(FLPe) containing four amino acid substitutions; these mutations were first identified in a cycling mutagenesis experiment performed using *Escherichia coli*.[20] Due to the protein's thermostability, FLPe has been reported to work as effectively as Cre in somatic cell lines[21-23] and in mouse,[24-26] one contrary report excepted.[27] However, in embryonic stem (ES) cell culture FLPe has been shown to be less efficient than Cre,[23] and so a mouse codon-optimized version of FLPe (FLPo) has been synthesized for use in ES cells.[28]

Another way to improve the virtual efficiency of FLP in mammalian cells is to increase the gene copy number of the transduced recombinase in the cells. The adenovirus vector (AdV) is one of the most efficient tools for gene transduction and expression in mammalian cells. Furthermore, Cre-expressing AdV has been widely applied for the regulation of gene expression and has been used as a "gene switch."[29-33] Although wtFLP is normally inefficient, wtFLP can excise 100% of the targets in mammalian cell cultures when expressed on AdV using a very potent CAG promoter.[34,35] Furthermore, FLP-driven excision was observed in the chromosomes of ES cell clones in nearly 100% of cells transformed with AdV without difficulty;[36] this efficiency in ES cells is extremely higher than that achieved by plasmid electroporation using FLPe or

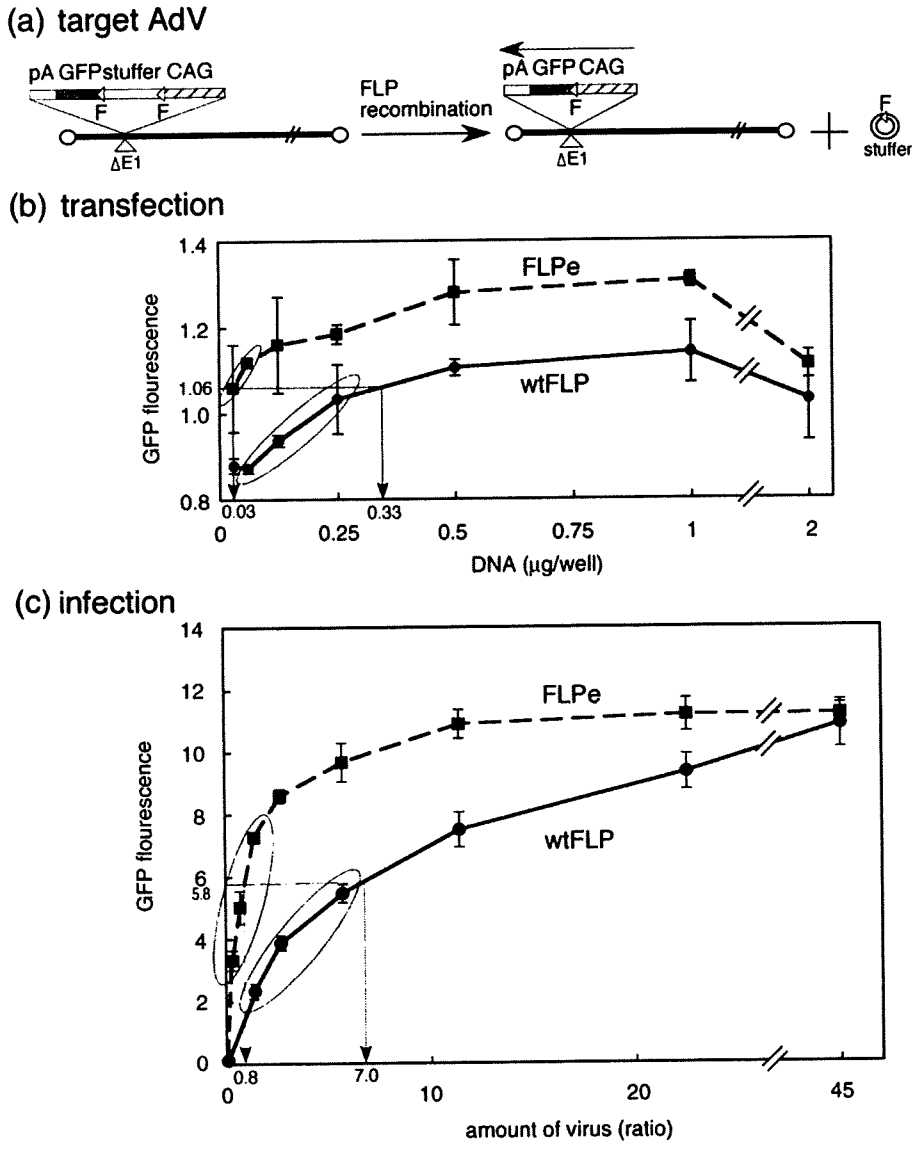

Fig. 1. Comparison of transduction between plasmid transfection and AdV infection. (a) Structure of the target AdV, AxCAFNFG. The stuffer consists of a neo gene and an SV40 pA sequence. CV-1 cells were infected with the target AdV AxCAFNFG. The wtFLP- or FLPe-expressing units needed to turn on GFP expression were transduced by the transfection of plasmids (b) or by infection with AdVs (c). pA, polyadenylation signal; F, FLP recognition site (FRT); E1, adenovirus E1 region. (b) Recombination efficiency using transfection. (c) Recombination efficiency using AdV. Each vertical line indicates 10× arbitrary units of GFP fluorescent intensity as measured using Fluoroskan AscentFL (Labsystems). The viral genome equivalent, indicated on the horizontal axis in (c) was defined using the amount of wtFLP genome at an MOI of 1 to represent a viral genome equivalent of 1 (see Materials and Methods). The areas defined as linear ranges are circled.

