Expression of Cre recombinase as a reporter of signal transduction in mammalian cells

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Background: Cell-based reporter assays, which rely on a reporter gene under the control of a regulated promoter, are widely used to screen chemical libraries for novel receptor ligands. Here, we describe a reporter system that is based on ligand-induced DNA recombination to express the reporter gene. This system converts a transient activation of a signal transduction pathway into an amplified, constitutive and heritable expression of the reporter gene.

Results: We constructed gene fusions of Cre recombinase and mammalian promoters regulated by calcium, nuclear receptors or cyclic AMP. Reporter systems, comprising a Cre gene fusion and a *loxP*/reporter gene, were used to study the kinetics and dose responses to compounds that activate or inhibit the corresponding signal transduction pathway. We compared these reporters with conventional reporter systems in which the reporter gene is under the direct control of the responsive promoter. Reporter gene expression of the Cre reporters was greater than that of conventional reporters and could be measured more than a week after adding the stimulus. For all pathways studied here, the dose responses of the Cre reporters are nearly identical to those of conventional reporter systems.

Conclusions: We have shown that Cre recombinase can be regulated by a variety of signal transduction pathways. It should therefore be possible to use receptor ligands to induce phenotypic conversion of mammalian cells for use in a variety of applications. One such application is high-throughput screening, and we developed *loxP*/luciferase reporter genes that provide an amplified and sustained luminescent response.

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Introduction

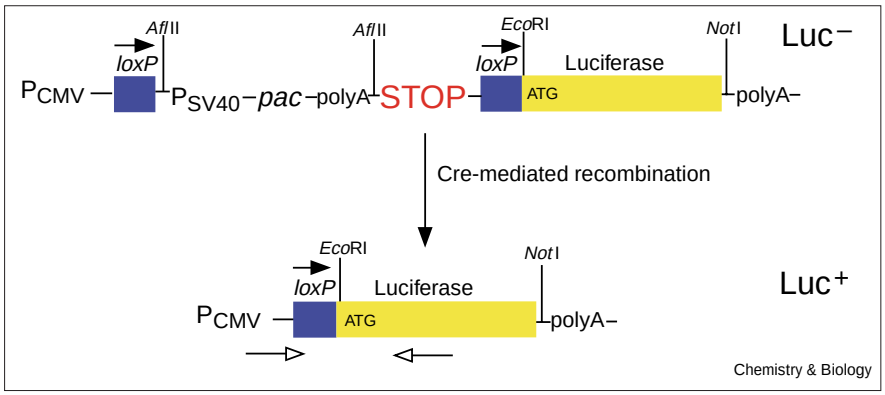
The use of reporter genes is a powerful and facile method for studying signal transduction pathways and for discovering new receptor ligands. To screen ligands, reporter genes are placed under the control of a regulated promoter in cell lines expressing a cell-surface receptor or intracellular target. Binding of an active compound to the target stimulates a signal transduction cascade, resulting in expression of the reporter gene. The use of such engineered cell lines for screening offers significant advantages over traditional competition binding assays, including the potential to identify active compounds that do not compete for binding with the natural ligand, and to distinguish receptor agonists from antagonists [1,2].

To adapt reporter-gene technology to drug discovery, it has become necessary to develop high-throughput assays because the recent introduction of combinatorial chemical libraries has resulted in a vast increase in the number of compounds to be screened [3,4]. A common high-throughput screening strategy is to miniaturize the assay by reducing the assay volume and increasing the density of wells per assay plate. Miniaturization results in fewer cells per well and a decrease in the signal strength, which can lead to potential problems for reporter genes under the control of weak promoters. A method for amplifying reporter gene expression would therefore be desirable for screening large numbers of compounds in a miniaturized format.

In addition to limited signal strength, the duration of reporter gene expression can be short lived, depending on the receptor type, signal transduction pathway and reporter protein stability. Factors such as receptor desensitization or compound degradation may also limit the time available for measuring a reporter gene response. Depending on the assay format, it may therefore be desirable to convert a transient activation of a promoter into a permanent and constitutive expression of the reporter gene.

Here we describe a strategy in which transient activation of a signal transduction pathway leads to an amplified, constitutive and heritable expression of a reporter gene. The method relies on DNA recombination by the Cre recombinase of bacteriophage P1. In mammalian cells, Cre can efficiently catalyze reciprocal DNA recombination between pairs of *loxP* sites [5]. Each *loxP* site consists of

Figure 1



Structure of the *loxP*/luciferase reporter plasmid pLM255 and activation of luciferase expression by recombination. The open arrows indicate the positions of PCR primers used to amplify the 750 bp fragment corresponding to recombined pLM255.

two 13 base pair (bp) inverted repeats flanking an 8 bp nonpalindromic core region [6]. If two directly repeated *loxP* sites flank a segment of DNA, Cre catalyzes deletion of the intervening sequence and one of the *loxP* sites.

The Cre/*loxP* system has become a powerful method for introducing precise genetic modifications in the mouse [7]. For example, specific gene ablation is possible by mating a transgenic mouse carrying a target gene flanked by *loxP* sites with a transgenic mouse carrying Cre under the control of a tissue-specific promoter. The target gene is deleted in those tissues of the double transgenic mouse in which the *cre* gene has been expressed.

To control recombination temporally, the *cre* gene has been placed under the control of the synthetic tetracycline promoter [8], or fused to the ligand-binding domain of a steroid receptor [9–11]. In the latter example, binding of ligand to the steroid receptor activates the recombinase function of the fusion.

In a previous study, we fused Cre to the ligand-binding domain of the glucocorticoid receptor and constructed a luciferase reporter gene that expresses luciferase after Cre-mediated recombination [12]. This reporter system, unlike a transcriptional reporter using the mouse mammary tumor virus promoter (MMTV), functions as a binding assay, and can be used to screen for steroid receptor agonists or antagonists.

To expand the use of the Cre/*loxP* system for screening receptor ligands, we placed the *cre* gene under the transcriptional control of the native MMTV, synthetic nuclear factor of activated T cells (NFAT), or cyclic AMP-responsive element (CRE)-dependent promoters. Our aim was to show the general utility of the Cre/*loxP* system for three distinct classes of signal transduction pathways: nuclear-receptor-mediated, calcium-regulated and cyclic-AMP-regulated pathways. Here we show the kinetics and dose responses of these gene fusions to compounds that activate or inhibit the corresponding signal transduction pathways. We discuss the results in the context of using these gene fusions in screening for receptor ligands and other transcriptional activators, and of their potential use as tools to study signal transduction pathways in development.

