# Efficient gene activation in cultured mammalian cells mediated by FLP recombinase-expressing recombinant adenovirus

**Masakazu Nakano[1,2], Kazuhiko Odaka[1,2], Masakazu Ishimura[1,2], Saki Kondo[1], Naoto Tachikawa[1,2], Joe Chiba[2], Yumi Kanegae[1] and Izumu Saito[1,*]**

[1]Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan and [2]Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 278-8510, Japan

## ABSTRACT

**A recombinant adenovirus (rAd) expressing Cre recombinase derived from bacteriophage P1 has already been extensively used for the conditional gene activation and inactivation strategies in mammalian systems. In this study, we generated AxCAFLP, a rAd expressing FLP recombinase derived from *Saccharomyces cerevisiae* and carried out quantitative comparisons with Cre-expressing rAd in both *in vitro* and in cultured cells to provide another efficient gene regulation system in mammalian cells. In the *in vitro* experiments, the relative recombination efficiency of FLP expressed in 293 cells infected with FLP-expressing rAd was approximately one-thirtieth that of Cre even at 30°C, the optimum temperature for FLP activity, and was approximately one-ninetieth at 37°C. Co-infection experiments in HeLa cells using a target rAd conditionally expressing *LacZ* under the control of FLP showed that an FLP-expressing rAd, infected at a multiplicity of infection (MOI) of 5, was able to activate the transgene in almost 100% of HeLa cells whereas the Cre-expressing rAd was sufficient at an MOI of 0.2. Since an MOI of 5 is ordinarily used in rAd experiments, these results showed that the FLP-expressing rAd is useful for gene activation strategies and is probably applicable to a sequential gene regulation system in combination with Cre-expressing rAd in mammalian cells.**

## INTRODUCTION

Site-specific recombinases have become a powerful tool for the regulation of the expression of transgenes (for reviews see 1–3). The site-specific recombinase Cre, derived from bacteriophage P1, has been widely applied to the gene activation and inactivation strategies in mammalian systems, in cell lines (e.g. 4,5) and in transgenic mice (e.g. 6–8). Moreover, a Cre-expressing recombinant adenovirus (rAd) has provided a 'molecular switch' to turn on or off the transgene expression in cell lines (9–18) or in mice (19–33). Despite their widespread application in many fields, these strategies of transgene regulation can only be carried out once, i.e. 'off to on' or 'on to off', when manipulated by Cre alone. However, 'off-on-off' or 'on-off-on' regulation is more attractive when verifying the function of a given gene, and would be valuable for stage-specific serial gene regulation in developmental studies. However, to regulate the expression of transgenes in serial steps, we need novel regulation systems mediated by recombinases other than Cre.

Another well-characterized site-specific recombinase FLP (34–38), derived from *Saccharomyces cerevisiae*, has also been applied to gene regulation in plants (39,40), *Drosophila* (41–43) and mammals (44–48). The use of FLP in combination with Cre may facilitate sequential gene regulation; however, only a few reports have been reported to date (49), probably due to the relatively low activity of FLP at 37°C, the optimum temperature of FLP being 30°C (50). In fact, a mutant FLP, FLPe, has been developed recently (51), which has overcome its low activity at 37°C.

In this report, we generated a rAd expressing wild-type FLP with the aim of using it as a 'second molecular switch' in cultured mammalian cells. We quantified the recombination efficiency of FLP expressed in 293 cells at 37°C and found that the activity of FLP was several-tens of times lower than that of Cre. However, we showed that such low activity can be readily overcome as long as an efficient rAd expression system under the control of a potent CAG promoter (52) was used. The FLP-expressing rAd was able to activate a transgene in virtually 100% of cultured mammalian cells, suggesting the usefulness of FLP-expressing rAd as a secondary regulation system in combination with the Cre-expressing rAd.

## MATERIALS AND METHODS

### Cell lines

The human embryonic kidney cell line, 293 (53), and the human cervical cancer-derived cell line, HeLa, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The 293 cells constitutively express adenoviral E1 genes and support replication of E1-substituted rAds. After the infection of rAds, cells were maintained in DMEM supplemented with 5% FCS.