Cre. Using wtFLP-expressing AdV, Takashima *et al.* efficiently removed a gene encoding neomycin resistance from its chromosomal location in ES cells during generation of Cre-conditional knockout mice.[36] However, AdVs expressing wtFLP and FLPe have not been fully characterized.

Another important feature of AdV is that it enables a quantitative assay much superior to transfection-based assays, although no report has appeared to date showing such a comparison clearly. Using plasmids and AdVs, we precisely compared the enzymatic activities of wtFLP and FLPe in mammalian cells. We confirmed that FLPe showed higher recombination efficiency on a gene molar basis than did wtFLP with regard to both transfection and AdV expression. However, counter to expectations based on wtFLP- and FLPe-catalyzed recombination in *E. coli*,[20] FLPe was less efficient than wtFLP in catalyzing recombination in mammalian cells. To obtain more efficient translation in human cells, we generated an altered FLPe coding gene, "humanized" FLPe (hFLPe), in which the codon usage was optimized for use in humans. The efficacy of transiently expressed hFLPe in catalyzing recombination makes it highly suitable for transfection experiments.

Results

Recombination efficiency of wtFLP and FLPe using FLP-expressing plasmids and AdVs

To compare wtFLP and FLPe and measure their recombination efficiencies precisely, both transfection and AdV infection were used. CV-1 cells were infected with the target AdV AxCAFNFG (Fig. 1a), and plasmids expressing either wtFLP or FLPe were transfected. The target AdV possesses an FLP target composed of a CAG promoter, a stuffer sequence flanked by a pair of FRTs, the green fluorescent protein (GFP) gene, and a polyadenylation (pA) sequence, in that order (Fig. 1a). When recombination occurs, expression of the GFP reporter gene is turned on. Three days after transfection, the recombination efficiency was detected as GFP fluorescence. The total fluorescence intensity of FLPe-transduced cells was higher than that of wtFLP-transduced cells (Fig. 1b). To obtain a GFP fluorescence intensity of 1.06, at which fluorescent intensity scaled nearly linearly with input FLPe or wtFLP plasmid DNA, a total of 0.03 and 0.33 μg of the FLPe- and wtFLP-expressing plasmids were