Results

Regulation of Cre by the NFAT signaling pathway

The transcription factor NFAT is activated in lymphoid cells in response to a rapid increase in the intracellular calcium concentration (reviewed in [13]). To study regulation of Cre recombinase by NFAT, we constructed the plasmid NFAT–Cre, which contains three copies of the minimal NFAT-binding site and an IL-2 minimal promoter. We also constructed the *loxP*/luciferase reporter plasmid pLM255 (Figure 1). Plasmid pLM255 does not express luciferase because a stop sequence, upstream of the luciferase gene, inhibits read-through transcription from the cytomegalovirus (CMV) promoter [14,15]. Two directly repeated *loxP* sites flank the stop sequence. Cre-mediated recombination excises the stop sequence and permits luciferase expression to be driven by the strong CMV promoter. To select for the presence of pLM255, we inserted the puromycin acetyltransferase (*pac*) gene adjacent to the stop sequence. The *pac* gene is excised by unregulated expression of Cre, so continuous growth in the presence of puromycin should select against clones that recombine in the absence of NFAT activation.

To test regulation of this system in a transient transfection assay, we cotransfected Jurkat cells with plasmids NFAT–Cre and pLM255. A control plasmid, NFAT–Luc, that expresses luciferase directly from the NFAT-dependent promoter, was used in a separate transfection. As shown in Table 1, luciferase activity is weakly stimulated when cells are incubated with the calcium ionophore ionomycin or the ionomycin or phorbol myristate acetate (PMA) alone. Costimulation of cells with PMA and ionomycin

Table 1

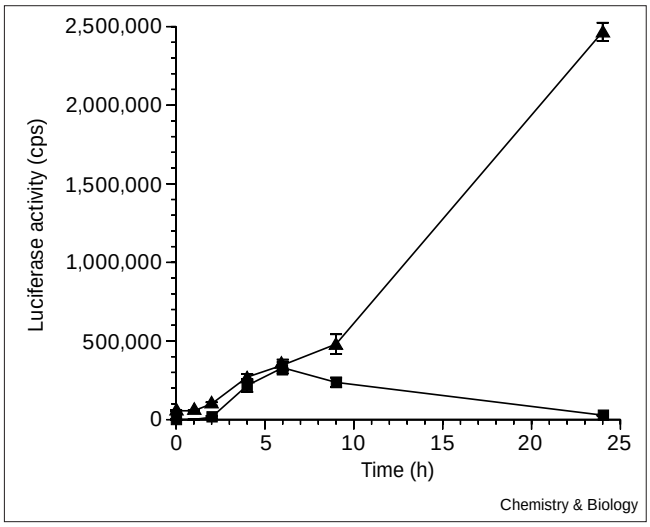
Luciferase activity of Jurkat cells after transient transfection with NFAT–Cre/pLM255 or NFAT–Luc plasmids.

	NFAT–Luc	NFAT–Cre/pLM255
Nonstimulated	8.5×10^2	1.7×10^4
Ionomycin	2.6×10^3 (3)	3.1×10^4 (2)
PMA	3.4×10^3 (4)	4.8×10^4 (3)
Ionomycin + PMA	4.4×10^4 (52)	2.1×10^6 (124)

Cells were stimulated as indicated for 20 h. Numbers in parentheses refer to x-fold stimulation relative to nonstimulated controls.

(P/I) results in a dramatic increase in luciferase expression for both transfected populations, but the NFAT–Cre population has a higher stimulated activity (nearly 50-fold at this time point of 20 hours) than NFAT–Luc. This is probably due to stronger expression of luciferase from the CMV promoter than that from the NFAT-dependent promoter, and indicates that the Cre system can amplify expression of a reporter gene. Although the nonstimulated level of luciferase is higher in the NFAT–Cre cell population, the fold stimulation is actually higher than in the NFAT–Luc population.

Figure 2



Time course of luciferase expression in stable Jurkat clones NFAT–Cre (triangles) or NFAT–Luc (squares). Cells were incubated in the presence of 2 μM ionomycin and 20 ng/ml PMA, and luciferase activity was measured at various times as indicated.

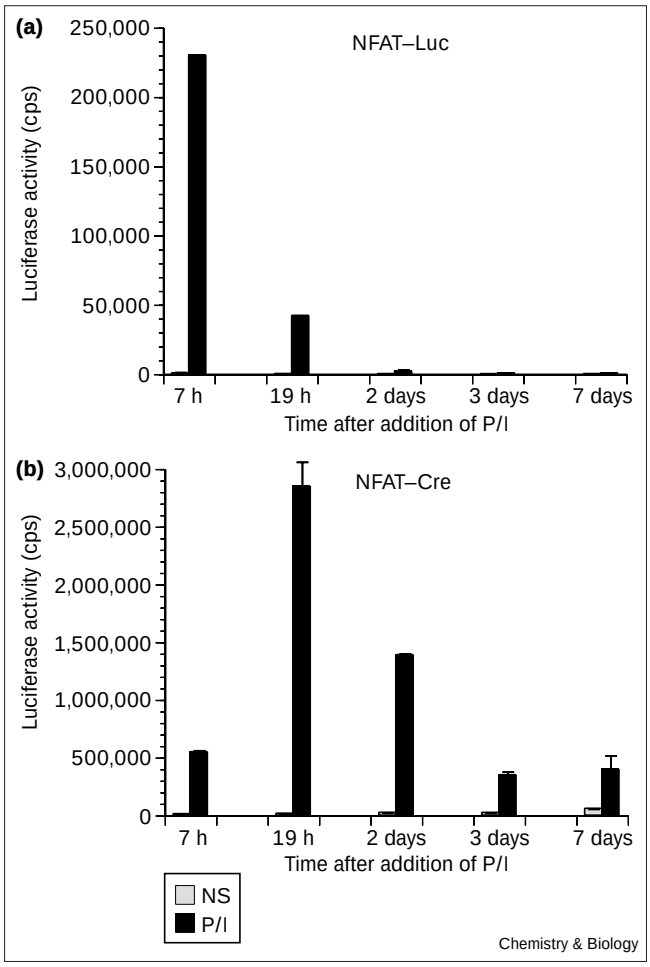
To study regulation of Cre in a stable clone, we transfected Jurkat cells with pLM255 and selected a puromycin-resistant population. The selected population was subsequently transfected with the NFAT–Cre–neo plasmid, carrying a neomycin resistance gene, and a puromycin- and neomycin-resistant population was selected. Clones were isolated by limiting dilution and tested for luciferase regulation by incubating with P/I. Clones ranged in phenotype from low activity regardless of stimulation to very high constitutive activity (data not shown). Clones that showed high inducible activity and low background were chosen for further analysis.

