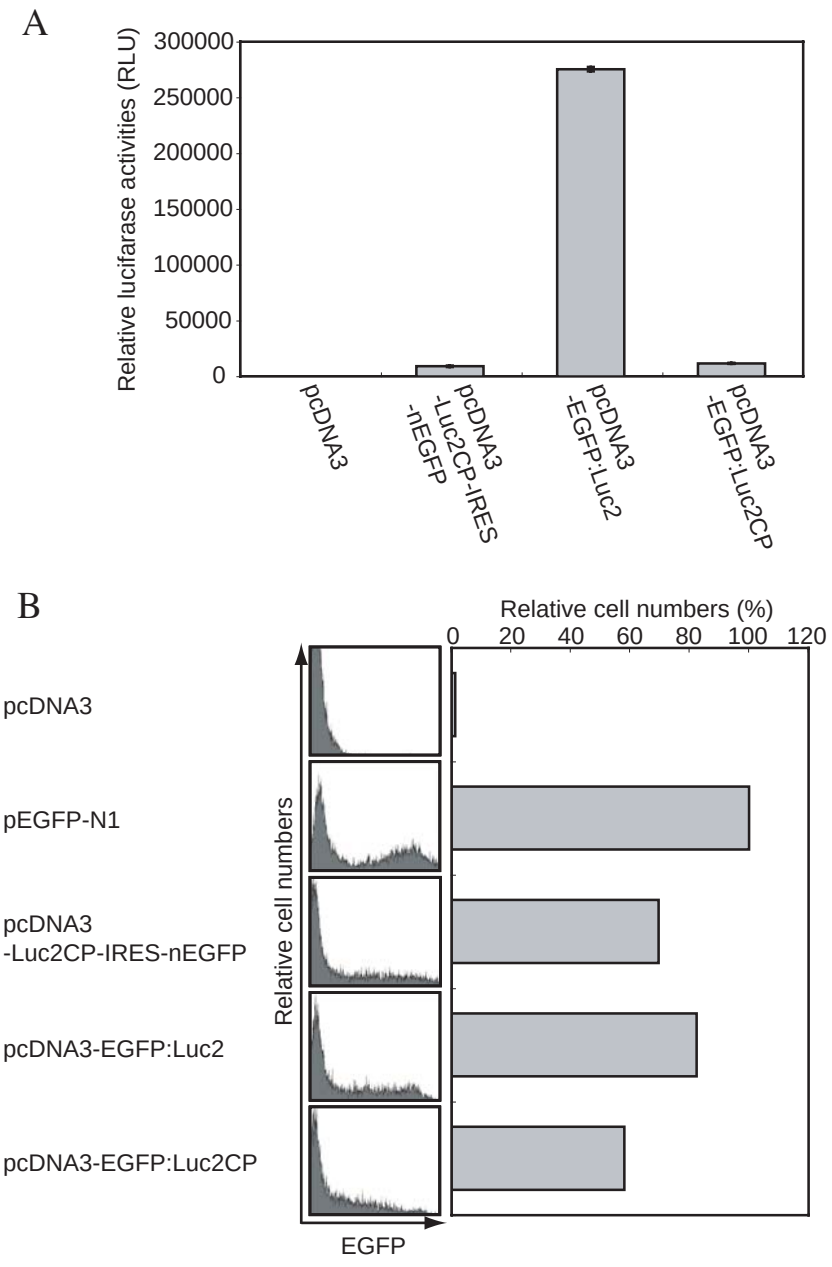

Figure 3, Matsuda

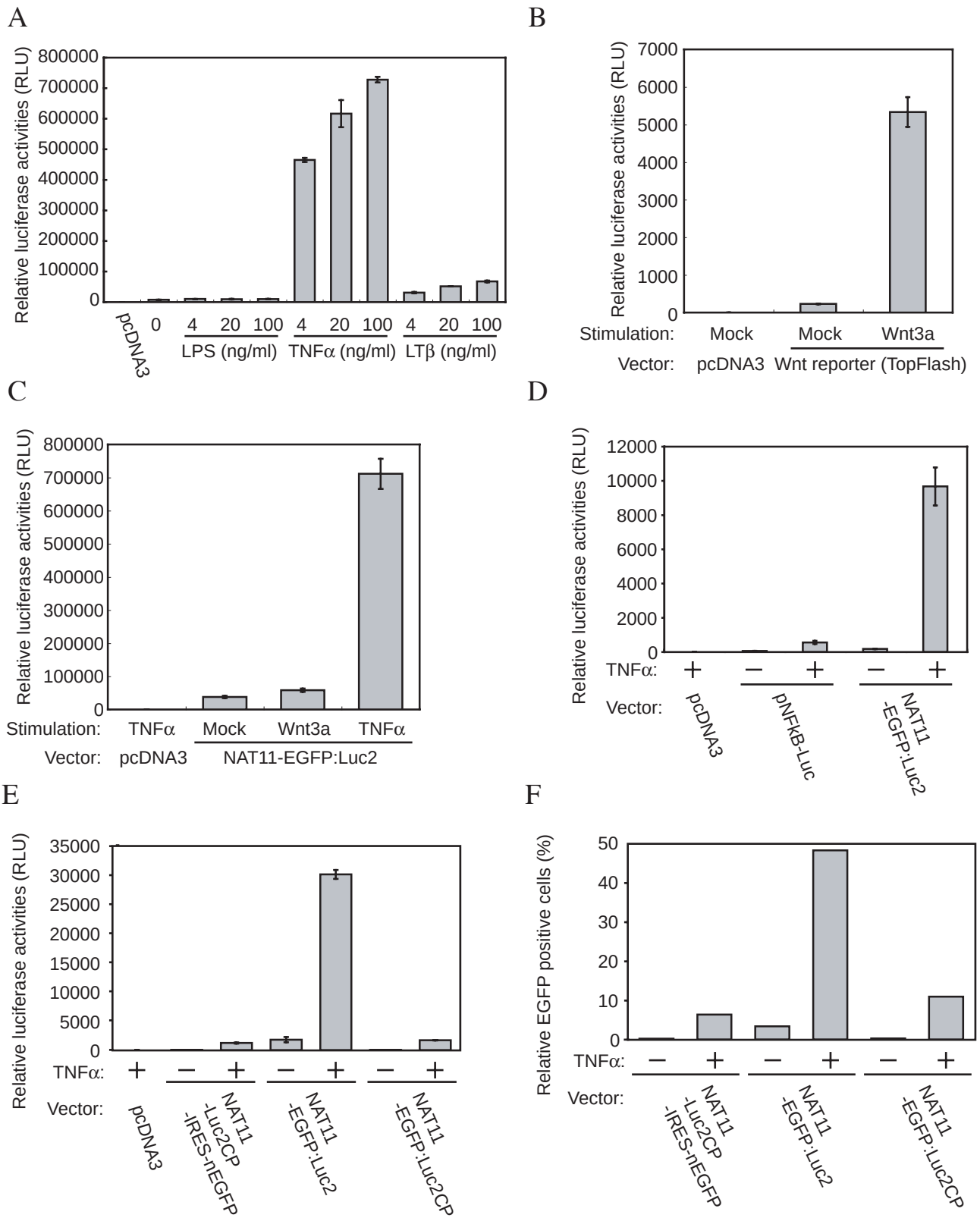