### Construction of the target unit CAFNF and the FLP expression unit

The FLP recognition target (FRT) sequence used in this work was a synthesized nucleotide of 54 nt (5′-AAATtccg-ga[*Bsp*EI]GAAGTTCCTATTCTCTAGAAAGTATAGGAA-CTTCgacgtc[*Aat*II]ATTT-3′), where the 34 nt functional FRT site (underlined) was flanked by *Bsp*EI and *Aat*II sites (lower-case letters) in that order. A head-to-tail dimer was cloned at the *Sma*I site of pUC18 (termed pUFwF) and used as a donor of a pair of FRT sequences. pCAFNF5 (Fig. 1A) was constructed as a cassette plasmid containing a target unit, CAFNF, consisting of the CAG promoter (52), the first FRT sequence, a *neo*-resistance gene (1.0 kb *Bgl*II–*Sma*I fragment derived from pSV2neo), the second FRT sequence and a rabbit β-globin polyadenylation [poly(A)] signal in that order followed by multi-cloning sites (MCS) (*Swa*I–*Eco*RI–*Sac*I–*Kpn*I–*Sma*I). pCAFNFG and pCAFNFZ, the target plasmids, were constructed by inserting green fluorescent protein (GFP) and *LacZ* genes, respectively, as the reporter gene at the MCS of pCAFNF5. The source of the GFP gene was pxCAEGFP, originally derived from pEGFP-C1 (EGFP vector; Clontech), introducing a termination codon at the *Bgl*II site. The coding sequence of *LacZ* was derived from pSRLacZ (54). pCAFNFG was also used as substrate DNA for the *in vitro* recombination assay of the FLP/FRT system.

The initiation codon of the coding region of FLP (1.5 kb *Sph*I–*Xba*I fragment from pUC19-Flp, a generous gift from Dr M. Jayaram) was altered to fit Kozak's rule (55). The *Sph*I digests at the initiation site including the initiation codon, so that the synthesized nucleotide (5′-agctt[*Hin*dIII end]ctgcag[*Pst*I]CAGAC-CGTGCATcatg[*Sph*I-compatible end]-3′) fitting Kozak's rule was ligated to the *Sph*I–*Xba*I fragment derived from pUC19-Flp. The resulting fragment was then cloned at the *Hin*dIII–*Xba*I site of pUC19 (termed pUkFLP). The pUkFLP was used as a donor of the FLP sequence.

Since our aim is to compare the recombination efficiency between FLP and Cre, we used Cre-expression unit as described (9) whose initiation codon has also been altered to fit Kozak's rule by tagging SV40 T antigen-derived nuclear localization signal (NLS, underlined) (5′-ctgcag[*Pst*I]CAGACCG-TGCATCATGAGCGGCCCTCCAAAAAAGAAGAGAAA-GGTAGAAGACCCGGgcggccgc[*Not*I]-3′).

### Generation of rAds

The rAds were generated by the COS-TPC method as described (54). Briefly, both FLP target units, CAFNFG and CAFNFZ, excised from pCAFNFG and pCAFNFZ, respectively, as a *Sal*I–*Hin*dIII fragment, were cloned into a cassette



**Figure 1.** Transgene activation strategy mediated by FLP recombinase. (**A**) Structure of the cassette plasmid, pCAFNF5, possessing the CAFNF target unit. CAG, CAG promoter; SpA, SV40 early poly(A) signal; MCS, multi-cloning site; GpA, rabbit β-globin poly(A) signal. The stuffer region is composed of the *neo* gene and the SpA flanked by a pair of FRT. (**B**) Structure of the FLP-expressing rAd, AxCAFLP, and the target rAds, AxCAFNFG and AxCAFNFZ. A triangle under the adenovirus genome represents the deletion of an adenovirus sequence. (**C**) Activation of the transgene by CAFNF target unit mediated by FLP recombinase. The horizontal arrows indicate the orientation of the transcription. FLP-mediated excisional deletion removes the stuffer region, resulting in the expression of the reporter genes, GFP or *LacZ*, under the control of the CAG promoter.