The kinetics of luciferase expression during incubation with ionomycin and PMA of a selected NFAT–Cre clone was compared with that of a control Jurkat clone carrying the NFAT–Luc plasmid. Figure 2 shows that the luciferase activity of the NFAT–Luc clone peaks at 6 hours, and declines dramatically after 24 hours. Luciferase activity of the NFAT–Cre clone, however, increases almost linearly during the 24 hour time period. By 24 hours, the luciferase activity produced by the NFAT–Cre cells is 7–8-fold greater than the peak luciferase activity from the NFAT–Luc cells. These results indicate that the pattern of luciferase gene expression differs markedly between NFAT–Cre and NFAT–Luc cells, and that amplification of gene expression in the Cre system is a delayed process.

To study the kinetics of luciferase gene expression over longer time periods, the NFAT–Cre and NFAT–Luc clones were stimulated for 7 hours, washed to remove P/I, and grown in medium lacking puromycin. Aliquots were removed at various times, and the luciferase activities of the stimulated and nonstimulated cells were compared. The activity of NFAT–Luc cells appears near maximal at 7 hours after stimulation as previously observed, and declines to background levels by 2 days (Figure 3a). The NFAT–Cre clone, however, continues to express luciferase 7 days following stimulation (Figure 3b). Expression of luciferase from the NFAT–Cre clone reaches a maximum of 12-fold greater than the peak activity of the NFAT–Luc cells at 19 hours, then declines and reaches a steady-state level after 3 days. The decline in luciferase activity from the NFAT–Cre cells probably results from removal of PMA during the wash step, as PMA is a strong stimulant of the CMV promoter in Jurkat cells (data not shown). These results indicate that expression of Cre through NFAT induction results in constitutive expression of a reporter gene.

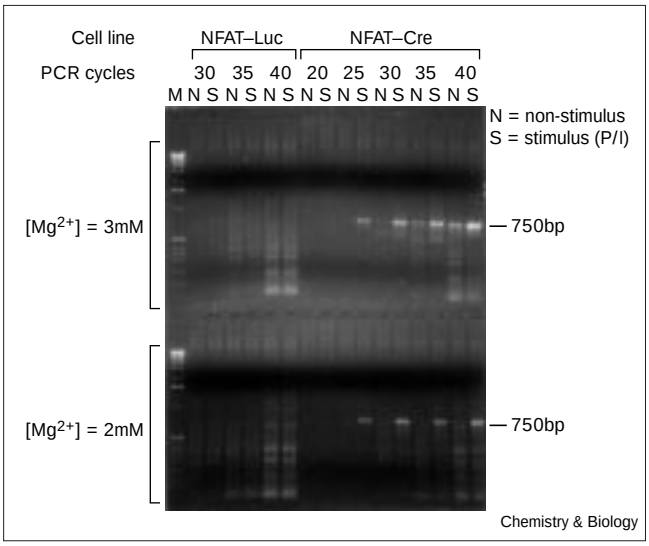
To confirm that expression of Cre results in DNA recombination, we analyzed genomic DNA by using the polymerase reaction (PCR). A 750 bp PCR fragment corresponding to the recombined form of pLM255 was generated from NFAT–Cre cells incubated with ionomycin and PMA in far greater abundance than in the nonstimulated cells. As a control, cells carrying the NFAT–Luc reporter do not contain the 750 bp fragment (Figure 4). The presence of small amounts of recombined pLM255 in the nonstimulated cells is consistent with the slightly increased nonstimulated luciferase levels in these cells as compared with NFAT–Luc cells (Figure 2). This basal

Figure 3



Time course of luciferase expression in Jurkat clones after removal of PMA and ionomycin (P/I). Jurkat clones (a) NFAT–Luc or (b) NFAT–Cre were incubated for 7 h in the presence (P/I) or absence (NS) of 20 ng/ml PMA and 2 μM ionomycin. Cells were then washed, cultured in growth medium lacking puromycin, and luciferase activities were determined and normalized to cell number at the indicated times after addition of stimulus.

Figure 4



PCR analysis of genomic DNA before and after expression of Cre in Jurkat cells. Jurkat clones NFAT–Luc and NFAT–Cre were incubated in the presence (S) or absence (N) of 20 ng/ml PMA and 2 μM ionomycin for 24 h, and genomic DNA was isolated using a Puregene™ kit (Gentra systems, Minneapolis, MN). PCR primers (indicated in Figure 1) were used to amplify a 750 bp fragment corresponding to recombined pLM255. PCR conditions included varying the cycle number and Mg^{2+} concentration as indicated. Lane M indicates molecular weight standards.

signal probably represents either a small proportion of cells that have recombined, or the presence of a least one recombined pLM255 construct out of multiple integrants in the NFAT–Cre clone.

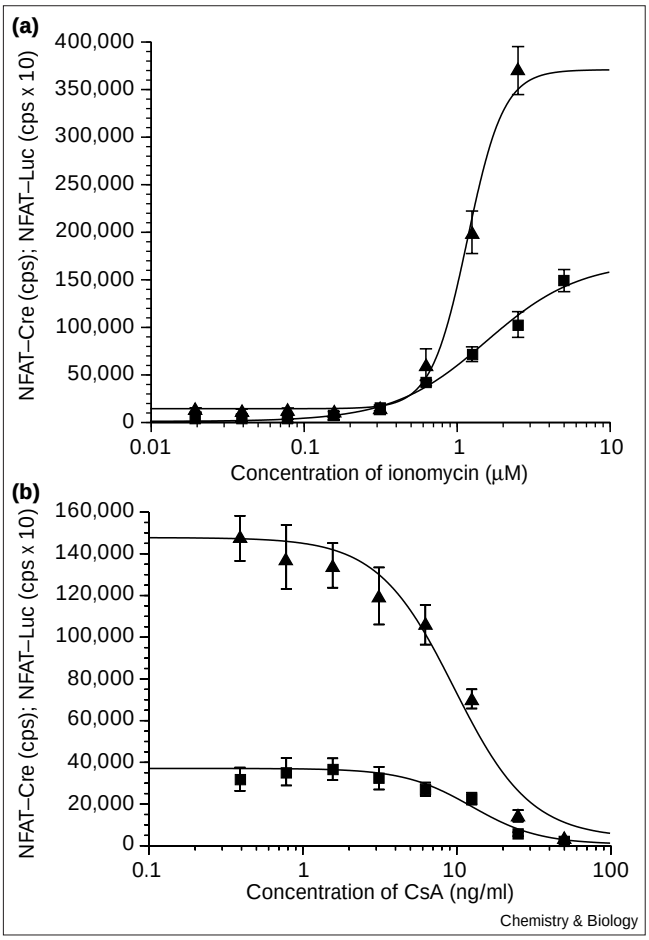
We next compared the dose responses of the clones to ionomycin or to the immunosuppressant cyclosporin A (CsA), which blocks translocation of NFAT to the nucleus [16]. Figure 5a shows that the EC_{50} value of ionomycin is essentially the same for both NFAT–Cre and NFAT–Luc cells (1.2 μM and 1.6 μM, respectively), but that the shapes of the curves are different (the NFAT–Cre dose-response curve is steeper). For CsA, the inhibition curves are similar in shape, and the IC_{50} values are essentially the same (12 ng/ml and 10 ng/ml for NFAT–Luc and NFAT–Cre, respectively, Figure 5b). The NFAT–Cre reporter system therefore gives the same EC_{50} for agonist and IC_{50} for antagonist as the conventional NFAT–Luc reporter system.