Figure 4, Matsuda

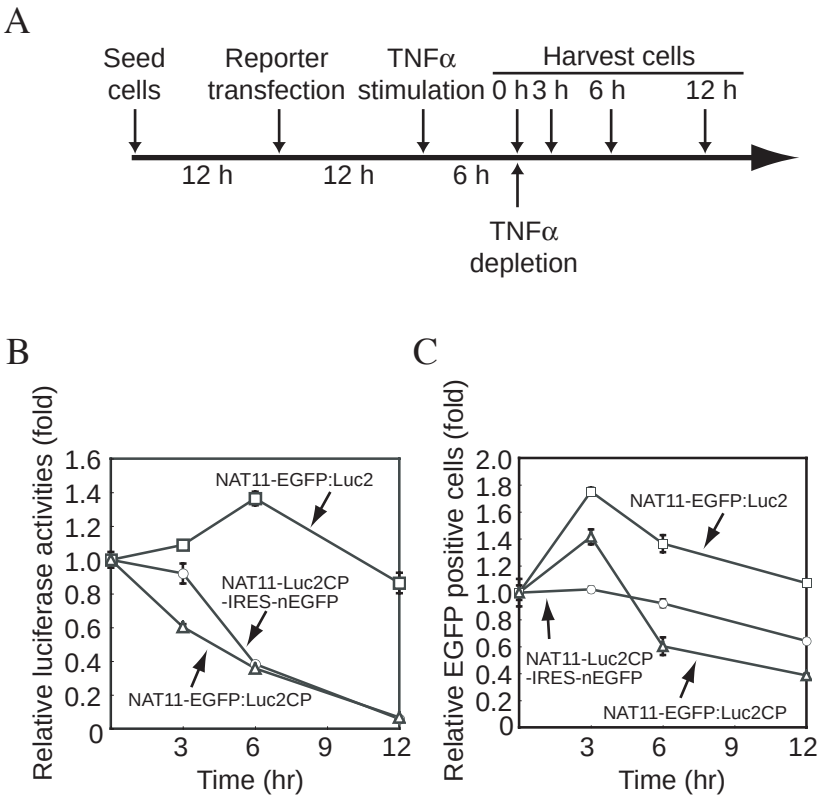

Figure 5, Matsuda