cosmid pAxcw (54). The FLP gene was excised from pUkFLP with *Pst*I and *Fsp*I and cloned into an expression cassette cosmid, pAxCAwt (termed pAxCAFLP), containing CAG promoter and poly(A) signal. Each cosmid was mixed with adenovirus DNA-terminal protein complex and transfected into 293 cells, resulting in the generation of rAds through homologous recombination. Target rAds containing the CAFNFG unit (AxCAFNFG), CAFNFZ unit (AxCAFNFZ) and FLP-expressing rAd (AxCAFLP) were generated (Fig. 1B). A target rAd, AxCALNLG, which expresses the GFP gene described above under the regulation of Cre, was also generated by inserting a target unit, CALNLG, into pAxcw. A

purified and concentrated virus stock was prepared as described (56).

### *In vitro* recombination assay

The *in vitro* recombination assay of the FLP/FRT system in this paper was essentially as described (57). FLP-containing lysate was prepared as follows. In total, $2 \times 10^7$ of 293 cells were infected with AxCAFLP at an MOI of 5. Twenty-two hours later, cells were harvested by scraping and collected by centrifugation at 1000 r.p.m. for 5 min at 4°C using Avanti HP-25I (Beckman-Coulter). The cell pellet was washed twice with PBS(–) and resuspended with 1 ml of FLP storage buffer (10% glycerol, 20 mM Tris–HCl pH 7.5, 300 mM NaCl, 1 mM EDTA and 0.02 mM phenylmethylsulfonyl fluoride). The cell suspension was then sonicated for 3 min (six cycles, 30 s each) using Bioruptor II (CosmoBio; Tokyo, Japan) at maximum power (200 W) and immediately cetrifuged at 10 000 r.p.m. for 20 min at 4°C. The supernatant was stored at –80°C.

The reaction was started by mixing *Hin*dIII-linearized pCAFNFG with FLP lysate (2.5–25 µl), and incubated for 30 min at 37 or 30°C in a 50 µl volume of FLP reaction buffer (25 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$ and 5 mM DTT). The reaction was terminated by phenol/chloroform extraction followed by two cycles of chloroform extraction and ethanol precipitation. The recovered DNA was then digested with *Fsp*I and DNA digests were detected by ethidium bromide staining after agarose gel electrophoresis. The recombination efficiency was calculated by quantifying the density of the DNA bands on the photograph based on densities of the quantification-standard DNA digests on the same gel as described (58). To compare the recombination efficiency of FLP and Cre, an *in vitro* recombination assay of Cre/*loxP* system was also carried out by using *Eco*RI-linearized pCALNLZ (9) and Cre lysate (0.1–5.0 µl) as described (58).

### GFP or *LacZ* gene activation by recombinase-expressing rAds

To compare the recombination efficiency of FLP and Cre recombinase in cultured mammalian cells, target rAds and the recombinase-expressing rAds were co-infected into HeLa cells. Either AxCAFLP or AxCANCre (NLS-tagged Cre-expressing rAd) (9) was infected to express each recombinase. Either AxCAFNFG or AxCAFNFZ was infected to introduce the reporter genes, GFP or *LacZ*, respectively, to assay the regulation of FLP. Similarly, either AxCALNLG or AxCALNLZ (9) was infected to assay the regulation of Cre. When each recombinase recognizes their target units, it excises the stuffer region, composed of the *neo* gene and the poly(A) signal flanked by a pair of recombinase target sequences, resulting in the expression of the transgene (Fig. 1C). The recombination efficiency of FLP and Cre recombinase was compared by measuring the fluorescence intensity of expressed GFP or by staining LacZ-expressed cells with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Three days after co-infection, the fluorescence intensity of GFP was quantified by Fluoroskan Ascent FL (Labsystems). For cell staining, 3 days after co-infection, cells were washed with PBS(–) twice, fixed with 0.25% glutaraldehyde and stained with 0.1% X-gal.