Regulation of Cre by the MMTV promoter

The MMTV promoter is activated by binding of a steroid receptor–ligand complex, such as the glucocorticoid receptor (GR) bound to the ligand dexamethasone (Dex), to specific sites on the DNA [17]. To study regulation of Cre by the MMTV promoter, we constructed plasmids MMTV–Cre and MMTV–Luc, which contain the MMTV long terminal repeat promoter regulating transcription of Cre and luciferase, respectively.

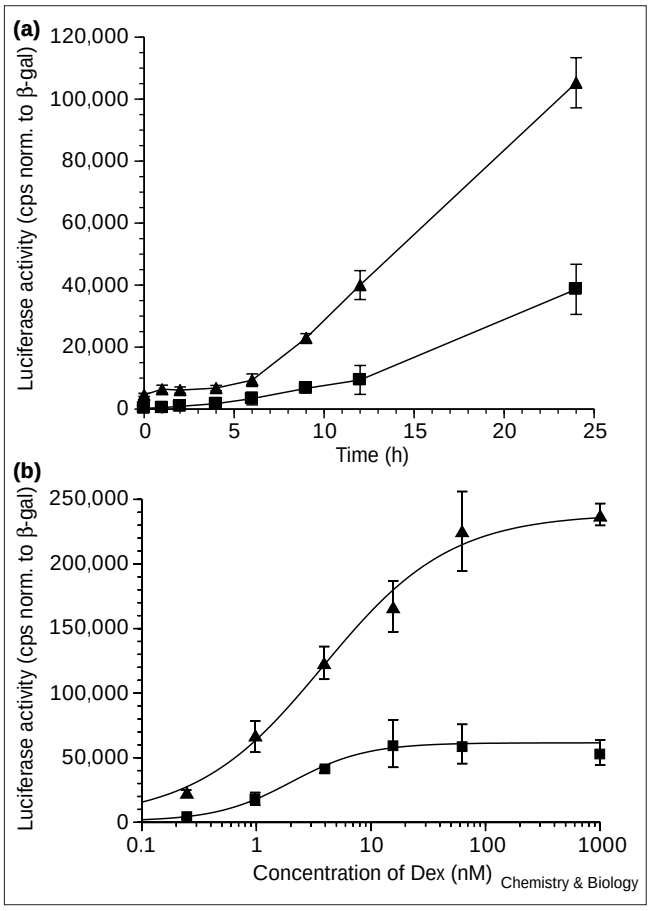
The time course of luciferase gene expression and dose response of dexamethasone was measured by transient transfections of CHO cells. Cells transfected with an expression construct for human GR and with either MMTV–Cre plus the *loxP*/luciferase reporter plasmid pLM255, or with the MMTV–Luc plasmid, were stimulated with Dex and luciferase activities were measured at various times. The time course of luciferase expression from the MMTV promoter, in contrast to that from the NFAT-dependent promoter, appears similar in both the MMTV–Cre and MMTV–Luc systems (Figure 6a). Both reporter systems show similar delayed kinetics, and

Figure 5



Dose responses of Jurkat clones NFAT–Luc (squares) and NFAT–Cre (triangles) to (a) ionomycin in the presence of 20 ng/ml PMA, or (b) cyclosporin A (CsA) in the presence of 1 μM ionomycin. NFAT–Luc cells were stimulated for 6 h and NFAT–Cre cells were stimulated for 24 h. For visual clarity, data are expressed as counts per second (cps) of luciferase activity for the NFAT–Cre cells and as cps × 10 for the NFAT–Luc cells. Therefore, the actual cps for the NFAT–Luc cells is tenfold less than indicated by the scale. EC_{50} and IC_{50} values and slopes were calculated using a nonlinear least squares regression to a four-parameter logistic equation [35].

Figure 6



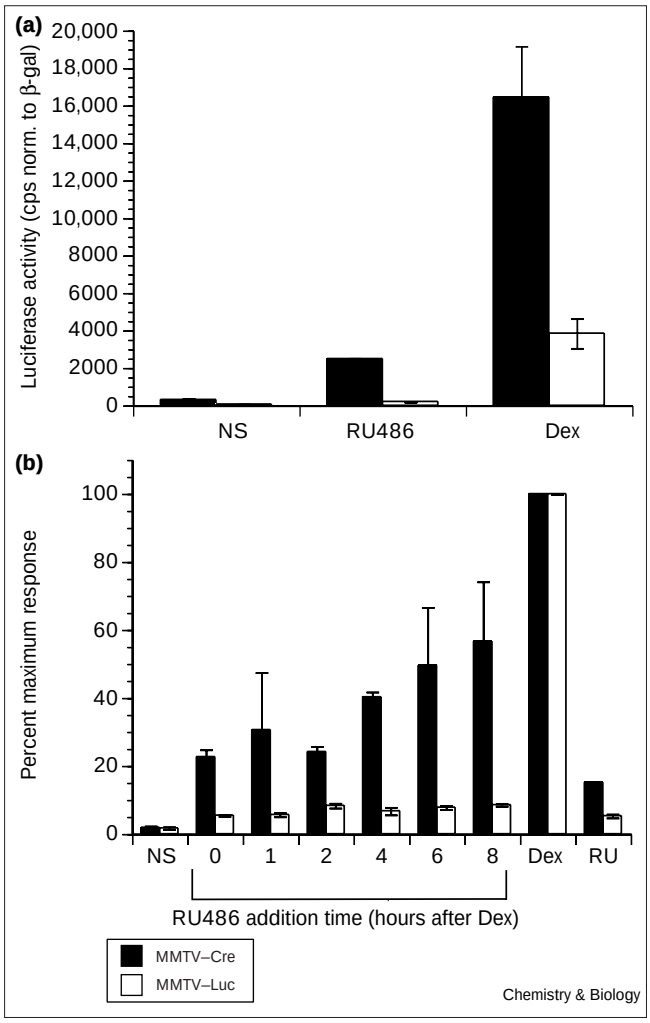
Luciferase expression in CHO cells transiently transfected with MMTV–Cre/pLM255 (triangles) or MMTV–Luc (squares) plasmids. (a) Time course of luciferase expression by cells incubated with 1 μM dexamethasone (Dex). (b) Dose-response curve for Dex. Cells were stimulated for 24 h. Luciferase activity is presented as counts per second (cps) normalized to β-galactosidase activity.

luciferase expression is not yet maximal after 24 hours of stimulation. The amplification of luciferase expression using the Cre/*loxP* system is less pronounced for the MMTV promotor than for the NFAT-dependent promoter (threefold at 24 hours). This may reflect the intrinsic relative strengths of these two promoter systems.