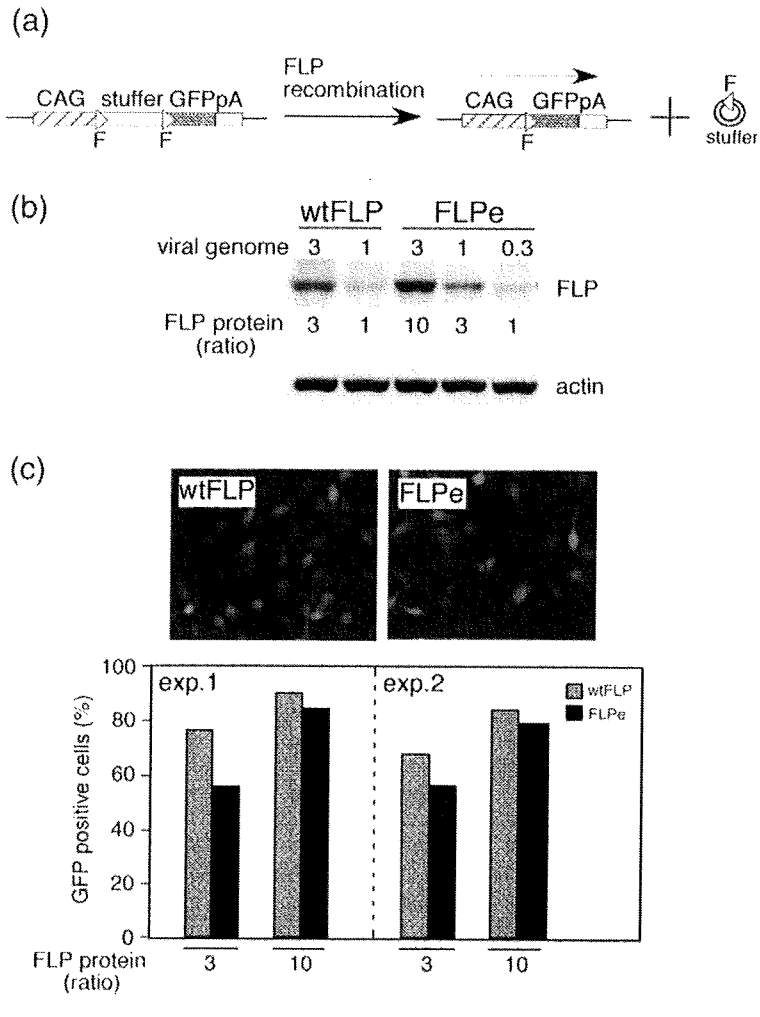

Fig. 2. Comparison between wtFLP- and FLPe-expressing AdVs in mammalian cells. (a) Structure of the chromosomally integrated target unit in CF cells. (b) Detection of FLP protein expressed in CF cells. Total protein was extracted from the infected cells, and FLP protein (top) and actin (bottom) were detected by Western blotting. At the bottom of each FLP band, the ratio of the amount of FLP protein is shown (see Materials and Methods). (c) Comparison of recombination efficiency. Top: fluorescent microscopic views of GFP-expressing cells 1 day after infection with the indicated FLP-expressing AdV. The cells were fixed 22 h after AdV infection, and the percentage of the GFP-expressing cells was measured using FACS (bottom, exp. 1, leftmost column). Exp. 1 and Exp. 2 were independent. 3 and 10 indicate the ratio of the amount of FLP protein when transduced as shown in (b). Because FACS counts very large number of cells, about 10,000 cells in each measurement, sampling effects were quite small.

required, respectively, showing that the recombination efficiency of FLPe was about 11-fold greater than that of wtFLP. Also, the linear response range upper cutoffs for FLPe and wtFLP input plasmid were approximately 0.1 and 0.25 μg, respectively, although the linear range was too narrow to compare precisely. These results were consistent with the previous *in vitro* results of Buchholz *et al.*[20] and *in vivo* results of Rodriguez *et al.*[26]

Next, wtFLP or FLPe was expressed through AdV infection instead of plasmid transfection (Fig. 1c). The recombination efficiency of FLPe was about nine times greater than that of wtFLP; that is, 0.8 and 7.0 viral genome equivalents of FLPe- and wtFLP-expressing AdVs, respectively, were required (estimated by Southern blot; see Materials and Methods) to obtain a GFP fluorescence of 5.8, where the amounts of both FLPe and wtFLP AdV vectors were within their linear ranges. The linear ranges of the FLPe- and wtFLP-expressing AdVs were less than 1.0 and 6.0 viral genome equivalents, respectively. These results show that both transfection and AdV infection provided similar quantitative measurements and that FLPe, on a gene molar basis, produced a fluorescent intensity that was about 10 times stronger than that produced by wtFLP. However, the AdV system gave much clearer results because the level of GFP expression after AdV infection was about 1 order of magnitude higher than that obtained using transfection, even when the maximum amount of FLPe-expressing plasmid was used. Furthermore, the linear response range for AdV infection was much wider than that for transfection; a comparison of Fig. 1b and c shows that transfection produced a linear response only for fluorescence readings between 0.85 and 1.15, while AdV achieved a linear response for readings between 1 and 8. Therefore, we concluded that AdV, a popular overexpression system, is also very useful for quantitatively analyzing precise measurements of enzymatic activity in mammalian cells. Therefore, the recombination efficiencies of wtFLP and FLPe were precisely compared with the use of the AdV system in all subsequent experiments.

Recombination efficiency of wtFLP and FLPe in an FRT-containing cell line

To compare the recombination efficiencies of the wtFLP and FLPe proteins on a molar basis in mammalian cells, wtFLP- and FLPe-expressing AdVs were infected into CF cells.[35] The CF cell line possesses a single copy of the FLP target unit integrated