## RESULTS

### Construction of the target unit CAFNF

We first constructed a cassette plasmid pCAFNF5 (Fig. 1A), which can activate the expression of a transgene inserted into its MCS mediated by FLP, based on the strategy mediated by Cre established previously (9,12). The stuffer region, composed of the *neo* gene and the SV40 early poly(A) signal (SpA) flanked by a pair of target sequences, hampers the expression of the foreign gene so that the target unit CAFNF initially expresses the *neo* gene. When functional FLP is supplied, the FLP-mediated excisional deletion removes the stuffer region, resulting in the expression of the transgene under the control of the CAG promoter (Fig. 1C). We confirmed whether the CAFNF unit works in cultured mammalian cells as described above by transfection experiments (see below).

### Establishment of FLP/FRT-mediated recombination assay *in vitro*

To compare the recombination efficiency of FLP and Cre produced in mammalian cells, an *in vitro* assay to detect and to quantify the excisional deletion mediated by FLP was established. We adopted the same strategy as the *in vitro* assay of Cre (58), using FLP expressed in mammalian cultured cells, since our aim was to assess whether FLP works efficiently in mammalian systems. When various doses of the FLP lysate were mixed with 1 µg of linearized substrate DNA, pCAFNFG, and incubated for 30 min at 30°C, which is the optimum temperature for FLP activity, recombined products were detected in a dose-dependent manner (Fig. 2A, lanes 10–16). As a result of the recombination, when the DNA recovered from the reaction mixture was digested with *Fsp*I, the recombined products were detected as 3.9 and 1.2 kb fragments derived from 2.8 and 2.4 kb substrates (Fig. 2B). The maximum rate of the recombination efficiency reached ~30–40% when the maximum amount of FLP lysate (25 µl) in our experimental conditions of 50 µl total reaction volume was used. We did not detect any bands of the 'intermediate' product, which was reported in the Cre/*loxP* system (58).

### Recombination efficiency of FLP and Cre *in vitro*

A comparative *in vitro* recombination experiment mediated by Cre/*loxP* was also carried out as described (58) using pCALNLZ as a substrate DNA. Cre lysate was prepared by same procedure used to prepare the FLP lysate. Cre showed lysate-dependent recombination at 37°C using 1 µg of linearized pCALNLZ (Fig. 3A, lanes 10–16), reaching a plateau level of ~40% with >1.3 µl of the lysate as described (58). As shown in Figure 3B, after *Nco*I digestion, the recombined products were detected as 4.4 and 1.2 kb fragments derived from 3.6 and 2.0 kb substrates, along with the 'intermediate' product as described previously (58).

Since the optimum temperature of FLP activity is 30°C (50), it has been considered that FLP does not work efficiently in mammalian expression systems (51). Therefore, we carried out the *in vitro* recombination assay at both 37 and 30°C, using FLP and Cre lysate expressed in 293 cells and the amount of the recombined products were quantified based on the band

**Figure 2.** *In vitro* recombination of the FLP/FRT system at 30°C. (**A**) Quantitation of the recombination products. M (lane 1), 1 kb DNA ladder marker (Gibco BRL); P and S, product and substrate, respectively. Quantitation standards (lanes 2–9) represent the *Nco*I fragment of the *Eco*RI-digested pCALNLZ. *Hin*dIII-linearized pCAFNFG and each denoted amount of the FLP lysate was incubated for 30 min at 30°C. The recovered DNA was then digested with *Fsp*I and the density of the recombined 3.9 kb products (P3.9) were quantified based on densities of the quantitation standards. One representative result out of three is shown. (**B**) Recombination process of the FLP/FRT system. F, *Fsp*I site.