We measured the dose response of Dex and found the EC_{50} values for MMTV–Cre and MMTV–Luc to be similar at 4 nM and 2 nM, respectively (Figure 6b). Unlike the NFAT system, the shape of the dose-response curves is similar between the MMTV–Cre and MMTV–Luc reporters. Differences between recombinase-based and conventional reporter systems in the kinetics of reporter gene expression and shape of the dose-response curve are therefore dependent on the promoter and signal transduction pathway being studied.

One additional advantage of the Cre/*loxP* system is the potential to amplify a short signal pulse into one that can be measured (albeit at a later time). To determine how long Cre must be synthesized for recombination to occur, we used the glucocorticoid receptor antagonist RU486, which competes with Dex for receptor binding and blocks transcription. CHO cells transfected with MMTV–Cre/pLM255 or MMTV–Luc plasmids were stimulated with Dex, washed, and RU486 was added at various times. Figure 7a shows that 24 hours of Dex treatment results in the usual induction and signal amplification in the Cre/*loxP* system, whereas RU486 alone for 24 hours results in a much smaller, but significant, induction. The induction by RU486 alone may be caused by RU486 acting as a

Figure 7



Effect of the receptor antagonist RU486 on luciferase expression in CHO cells transiently transfected with MMTV–Cre/pLM255 or MMTV–Luc plasmids. (a) Cells were incubated in the absence (NS) or presence of 1 μM RU486 or 1 μM dexamethasone (Dex) for 24 h. Luciferase activities were normalized to β-galactosidase activity. (b) Cells were incubated with 5 nM Dex, and RU486 was added (final concentration 1 μM) at the times indicated. After 24 h, luciferase activities were measured. Luciferase activities were normalized to β-galactosidase activity and expressed as a percentage of the signal obtained with Dex alone for 24 h. RU, RU486 treatment alone for 24 h.

partial agonist of the MMTV promoter, and small amounts of Cre may be sufficient to recombine the luciferase reporter gene.

To vary the amount of Cre, RU486 was added at various times after addition of Dex. In the MMTV–Luc system, addition of RU486 at 8 hours after addition of Dex resulted in less than 10% of the luciferase activity observed for cells incubated with Dex alone (Figure 7b). The MMTV–Cre system, however, resulted in nearly 60% maximal activity after 8 hours of Dex stimulation, although the partial agonist activity of RU486 may also be contributing to the synthesis of Cre under these conditions. It therefore appears that limited transcription of Cre results in nearly maximal induction of luciferase.

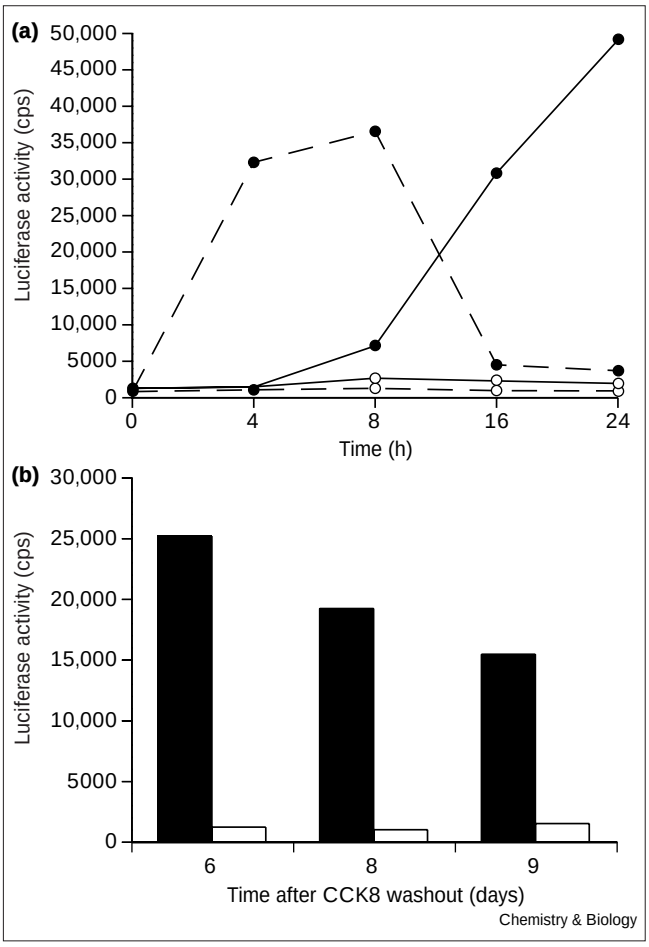
Regulation of Cre by the cyclic AMP response pathway

The elevation of the intracellular cyclic AMP concentration results in phosphorylation of the transcriptional transactivator CREB, which is then competent to bind the cyclic AMP response element (CRE) and stimulate transcription [18]. To study regulation of Cre recombinase by the cyclic AMP response pathway, we constructed plasmid 6CRE–Cre that contains the Cre gene under the transcriptional control of six CREs and a minimal thymidine kinase promoter (6CRE promoter). Plasmid 6CRE–Cre also carries a hygromycin selectable marker for selection.

To isolate stable clones, plasmid 6CRE–Cre and the *loxP*/luciferase reporter plasmid pLM255 were cotransfected into CHO cells expressing the cholecystokinin B (CCKb) receptor, and selected in medium containing hygromycin and puromycin. The CCKb receptor is a G-protein-coupled receptor that normally couples to Gq, resulting in production of IP3 and release of calcium from intracellular stores [19]. We found, however, that the CCKb receptor in CHO cells also couples to G_S-proteins, and causes an increase in the intracellular cyclic AMP concentration after binding of the ligand CCK8 (T. Chernov-Rogan and L.M., unpublished observations). Individual clones were isolated using a fluorescence-activated cell sorter (FACS) and tested for luciferase production after incubation with CCK8. The frequency of CCK8-regulated clones was rare (only three clones out of ~100 screened). The clone with the highest induced activity of luciferase (6CRE–Cre 2D7) was chosen for further study. As a control, we isolated a CHO clone expressing the CCKb receptor and carrying the luciferase gene under the control of the 6CRE promoter (6CRE–Luc 3B6).