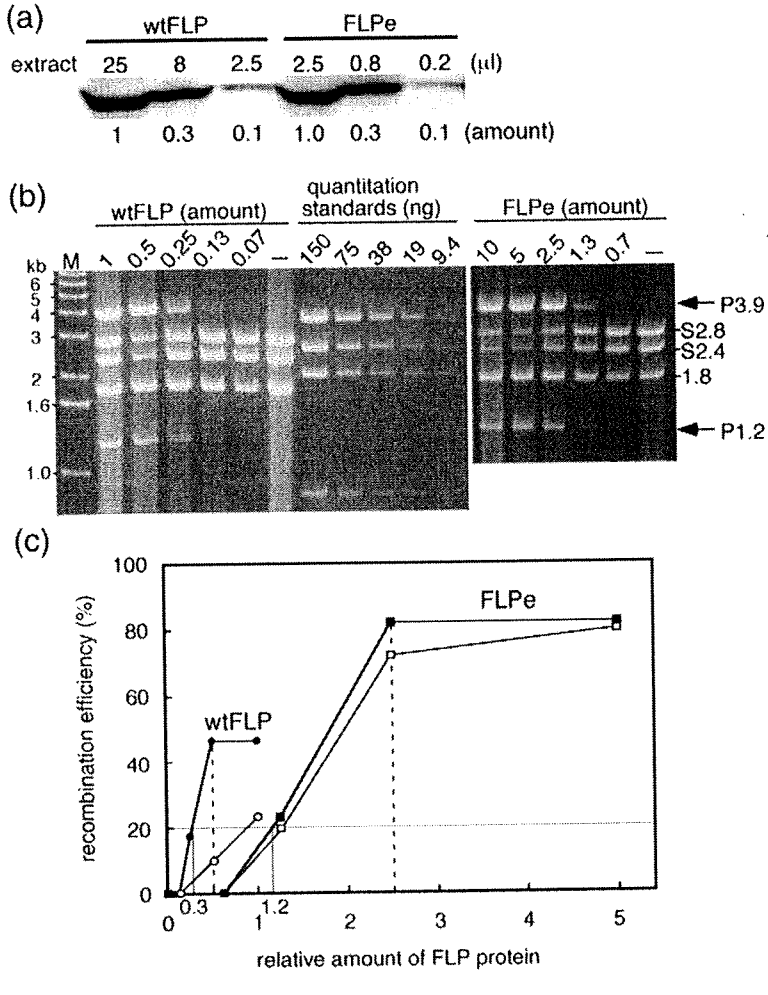

Fig. 3. *In vitro* recombination assay. (a) FLP protein contained in each extract was detected using Western blotting. The FLPe extract was diluted 10-fold with the reaction buffer. The amount of each lysate is shown above the band; the observed amounts of FLP protein are shown below the band. One aliquot unit was defined as the amount of FLP protein detected in the maximum reaction volume of wtFLP lysate (25 μL). (b) Quantification of *in vitro* recombination products. M, 1-kb Ladder marker (Gibco). The relative amounts of FLP protein are shown above the gel; the symbol '–' indicates the negative control. S, substrate; P, product. (c) Recombination efficiency of wtFLP (circle) and FLPe (square) in an *in vitro* assay at 30 °C (filled) and at 37 °C (open). The substrate DNA was reacted with wtFLP or FLPe *in vitro*, digested with FspI, and electrophoresed in an agarose gel. The original substrate DNA produced bands of 2.4 and 2.8 kb, while the recombined DNA generated bands of 3.9 and 1.2 kb.

in one of its chromosomes (Fig. 2a); GFP gene expression is turned on through the excision of the stuffer sequence mediated by FLP recombination. When the same amounts of FLPe- and wtFLP-expressing AdV were transduced into CF cells, the steady-state FLPe protein level was about threefold higher than that of wtFLP, as detected by Western blot analysis (Fig. 2b). This result is consistent with the thermostability of FLPe at 37 °C.

To examine the recombination efficiency of FLP, the GFP-expressing cells were observed with fluorescence microscopy (Fig. 2c, top panels). Notably, under conditions where the steady state amounts of FLPe and wtFLP enzymes were adjusted to identical intracellular levels, as determined by Western blot analysis, fewer GFP-expressing cells were observed in the visual field after FLPe transduction than after wtFLP transduction; this result was further confirmed quantitatively by fluorescence-activated cell sorter (FACS) analysis (Fig. 2c, bottom). The observation of fewer GFP-expressing cells after FLPe transduction than after wtFLP transduction was consistent in four pairwise comparisons over two independent experiments. One possible explanation for these results is that the recombination efficiency of the FLPe enzyme is less, not greater, on a molar basis than that of the wtFLP enzyme, with the activity of FLPe corresponding to about 70% of the activity of wtFLP in mammalian cells. This explanation also assumes that in a substantial percentage of cells, the expression of FLPe was insufficient to cause recombination even though the presence of FLPe protein was confirmed by Western blotting.

Recombination efficiency of FLP enzymes *in vitro*

Our result that the recombination efficiency of FLPe was lower on a protein molar basis than that of wtFLP in mammalian cells was unexpected because the opposite was believed to be true based on the results of a previous report using *E. coli*-produced FLPe and wtFLP *in vitro*.[20] Therefore, we decided to examine the recombination efficiencies of FLPe and wtFLP at their optimal temperature in an assay system using AdV-expressed FLPs. A comparative *in vitro* recombination experiment was performed at 30 °C using FLPe and wtFLP; recombination was performed with 1 μg of linearized pCAFNFG plasmid as a DNA substrate and the extracts of cells infected with AdVs expressing wtFLP or FLPe according to a previously described method.[34] Before assaying recombination, we measured the concentration of recombinase protein in the extracts by Western blotting, with the result that the FLPe extract contained a concentration of FLP protein that was about 10-fold higher than that of the wtFLP extract (Fig. 3a), presumably due to differences in thermostability. Therefore, the amounts of extract were normalized for FLP protein content by diluting the extract by an FLP reaction buffer. To measure recombination efficiency, the DNA products produced by recombination were quantified by comparing the corresponding bands with the intensity of a quantitation standard run on the same gel (Fig. 3b).