**Figure 3.** *In vitro* recombination of the Cre/*loxP* system at 37°C. (**A**) Quantitation of the recombination products. M (lane 1), 1 kb DNA ladder marker (Gibco BRL); P and S, product and substrate, respectively; i, recombination intermediate; *, unexpected bands (may be due to the site preference of *Nco*I). The same quantitation standards (lanes 2–9) as used in the FLP/FRT system were applied. *Eco*RI-linearized pCALNLZ and each denoted amount of the Cre lysate was incubated for 30 min at 37°C. The recovered DNA was then digested with *Nco*I and the densities of the 4.4 kb products (P4.4) were quantified based on those of the quantitation standards. One representative result out of three is shown. (**B**) Recombination process of the Cre/*loxP* system. N, *Nco*I site.

intensity of the quantitation standards (Figs 2A and 3A, for example). As shown in Figure 4, Cre showed almost the same recombination efficiency at 37 and 30°C. In contrast, FLP showed significantly higher recombination efficiency at its optimum temperature, 30°C. The kinetics of recombination efficiency of Cre appeared to be a three-phase or sigmoid curve; a gradual increase up to ~2%, linear dose-dependency from 2 to 35%, and saturation at ~35%. The kinetics of FLP at 30°C appears similar at least up to the second phase. Since the maximum recombination efficiency of FLP at 37°C in our experiment was ~2%, we compared the relative recombination efficiency as the reciprocal number of the lysate amount yielding 2% recombination. The relative recombination efficiency of FLP at 30°C was approximately one-thirtieth of Cre at 30°C, and was approximately one-ninetieth of Cre at 37°C. Therefore, the recombination efficiency of FLP at 37°C was only 3-fold greater than that at 30°C. The large difference in the relative recombination efficiency between FLP and Cre seems to be due to the enzymatic activity, but we cannot rule out the possibility that it is due to the expression level of these proteins in cultured mammalian cells.

## Quantitative gene activation by FLP-expressing rAd

To assess whether the FLP/FRT system works efficiently in cultured mammalian cells, we generated three rAds (Fig. 1B); one expressing FLP (AxCAFLP), and the others bear the target unit with reporter genes, GFP and *LacZ* (AxCAFNFG and AxCAFNFZ, respectively). Corresponding rAds using the Cre/*loxP* system instead of the FLP/FRT system were also prepared. AxCAFLP was infected into HeLa cells together with the target rAd. The infection titer of the target rAd was kept constant at MOI of 5 and AxCAFLP was co-infected at various MOIs.

**Figure 4.** Recombination efficiency of FLP and Cre *in vitro*. The recombination efficiency per lysate of FLP at 37°C (filled circle) or 30°C (open circle) and that of Cre at 37°C (filled square) or 30°C (open square) were quantified as described. The maximum amount of the reacted lysate was 25 µl in our experimental condition of 50 µl total reaction volume. The quantitative results were normally reproducible within ±5%. One representative result out of three is shown.



**Figure 5.** GFP gene activation mediated by recombinase-expressing rAds. Various MOIs of recombinase-expressing rAds, AxCAFLP or AxCANCre, and target rAds, AxCAFNFG or AxCALNLG kept constantly at an MOI of 5, were co-infected into HeLa cells. Three days later, the fluorescence intensity of GFP was quantified by Fluoroskan Ascent FL (Labsystems). All data represent means ± SD of several regions of the wells measured.

To measure the expression level of the activated transgene, we first compared the fluorescence intensity of GFP infected with the recombinase-expressing rAds, AxCAFLP or AxCANCre, and the target rAds, AxCAFNFG or AxCALNLG, respectively (Fig. 5). Each recombinase-expressing rAd showed MOI-dependent expression of GFP, while no fluorescence was detected after infection of the target rAd alone. The fluorescence intensity of GFP of AxCAFLP infection at MOI of 5 almost corresponded to that of AxCANCre infection at MOI of 0.07, suggesting that the recombination efficiency of FLP in mammalian cultured cells was approximately one-seventieth of that of Cre. This result agreed well with that of the *in vitro* recombination assay compared at 37°C described above.