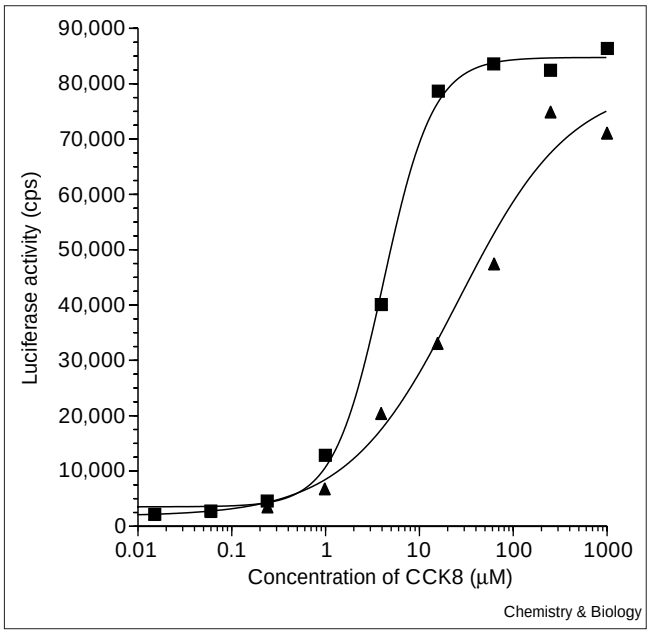
The time course of luciferase expression was determined by incubating the 6CRE–Cre and 6CRE–Luc clones with the ligand CCK8 for 24 hours. As was observed for the NFAT–Cre and MMTV–Cre reporter systems, the luciferase activity of the 6CRE–Cre clone increases throughout the 24 hour time period, whereas the activity of 6CRE–Luc peaks at 8 hours and returns to near background levels by 24 hours (Figure 8a). The recombination rate in the 6CRE–Cre clone appears to be tightly regulated, because luciferase activity in the absence of ligand is very low (Figure 8a). The activity of 6CRE–Cre at 24 hours is only 30% higher than the peak activity of 6CRE–Luc at 8 hours. Thus, although absolute amplification of reporter expression in the CRE-based promoter system is similar to the MMTV promoter system, the time course of reporter induction is similar to the NFAT-dependent promoter system.

Figure 8



Time course of luciferase expression in CHO clones expressing the CCKb receptor. (a) Clones 6CRE–Cre (solid line) or 6CRE–Luc (dashed line) were incubated in the presence (closed circles) or absence (open circles) of 0.5 μM CCK8 for 24 h, and luciferase activities measured at the indicated times. (b) Clone 6CRE–Cre was incubated in the presence (solid bar) or absence (open bar) of 0.5 μM CCK8 for 24 h, then washed to remove ligand and cultured for the indicated days. Luciferase activity was determined and normalized to total cell number.

Figure 9



Dose-response curves of 6CRE–Cre (triangles) and 6CRE–Luc (squares) clones after incubating with the CCKb agonist CCK8 for 24 h and 6 h, respectively.

To study the regulation of 6CRE–Cre at longer time intervals, we incubated cells in the presence or absence of CCK8 ligand for 24 hours, then washed to remove ligand, and further cultured the cells for 6, 8 and 9 days. Even at 9 days after exposure to ligand, the activity of the stimulated cells is more than tenfold greater than that of the unstimulated cells (Figure 8b). These results are consistent with those obtained with the NFAT–Cre clone, and demonstrate that the Cre system is able to record signal transduction events occurring more than a week after stimulation with ligand. Dose-response curves were constructed and the EC_{50} value of the ligand CCK8 was measured at 21 nM for the 6CRE–Cre clone and 4 nM for the 6CRE–Luc clone (Figure 9). These results suggest that minor differences in the reporter response curves between conventional and the Cre/*loxP* systems can occur, and may depend on the particular signaling system and cell clones used.

Discussion

Cre recombinase has been expressed in a variety of cell types and is a powerful tool for introducing precise genetic alterations in a genome [20–22]. In this study, we placed Cre under the control of several promoters that are regulated by distinct signal transduction pathways. We developed reporter systems for studying the regulation of Cre by these pathways, and compared them with conventional transcriptional reporter systems for assaying receptor ligands.

To screen for receptor ligands, the Cre/*loxP* reporters described here have potentially two important advantages over conventional transcriptional reporters. First, the Cre/*loxP* system can amplify expression of the reporter gene, because any strong promoter can be used to transcribe the reporter. The extent of amplification, however, appears to depend somewhat on the particular signal transduction pathway, but a variety of strong promoters can be selected to gain optimal amplification in a given cell type. Second, expression of the reporter gene is constitutive once cells have undergone recombination. This allows reporter gene expression to be assayed days or weeks after addition of the ligand. In theory, if the background rate of recombination is negligible, the induced reporter signal has an unlimited time frame of

readability because expression of the reporter gene is a heritable trait.

The utility of the Cre/*loxP* system for screening may ultimately depend on the screening format. For example, it may be possible to assay reporter activity of a single cell by incubating with compound and waiting for the signal to amplify exponentially during cell growth. Other screening formats, such as compounds overlaid on cells embedded in agar [23], may benefit from a Cre-based system because the diffusion of a compound through agar can limit the time cells are exposed to an effective concentration of the compound. A short exposure time to the compound may only induce Cre transcription in a few cells, but this may be sufficient to trigger recombination and therefore amplification of expression of the reporter gene. It may also be possible to substitute luciferase with a selectable marker and use growth selection to report binding of a ligand. The Cre/*loxP* system may therefore be most useful in screening formats that are not suitable for the transient signals generated by conventional reporter systems.

An important feature of any cell-based reporter system is the dose response of receptor agonists and antagonists. The Cre reporters are two-component systems comprised of the recombinase and the *loxP* reporter, yet they appear to give similar EC_{50} values to the conventional systems for the three different promoters studied here. The EC_{50} value is therefore determined by transcription initiation of the promoter responding to the stimulus, and appears to not be greatly affected by other downstream events innate to the Cre/*loxP* system or by parameters that can affect reporter gene expression, such as mRNA stability or translation initiation.

Reporter gene expression by recombination is a multistep process, and increasing luciferase activity in the Cre reporter systems is probably caused by an increasing percentage of cells within the population undergoing recombination and thus expressing the reporter gene. In NFAT–Cre cells, two potential concentration thresholds, NFAT and recombinase, probably need to be overcome to recombine the reporter gene. Transcription from an NFAT-dependent promoter has previously been shown to respond only to a tight threshold of NFAT concentration in the nucleus [24]. This property of two thresholds may be responsible for the steeper ionomycin dose-response curve for NFAT–Cre cells than that for NFAT–Luc cells. In contrast, the shape of the dose-response curves in the MMTV and 6CRE systems are similar in the Cre-based and corresponding traditional reporters. This is probably due to intrinsic properties of these promoters as compared with the NFAT-dependent promoter. All of the Cre reporters, however, have a delay in reporter gene expression, presumably caused by the extra time needed for Cre to translocate into the nucleus and recombine the *loxP* sites.