Lysate-dependent recombination was observed, reaching a plateau level of about 50% for wtFLP (Fig. 3c, filled circles). In contrast, recombination mediated by FLPe (filled squares) became detectable only after wtFLP-mediated recombination had reached its plateau value. Next, the recombination efficiencies were compared by titrating the amount of enzyme-normalized extract needed to achieve 20% recombination (Fig. 3c). The recombination activity of FLPe was only about one-fourth that of wtFLP (1.2 and 0.3 amounts corresponding to 1.2 and 0.3 μl of FLP protein-normalized extract, respectively, were required). These results showed that FLPe protein was considerably less efficient at recombination than the wtFLP protein when examined *in vitro* for enzymatic activity at their optimum temperature (30 °C). Note that wtFLP is thermolabile, while FLPe is thermostable; thus when the reaction starts with the same amount of each enzyme, the relative amount of wtFLP probably decreases over the course of the reaction. Therefore, if wtFLP is thermolabile even at 30 °C, the true recombination efficiency of wtFLP may be underestimated. Regardless, the activity of FLPe was lower than that of wtFLP at 30 °C. The recombination activity of FLPe was nearly the same at 30 and 37 °C (open squares), while the activity of wtFLP was extremely low at 37 °C (open circles). This result confirms the thermostability of the FLPe mutant and does not contradict the results described above.

Recombination efficiency of FLPe and hFLPe

To optimize the translational efficiency of the FLPe gene in mammalian cells, the FLPe gene was modified to coincide with mammalian codon usage. Because fully 19% of the FLPe coding sequence had to be altered, sixty-six 40-mer oligonucleotides were synthesized, annealed, and ligated to generate a humanized FLPe (hFLPe) gene (DNA Data Bank of Japan; AB434280). To measure the level of hFLPe gene expression in mammalian cells, FLPe- and hFLPe-expressing plasmids were transfected into the CF cell line and examined by Western blot analysis (Fig. 4). In fact, the amount of protein expressed by the hFLPe gene was about 10-fold greater than that expressed by the FLPe gene (Fig. 4a), suggesting that hFLPe was more efficiently translated in mammalian cells as a result of its optimized codon usage. Recombination efficiency was monitored as the fluorescent intensity of GFP expression. hFLPe was found to be more active; that is, the number of CF cells that became GFP-positive as a result of the hFLPe plasmid was about three times higher than the number that became GFP-positive as a result of the FLPe plasmid (Fig. 4b and c). The observation of only a threefold increase was probably attributable to the all-or-nothing nature of the assay; CF cells contain only a single copy of the recombination unit and cannot express GFP in a

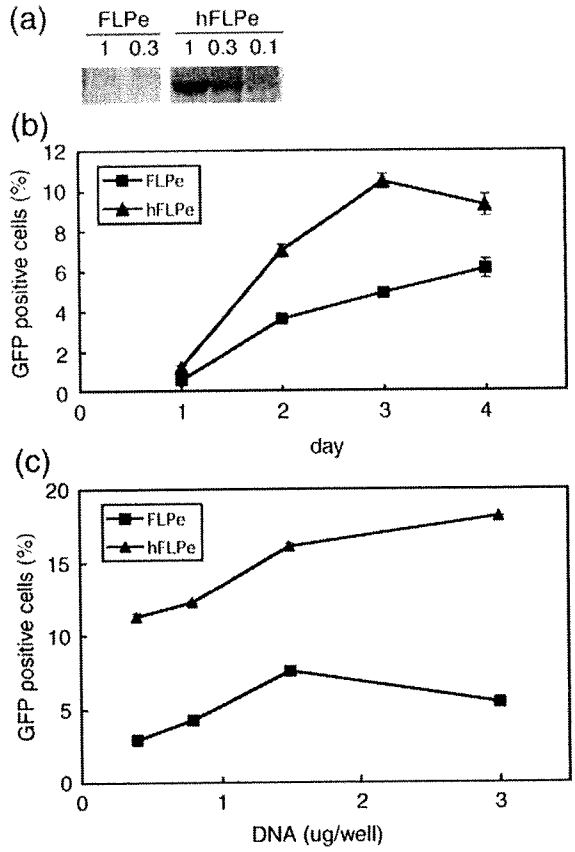

Fig. 4. Comparison of recombination efficiency between FLPe and hFLPe in mammalian cells. (a) Detection of FLP protein expressed in CF cells. Total protein was extracted from the transfected cells, and the FLP protein was detected by Western blotting. The relative amount of each cell extract is shown above the band. (b and c) Comparison of recombination efficiency. The cells were fixed after each plasmid was transfected, and the percentage of GFP-expressing cells was measured using FACS. Most error bars are difficult to see because the three measured values obtained by FACS were very similar.

dose-dependent manner. These results suggested that the hFLPe gene could successfully increase the translation of FLP in mammalian cells by about 10-fold compared with the FLPe gene. We also attempted to generate an hFLPe-expressing AdV to compare the expression levels of FLP and the recombination efficiency of FLPe and hFLPe further. However, only deleted viruses that did not express intact hFLPe were obtained in our attempts to construct an AdV vector expressing the hFLPe gene under the very potent CAG promoter.