### Frequency of FLP-mediated gene activation in mammalian cultured cells

While confirming whether CAFNF works as a target unit of FLP by co-transfecting plasmids, pCAFNFZ and pxCAFLP, into 293 cells, we also compared the number of *LacZ*-expressing cells by co-transfecting pCALNLZ and pxCANCre

(9). The number of *LacZ*-expressed cells of FLP/FRT was no less than 25% of that obtained with the Cre/*loxP* system (data not shown), apparently different from when GFP-expressing rAd was infected into HeLa cells.

Since gene transfer mediated by rAd is rather uniform and more quantitative than transfection methods, we then co-infected AxCAFLP or AxCANCre with *LacZ*-expressing target rAds, AxCAFNFZ or AxCALNLZ, into HeLa cells and compared the *LacZ*-expression by X-gal staining (Fig. 6). When only each target Ad was infected, very few cells, if any, were stained blue (Fig. 6C and F). To obtain staining of 100% of HeLa cells, the FLP-expressing rAd, AxCAFLP, had to be infected at least at MOI of 5 (Fig. 6A) while an MOI of 0.2 of Cre-expressing rAd, AxCANCre was sufficient (Fig. 6E). Since AxCAFLP can be prepared as a purified high titer stock at >10$^{10}$ plaque forming units (p.f.u.)/ml, infection at MOI of 5 is readily obtained in cultured cells. Therefore, the FLP-expressing rAd could activate the transgene in nearly 100% of cultured mammalian cells.

### DISCUSSION

Here we demonstrated that the rAd expressing FLP worked efficiently in the gene-activation strategies in cultured mammalian cells. This conclusion is based on the fact that the activation of the *LacZ* gene was observed in nearly 100% of cultured cells by co-infection of the FLP-expressing rAd, AxCAFLP, and the target rAd, AxCAFNFZ.

We showed that the infection of the Cre-expressing rAd, AxCANCre, at an MOI of 0.2 gave transgene activation in almost 100% of cultured cells (Fig. 6E) (9). Since one p.f.u. generally corresponds to ~10 infectious viral particles (9), the results showed that the Cre-expressing rAd is so effective that only a few Cre-expressing DNA copies per cell are sufficient for successful gene activation. On the other hand, the FLP-expressing rAd required an MOI of 5 for gene activation in nearly 100% of cultured cells, suggesting that ~50 copies of FLP-expressing DNA per cell are required. Transduction of >50 copies per cell in 100% of cultured cells is easily achieved by adenovirus vector, but not necessarily by other methods of gene transfer. Therefore, the FLP-expressing rAd described here may be a useful tool for the gene regulation strategies by FLP. In this work, the target CAFNF unit was present extra-chromosomally on the adenovirus genome. We also observed that the FLP-expressing rAd was able to activate the CAFNF unit located on the cell chromosome after isolating *neo*-resistant cell clones in nearly 100% of cultured cells (Y.K., M.N. and I.S., unpublished data).

Although the efficiency of gene activation by FLP is much lower than by Cre, a number of groups successfully applied FLP-mediated gene regulation in cultured mammalian cells as long as the transfection method was used (44,45,59). We also observed that, in transient co-transfection experiments using target and recombinase-expressing plasmids, the number of conditionally *LacZ*-expressing cells mediated by FLP was as much as 25% of that mediated by Cre, and not more than 10-fold lower. This may be because, in transient transfection, most of the cells having received plasmids tend to accept many copies, >50 copies for instance, of plasmid DNA. However, this has meant that FLP-mediated gene activation has been successfully used in transfection experiments as long as gene

**Figure 6.** *LacZ* gene activation mediated by recombinase-expressing rAds. Denoted MOI of AxCAFLP (**A–C**) or AxCANCre (**D–F**) and target rAds, AxCAFNFZ or AxCALNLZ kept constant at an MOI of 5, were co-infected into HeLa cells. Three days later, cells were fixed and stained with X-gal. The results of MOI 5 (A and D), MOI 0.2 (B and E) and non-infected (C and F) of recombinase-expressing rAds are shown.

activation is sufficient for only a fraction of cultured cells. The approach using FLP-expressing rAd, therefore, will be especially valuable when gene activation is desirable in almost all cultured cells.