A second potential application of Cre-based reporters is their use in the study of signal transduction pathways during development. Current reporter constructs used in transgenic animals, for instance, provide a readout signal only when there is an actual transcriptional stimulus and do not track the fate of cells afterwards. The ability to convert a transient signal into a permanent readout of gene expression may make Cre-based reporter systems valuable in this context. For example, the role of NFAT-dependent signaling during cell fate decisions in thymic development could be studied by using NFAT–Cre and a visible *loxP*-based reporter gene such as green fluorescent protein. Moreover, the effects of a drug or pathogen on developmental processes could be studied weeks or months after a single exposure.

The success of isolating suitable stable cell lines carrying Cre gene fusions is largely dependent on the basal activity of the promoter and signal transduction pathway being studied. We used puromycin selection to isolate stable clones, because unregulated expression of Cre results in excision of the puromycin resistance cassette and elimination of these clones from the population. We found that with promoters having low basal activity, such as NFAT in Jurkat cells, it was relatively easy to find many clones that were appropriately regulated. In contrast, only a few useful clones of the 6CRE–Cre reporter were isolated, presumably because relatively high intracellular cAMP concentrations results in high basal activity of this promoter unless integration occurs in a particularly quiescent area of the genome. Cre recombinase is a stable enzyme [25], and it is likely that small amounts can eventually catalyze recombination. Our results are consistent with this hypothesis because reporter expression still occurs in the MMTV–Cre system, even if transcription is blocked by RU486 soon after induction with Dex.

Another method for isolating a stable clone carrying Cre under the control of a leaky promoter may be to mutagenize the *loxP* sites. Previous results have shown that the rate of Cre-mediated recombination is dependent on the nucleotide sequence of the *loxP* sites [26,27]. It should be possible, therefore, with a library of *loxP* sites, to screen the library using puromycin selection for sites that are resistant to the background levels of Cre. Thus, one could find classes of *loxP* sites whose recombination potential is matched to the basal activities of different classes of promoters. Such improvements could extend the use of Cre reporters to include many additional promoters and signal transduction pathways.

Significance

We have developed a system for regulating DNA recombination in mammalian cells using chemical ligands. The Cre reporter system links signal transduction to DNA recombination. Receptor ligands can therefore be used to

alter mammalian cell phenotypes. The Cre reporters can also be used to study signal transduction under conditions that are not suitable for the transient signals generated by conventional reporter systems. For ultra-miniaturized screening formats, it may be possible to use the Cre reporter system to measure effects of ligands on single cells. An active compound will trigger a genetic switch in a single Cre reporter cell to express the reporter gene, and inheritance of the reporter gene phenotype will cause the reporter signal to amplify through cell division. If the background rate of recombination remains low, there is also no time limit for assaying the cells after compound addition. Thus, compared with conventional reporter systems, the Cre reporters offer the advantage of signal amplification and an unlimited time frame for signal detection. Other applications of Cre reporters may include studying the fate of transcribed genes during development. The ability to convert a transient activation of a signal transduction pathway into a permanent readout of gene expression means that a compound's effect on transcription could be tracked for weeks or months after a single exposure.

Materials and methods

Plasmid constructions

To construct the *loxP* luciferase reporter plasmid pLM255, plasmid pBS302 [15] was cut with *Eco*RI/*Sca*I and the 1.5 kb fragment containing the *loxP*/stop/*loxP* cassette was ligated to the *Eco*RI/*Bbs*I sites of pCMVSport 1 (Life Technologies, Gaithersburg, MD) to generate pCLSport 1. Plasmid pSRα lux (ARI 1819) was digested with *Not*I/*Xho*I and the 1.7 kb fragment containing the luciferase gene was ligated to the *Not*I/*Sal*I sites of pCLSport 1 to generate pLM252. The 1.1 kb *pac* gene and SV40 promoter from pPUR (Clontech, Palo Alto, CA) was amplified using PCR and ligated to the *Afl*II sites of pLM252 to generate pLM255.

The NFAT–Luc reporter plasmid was a gift from Peter Kao and contains three copies of the NFAT binding site (–286 to –257 of the human IL-2 gene) linked to the human IL-2 promoter (–72 to +47) driving luciferase [28]. The NFAT–Cre plasmid was constructed by combining a blunted *Hind*III to *Dra*III fragment from pCMVCre (pLM208), containing the Cre coding region and a polyadenylation signal, with a blunted *Hind*III to *Eco*RI fragment from the plasmid NFAT–SEAP [29] containing the NFAT-responsive elements and IL-2 promoter described above. Subsequently, a neomycin resistance cassette (*Nde*I to *BamH*I fragment from pSV2neo [30] was inserted into NFAT–Cre to form NFAT–Cre–neo.

Plasmid MMTV–Luc (pLM253) was constructed as described previously [12]. To construct MMTV–Cre (pLM269), the MMTV–Luc plasmid was digested with *Nco*I/*Sal*I to remove the luciferase gene and religated. The resulting plasmid was digested with *Xho*I/*Hind*III, and ligated to an *Xho*I/*Hind*III restriction fragment containing the Cre gene from pBS185 [31].

Plasmid 6CRE–Luc (6CRE–TK–Luc/Hyg) was constructed as described previously [32]. To construct 6CRE-Cre (pLM266), the Cre gene was amplified from pBS39 [33] and cloned into the *Xho*I/*Hind*III sites of a derivative of 6CRE-Luc in which a *Xho*I linker was inserted at the unique *Sma*I site.

Cell growth, transfection and isolation of stable transfectants

CHO-K1 (ATCC, Rockville, MD) or Jurkat cells were incubated at 37°C under 5% CO_2 and cultured in growth medium consisting of Dulbecco's modified Eagle medium/Nutrient Mixture F12 (D-MEM/F12) containing 5% FetalClone II (Hyclone, Logan, UT) or RPMI 1640 containing 10% fetal bovine serum (FBS) respectively, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. To isolate CHO transfectants, 10^7 cells suspended in 0.8 ml of D-MEM/F12 were combined with 20 μg each of plasmid DNA in a 0.4 cm cuvette and electroporated with a BioRad Gene Pulser set at 400 V and 250 μF. To isolate Jurkat stable transfectants, 3×10^7 cells were suspended in 0.3 ml of RPMI 1640 containing 10% FBS and combined with 20 μg linearized plasmid DNA in a 0.4 cm cuvette. Cells were electroporated as above except with 250 V and 960 μF settings. Cells were grown for 48 h before selecting with 1 mg/ml G418, 10 μg/ml puromycin, or 800 U/ml hygromycin. After 10 days of selection, the population was cloned by limiting dilution or by a FACS into wells of 96-well master plates. Clones were grown in the presence of G418 and puromycin, and replica plated to wells of two 96-well black-view bottom plates (Packard, Meriden, CT). Clones expressing Cre were always grown in medium containing puromycin to prevent unregulated recombination. To assay for luciferase inducibility in individual clones, cells were washed and incubated in growth medium lacking puromycin in the presence or absence of stimulating agent(s) for the indicated times.