Discussion

We observed that (i) an AdV system is useful for quantitative analysis involving precise activity measurements of enzymes expressed in mammalian cells, (ii) the recombination efficiency of FLPe is unexpectedly lower than that of wtFLP on a protein molar basis, (iii) hFLPe was generated, and in fact, hFLPe yielded substantially more recombined targets than did FLPe following transfection, and (iv) hFLPe-expressing AdV could not be generated, showing that it is difficult to generate an AdV construct expressing FLP showing high recombination efficiency; consequently, the FLPe-expressing AdV construct was the best AdV construct for FLP expression that could be produced in our study.

In general, the transfection of FLP-expressing plasmids is the most common method for comparing the recombination efficiencies of wtFLP and FLPe. Here, we showed that both plasmid transfection and AdV infection produced essentially identical results with regard to the characterization of wtFLP and FLPe recombination efficiencies; the recombination efficiency achieved by FLPe constructs was about 10-fold higher than that by wtFLP constructs. The second important result was that the values obtained by AdV constructs were several times higher than those obtained using transfection, and that AdV infection enables a more quantitative analysis to be performed. Furthermore, once FLP-expressing AdVs were prepared, AdV-mediated expression produced a linear range above the GFP background level that was severalfold greater than that produced by transfection, thus enabling more quantitative data to be obtained. Therefore, the AdV system appeared to be more suitable for characterizing enzymatic activity in mammalian cells. In the present study, the AdV system also compensated for the low activity of FLP and showed the differences in FLP recombination efficiencies more clearly. Therefore, in the present study at least, the results obtained using the AdV method were more reliable and more quantitative than those obtained using the transfection method. In general, the AdV method should enable one to obtain the superior quantitative data due to the above differences.

Here, we showed that on a protein molar basis, FLPe displayed lower recombination efficiency than did wtFLP at both 37 °C and its optimal temperature of 30 °C in an *in vitro* analysis (Figs. 2 and 3). Not only is the former observation novel, but the latter observation also apparently contradicts the results obtained by expression in *E. coli*,[20] where the purified FLPe protein catalyzed recombination more efficiently than did the purified wtFLP protein. In the previous study, the authors acknowledged the possibility that their results may have been influenced by their purification method, since sufficient active FLP enzyme was more difficult to obtain for wtFLP than for FLPe.[20] Because we were able to prepare a large amount of FLP enzyme from 293 cell extract using replicating AdV, we were able to avoid difficulties in the purification of wtFLP and were able to measure its recombination efficiency directly. In fact, it may be more reasonable to suggest that the four amino acid mutations in FLPe may have reduced recombination efficiency of the enzyme, compared with that of authentic wtFLP.

The observation that the recombination efficiency of FLPe on a protein molar basis was lower than

that of wtFLP at 30 °C was also true at 37 °C in mammalian cells (Fig. 2c). This finding was somewhat surprising, since the efficiency of FLPe has been demonstrated in mammalian cells,[21–23] in animals,[24–26] and in the present study (Fig. 1b and c). To explain this apparent contradiction, we hypothesize that the thermostability of FLPe outweighs the impact of the reduction in recombination efficiency of FLPe per protein. Fig. 1c clearly shows that FLPe-expressing AdV gave rise to more recombination than wtFLP-expressing AdV, although wtFLP-AdV has been previously applied to mammalian cells and has been shown to function efficiently in regulating transgene expression and catalyzing intentional chromosome rearrangement.[34–36] Because the apparent higher recombinant efficiency of FLPe was due solely to its higher steady-state levels resulting from its thermostability at 37 °C, we tried to enhance expressed levels of FLPe in mammals by generating hFLPe, in which the codon usage was optimized for expression in mammals. Approximately 19% of the FLPe coding nucleotides were substituted to produce the hFLPe gene. The codons most frequently used in humans were selected in all instances for the design of the hFLPe gene. No less than 14.4% of the gene sequence differed from that of FLPo,[28] another previously reported codon-optimized variant of FLPe. We were able to show that the expression of hFLPe increased by about 10-fold and that the hFLPe-expressing plasmid yielded about 2.3-fold more recombinants than did the FLPe-expressing plasmid in a transfection experiment. The lack of a greater increase seems to be due to the all-or-none nature of the assay in which the transfected CF cell line contained the FLP target unit present only in a single copy number. On account of its high expression, the hFLPe-expressing plasmid took only 2 days to yield the same extent of recombination attained by the FLPe-expressing plasmid 4 days after transfection (Fig. 4b). This quick, high-yield recombination obtained with hFLPe should be advantageous for various experiments, especially for application to ES cells, although no hFLPe-expressing AdV could be generated.