Buchholz *et al.* (50) demonstrated lower recombination efficiency by FLP above 35°C in mammalian cultured cells. Recently, a thermostabilized FLP, FLPe, which retains its activity even at 37°C, was developed (51) and was reported to work efficiently in mice (60). Our *in vitro* results, however, suggested that the temperature sensitivity of FLP may not be the major reason for the poor recombination efficiency, since the recombination efficiency of FLP at 37°C was only 3-fold different from that at 30°C, while the relative recombination efficiency of FLP expressed by infecting AxCAFLP to 293 cells was approximately one-thirtieth of Cre even at 30°C, the optimum for FLP. Other reasons, such as lower activity of the FLP enzyme itself or a lower expression rate of FLP in mammalian cells than Cre, may be major reason(s) for such low recombination efficiency of FLP. Ringrose *et al.* reported (61), based on their *in vitro* recombination assay, that ~10-fold more FLP was required than Cre to recombine a given quantity of substrate. Therefore, although the use of a thermostabilized FLP is undoubtedly important for achieving efficient recombination by FLP, a strategy of high-level expression of FLP in mammalian cells, for instance using an adenovirus vector with a potent CAG promoter, may be more effective, enabling the use of FLP for sequential gene regulation in combination with Cre.

Since an appropriate FLP antibody to detect the FLP protein has not been available, we could not quantify the amount of each recombinase contained in the lysate. However, the difference of the recombination efficiency between FLP and Cre observed in this study does not seem to be due to the difference of expression level of each recombinase-encoding mRNA, since we normally observed similar mRNA expression levels among several genes as long as expressed by the identical rAd under the control of CAG promoter (data not shown). Moreover, although the NLS sequence tagged to Cre improved the efficiency by ~3-fold (9), it does not seem to be the major

reason for the difference in apparent activity between FLP and Cre.

Recent advances in conditional gene activation or inactivation strategies mediated by Cre-expressing rAd have identified the functions of a number of key molecules in several signal transduction cascades (13–16,24,29,33). The strategy using Cre-expressing rAd has advantages such as stable regulated effects, very low background and a wide selection of promoters, including tissue-specific promoters, over other strategies (tetracycline-mediated regulation for example). However, it can only activate or inactivate a given gene once, i.e. 'off to on' or 'on to off'. To better understanding the contribution of such molecules to signal cascades, sequential gene regulation such as a novel 'off-on-off' or 'on-off-on' strategy mediated by FLP-expressing rAd in combination with Cre-expressing rAd may be a valuable tool, especially in time-course experiments, to obtain further functional information.

Cre-expressing rAd has facilitated extensive manipulations in transgenic mice (19–26,28,29,31). Since the recombination efficiency of FLP at 37°C was markedly lower than that of Cre, FLP is probably not appropriate for gene regulation strategies in mice. FLP was reported, however, to be useful for gene activation or for removal of the selectable marker in murine ES cells in transfection experiments (59,62). FLP- and Cre-expressing rAds may therefore have complementary functions through the generation of transgenic mice; FLP for the removal of the selectable marker in ES cells and Cre for the conditional gene regulation in mature transgenic mice. Especially, FLP-expressing rAd probably serves as an attractive tool for removing selectable markers.

Regulation of the expression of foreign genes introduced by a viral vector is necessary for both safety and efficacy of clinical gene therapy. For example, we have established a specific- and a high-level expression strategy using a tissue-specific promoter for controlled Cre production (27). Sequential gene regulation mediated by FLP- and Cre-expressing rAds will provide further sophisticated strategies for gene therapy. Whenever the therapeutic gene may cause side effects harmful to the patient, we could immediately remove the

expression unit, and when transgene expression was insufficient, further therapy could be continued with an another therapeutic gene using a secondary regulation system.

FLP-expressing rAd, AxCAFLP, and FRT-containing plasmids, pUFwF and pCAFNF5, will be available from the Riken DNA Bank (http://www.rtc.riken.go.jp).

## ACKNOWLEDGEMENTS

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