Transient transfections

Jurkat cells were transiently transfected by electroporation essentially as previously described [34] using 4 μg each plasmid with 1.5×10^7 cells in 0.3 ml growth medium. After electroporation, cells were recovered for 18–24 h in 8 ml growth medium before stimulations with ionomycin (2 μM), PMA (20 ng/ml), or both. CHO cells were transiently transfected by lipid-mediated transfection as previously described [12] using the β-galactosidase reporter plasmid, pCH110, as an internal control. Luciferase assays were performed as described above and β-galactosidase assays were performed as previously described [12]. Luciferase activity in each well is normalized to the β-galactosidase activity in that well by dividing the luciferase activity by the β-galactosidase activity.

Luciferase assay

To measure luciferase activity in stable clones, 5×10^4 CHO cells or 2×10^5 Jurkat cells were added to each well of a 96-well black-view bottom plate and incubated in growth medium lacking puromycin as indicated. Each well was washed and filled with 100 μl of DMEM/F12 medium lacking phenol red and 100 μl of LucLite reagent (Packard, Meriden, CT). The plate was agitated for a few minutes and luminescence was measured in a 96-well TopCount scintillation counter (Packard). Luciferase activity was measured as luminescent counts per second (cps).

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References

1. Jayawickreme, C.K. & Kost, T.A. (1997). Gene expression systems in the development of high-throughput screens. *Curr. Opin. Biotechnol.* 8, 629-634.
2. Silverman, L., Campbell, R. & Broach, J.R. (1998). New assay technologies for high-throughput screening. *Curr. Opin. Chem. Biol.* 2, 397-403.
3. Gallop, M.A., Barrett, R.W., Dower, W.J., Fodor, S.P.A. & Gordon, E.M. (1994). Applications of combinatorial technologies to drug discovery. 1. Background and peptide combinatorial libraries. *J. Med. Chem.* 37, 1233-1251.
4. Gordon, E.M., Barrett, R.W., Dower, W.J., Fodor, S.P.A. & Gallop, M.A. (1994). Applications of combinatorial technologies to drug discovery. 2. Combinatorial organic synthesis, library screening strategies, and future directions. *J. Med. Chem.* 37, 1385-1401.
5. Sauer, B. & Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl Acad. Sci. USA* 85, 5166-5170.
6. Hoess, R.H. & Abremski, K. (1985). Mechanism of strand cleavage and exchange in the Cre-*lox* site-specific recombination system. *J. Mol. Biol.* 181, 351-362.

7. Sauer, B. (1998). Inducible gene targeting in mice using the Cre/*lox* system. *Methods* **14**, 381-392.
8. St-Onge, L., Furth, P.A. & Gruss, P. (1996). Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res.* **24**, 3875-3877.
9. Metzger, D., Clifford, J., Chiba, H. & Chambon, P. (1995). Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proc. Natl Acad. Sci. USA* **92**, 6991-6995.
10. Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K. & McMahon, A.P. (1998). Modification of gene activity in mouse embryos *in utero* by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8**, 1323-1326.
11. Kellendonk, C., Tronche, F., Casanova, E., Anlag, K., Opherk, C. & Schütz, G. (1999). Inducible site-specific recombination in the brain. *J. Mol. Biol.* **285**, 175-182.
12. Dias, J.M., Go, N.F., Hart, C.P. & Mattheakis, L.C. (1998). Genetic recombination as a reporter for screening steroid receptor agonists and antagonists. *Anal. Biochem.* **258**, 96-102.
13. Rao, A., Luo, C. & Hogan, P.G. (1997). Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* **15**, 707-747.
14. Lakso, M., *et al.*, & Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl Acad. Sci. USA* **89**, 6232-6236.
15. Sauer, B. (1993). Manipulation of transgenes by site-specific recombination: use of Cre recombinase. *Methods Enzymol.* **225**, 890-900.
16. Flanagan, W.M., Corthesy, B., Bram, R.J. & Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **352**, 803-807.
17. Lee, F., Mulligan, R., Berg, P. & Ringold, G. (1981). Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. *Nature* **294**, 228-232.
18. Montminy, M. (1997). Transcriptional regulation by cyclic AMP. *Annu. Rev. Biochem.* **66**, 807-822.
19. Galas, M.C., Bernad, N. & Martinez, J. (1992). Pharmacological studies on CCK_B receptors in guinea pig synaptoneurosomes. *Eur. J. Pharmacol.* **226**, 35-41.
20. Gu, H., Marth, J.D., Orban, P.C., Mossmann, H. & Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103-106.
21. Tsien, J.Z., *et al.*, & Tonegawa, S. (1996). Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* **87**, 1317-1326.
22. Lewandoski, M., Wassarman, K.M. & Martin, G.R. (1997). Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. *Curr. Biol.* **7**, 148-151.
23. Quillan, J.M., Jayawickreme, C.K. & Lerner, M.R. (1995). Combinatorial diffusion assay used to identify topically active melanocyte-stimulating hormone receptor antagonists. *Proc. Natl Acad. Sci. USA* **92**, 2894-2898.
24. Fiering, S., Northrop, J.P., Nolan, G.P., Mattila, P.S., Crabtree, G.R. & Herzenberg, L.A. (1990). Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor. *Genes Dev.* **4**, 1823-1834.
25. Buchholz, F., Ringrose, L., Angrand, P.O., Rossi, F. & Stewart, A.F. (1996). Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. *Nucleic Acids Res.* **24**, 4256-4262.
26. Hoess, R.H., Wierzbicki, A. & Abremski, K. (1986). The role of the *loxP* spacer region in P1 site-specific recombination. *Nucleic Acids Res.* **14**, 2287-2300.
27. Lee, G. & Saito, I. (1998). Role of nucleotide sequences of *loxP* spacer region in Cre-mediated recombination. *Gene* **216**, 55-65.
28. de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. & Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725-737.
29. Clipstone, N.A. & Crabtree, G.R. (1992). Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **357**, 695-697.
30. Southern, P.J. & Berg, P. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1**, 327-341.
31. Sauer, B. & Henderson, N. (1990). Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biol.* **2**, 441-449.
32. Stables, J., *et al.*, & Rees, S. (1999). Development of a dual glow-signal firefly and *Renilla* luciferase assay reagent for the analysis of G-protein coupled receptor signalling. *J. Recept. Signal Transduct. Res.* **19**, 395-410.
33. Sauer, B. (1987). Functional expression of the *Cre–lox* site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**, 2087-2096.
34. Northrop, J.P., Ullman, K.S. & Crabtree, G.R. (1993). Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. *J. Biol. Chem.* **268**, 2917-2923.
35. DeLean, A., Munson, P.J. & Rodbard, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **23**, 97-102.

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