In fact, a limited deleterious effect was observed when wtFLP was expressed with the use of AdV in 293 cells during titer determination. When titration was performed with an end-point cytopathic effect assay in 293 cells,[37] cell death occurred much earlier than usual, and as a result, the wtFLP AdV acted as if its titer were about threefold higher than its actual value, as determined using Southern blot analysis to detect the transduced virus genome in CV-1 cells, for example (data not shown). This observation was specific for AdV expressing wtFLP and was rarely seen for AdV expressing-FLPe or Cre. Therefore, when we compared FLPe with wtFLP using AdV in the present study, Southern-blot-determined transduced viral genome equivalents were used instead of multiplicity of infection (MOI). We think that the wtFLP-expressing AdV may have had a very mild deleterious effect that was manifested only when the viral genome was amplified by more than 10,000 copies per cell in 293 cells during wtFLP-expressing AdV replication. Probably, this deleterious effect is so subtle that it is not discernible in typical plasmid transfection experiments or, more importantly, in the present study using FLPe, which shows lower recombination efficiency on a protein molar basis; nevertheless, such a deleterious effect magnified by the greater protein expression of hFLPe offers one potential explanation for the instability of hFLPe-expressing AdV. Therefore, if the subtle deleterious effect in 293 cells is correlated with the high molar recombination efficiency of wtFLP enzyme, the above observations could be consistently explained.

Our results showed that when one attempts to generate an effective FLP AdV, it is probably impossible to obtain a better FLP AdV than FLPe AdV in mammalian cells because the FLPe AdV had no perceptible deleterious effect and showed the best recombination efficiency practically achievable in mammalian cells. To obtain the same recombination efficiency in cells, FLPe AdV required a smaller viral dose than wtFLP AdV. The advantage of FLPe AdV in mammalian cells is that the recombination efficiency of FLPe is maintained at 37 °C not only due to its thermostability, but also owing to minimal deleterious effects on 293 cells during preparation, despite its lower recombination efficiency per enzyme molecule. wtFLP-expressing AdV was a less ideal choice in mammalian cells not only due to instability of wtFLP protein at 37 °C, but also due to deleterious effect of the construct in preparing AdV; finally, an hFLPe-expressing AdV could not be generated because the FLPe protein expression was too high.

Although an AdV expressing hFLPe under the control of the very potent CAG promoter could not be generated, hFLPe might be useful if it were to be expressed in an AdV containing a weaker promoter, such as a tissue-specific promoter for tissue-specific expression. Moreover, we showed here that the translational efficiency of the hFLPe-expressing plasmid was about 10-fold greater than that of an FLPe-expressing plasmid after transfection. Thus, we strongly recommend the use of hFLPe in situations where AdV infection is not suitable, such as in transfection experiments or studies using transgenic mice. A plasmid, pxCAhFLPe, expressing hFLPe gene under control of the CAG promoter, is available from RIKEN DNA bank†.

Materials and Methods

Cell lines and AdVs

Human embryo kidney 293 cell line[38] and monkey kidney cell line (CV-1) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal

† http://www.brc.riken.go.jp/lab/dna/ja/

calf serum. The CF cell line[35] derived from HeLa cells was cultured in the same culture medium with 10% fetal calf serum plus geneticin (0.75 mg/mL). After the cells had been infected with a recombinase-expressing AdV, they were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum without geneticin. The wtFLP-expressing AdV (AxCAFLP) and the target AdV (AxCAFNFG) were described previously.[34] The FLPe-expressing AdV (AxCAFLPe) was generated with the intact-genome transfection method.[39] Briefly, the FLPe recombinase gene was derived from pBSSK-FLPe, which was prepared from pCAGGS-FLPe (Gene Bridges). The FLPe gene used here contained an initiation codon that complies with Kozak's rule[40] and lacked a nuclear localization signal. The genomes of AxCAFLP and AxCAFLPe were identical, except for the FLP gene. The wtFLP and the FLPe recombinases were expressed under the control of a CAG promoter,[41] consisting of the cytomegalovirus IE enhancer, chicken β-actin promoter, and rabbit β-globin poly(A) signal. AxCAFNFG contained a CAFNFG unit, which was composed of a CAG promoter,[41] a first FRT sequence, a stuffer, another FRT sequence, the GFP gene, and GpA, in that order.

Quantitation of AdVs

The amount of each viral genome transduced to the cells was measured using a Southern blot analysis. When wtFLP-expressing AdV was used: the MOI did not correspond to the copy number: to obtain the same amount of genome, the wtFLP-expressing AdV required an MOI that was three times higher than that of any other AdV, including the FLPe-expressing virus. Thus, in this study, the expression of FLP and the recombination efficiency of each AdV were described not with MOIs but the amount of transduced viral genomes. In this study, a transduced viral genome equivalent of 1 corresponds to an MOI of 1 of wtFLP-expressing AdV and MOI of 0.3 of FLPe-expressing AdV and AxCAFNFG.

Complete synthesis of hFLPe Gene

To obtain an FLPe codon usage suitable for human cells,[42] 19% of the FLPe gene was substituted to produce the hFLPe gene. To generate the hFLPe gene, sixty-six 40-mer oligonucleotides were synthesized in a manner such that each oligonucleotide overlapped with two antisense oligonucleotides for a length of 20 nucleotides. These oligonucleotides were phosphorylated with T4 polynucleotide kinase, annealed with each other, and ligated. The hFLPe gene was synthesized in three parts, and the sequence was verified using nucleotide sequencing. The 1.3-kb full-length hFLPe sequence was then constructed by ligating the three fragments in correct order and orientation and cloning in pBluescript II SK(-) (Stratagene), yielding pBSSK-hFLPe. The hFLPe sequence is available at DNA Data Bank of Japan (AB434280).

FLP-expressing plasmids

pxCAwtFLP, pxCAFLPe, and pxCAhFLPe are pBR327-derived vectors containing wtFLP-, FLPe-, and hFLPe-expressing units, respectively, under the control of the CAG promoter. Each recombinase gene has an initiation codon that complies with Kozak's rule.[40] Three days after each plasmid was transfected into CV-1 cells (which had been infected with the target AdV, AxCAFNFG, 1 day before transfection) using Transfast (Promega), the culture medium was changed to Hanks' balanced salt solution, and the GFP fluorescent intensity was measured using Fluoroskan AscentFL (Labsystems). Each day after each plasmid had been transfected into the CF cells, the cells were washed twice with phosphate-buffered saline (PBS) (−). Then, the cells were collected, and the total DNA of a portion of the cell population was extracted; real-time PCR was performed to confirm that a similar amount of DNA had been transfected in the FLPe and hFLPe experiments (data not shown). The remaining portion of transfected cells was used for the detection of FLP protein and to count the number of GFP-positive cells by FACS analysis.

In vitro recombination assay

The recombinase-containing lysates were prepared as described previously.[34] In total, 2×10^7 of the 293 cells were infected with AxCAFLP and AxCAFLPe at an MOI of 75 and 5, respectively (an MOI of 1 corresponds to about 13 and 53 virus particles per cell, respectively). Twenty-two hours later, the cells were harvested by scraping and collected by centrifugation at 1000 rpm for 5 min at 4 °C using an Avanti HP-25I (Beckman-Coulter). The cell pellet was washed twice with PBS(−) and resuspended in 1 mL of FLP storage buffer [10% glycerol, 20 mM Tris–HCl (pH 7.5), 300 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), and 0.02 mM phenylmethylsulfonyl fluoride]. The cell suspension was then sonicated for 3 min (six cycles, 30 s each) using a Bioruptor II (CosmoBio; Tokyo, Japan) at maximum power (200 W) and was then immediately centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was stored at −80 °C.

The *in vitro* recombination assay in this study was performed as described previously.[34] Briefly, the reaction was started by mixing HindIII-linearized pCAFNFG with each lysate (2.5–25 μL) and then incubating for 30 min at 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 FspI and the DNA digests were detected using 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 the densities of the quantification-standard DNA digests run on the same gel as described previously.[34] To compare the recombination efficiency of FLP and FLPe per FLP protein molecule, the FLPe lysate was diluted 10-fold with the FLP reaction buffer.

Detection and quantitative determination of GFP gene expression

A fluorescence microscope (IX70, Olympus) was used to observe GFP expression. To determine the percentage of GFP-expressing cells among the transfected cells and the infected cells, a flow cytometry analysis was performed. One day after infection, one-third of the cells in a 10-cm dish were fixed using 4% paraformaldehyde for 15 min at room temperature. The cells were washed twice with PBS (−), and their fluorescence emission was measured using a band-pass filter at 525 nm for GFP in a FACSCalibur with Cell Quest software (Becton Dickinson). A gate was set on a homogeneous cell population, as determined by the scatter characteristics, and 10,000 events were monitored.

The marker gates were set to calculate the percentage and the mean fluorescence intensity of the positive cells.

Polyclonal antibodies

FLP peptide was synthesized using a cysteine residue followed by the amino acid residues of RYPAWNGIIS-QEVLDYLSSYINRRI (R399–I423), including helices P′ and Q (underlined) at the C-terminal domain of the FLP protein.[43] Before immunization, the C terminus of the peptide was covalently conjugated with keyhole limpet hemocyanin (KLH) to increase its antigenicity. Polyclonal antisera against the peptide were raised in rabbits using the standard immunization protocol at Yanaihara Institute Inc. (Shizuoka, Japan). Briefly, two Japanese white rabbits (body weight, 2.5 kg) were subcutaneously immunized with 1.3 mg (for the initial immunization) and 0.65 mg (for the booster immunizations) of the peptide emulsified with an equal volume of Freund's complete adjuvant. The immunization was carried out five times at 2-week intervals. Ten days after the last immunization, blood was collected, allowed to coagulate at 37 °C for 2 h, and stored at 4 °C overnight. The sera were then recovered in the supernatant after centrifugation. Finally, the sera were affinity-purified using a HITrap NHS-activated column with an immobilized FLP peptide.

Western blotting

One day and 3 days after infection, one-third of a 10-cm dish of CF cells and six-well plate of 293 cells, respectively, were harvested and the total protein was extracted using NP-40 lysis buffer [50 mM Tris–HCl (pH 8.0), 0.15 M NaCl, 5 mM EDTA, 1% NP-40]. The lysates were mixed well in a rotator for 2 h at 4 °C, then centrifuged at 15,000 rpm for 5 min at 4 °C, and the supernatants were collected. Detection of the FLP protein in the lysate was performed for the *in vitro* assay using the lysate as it was. Western blotting was performed as described previously.[18] The membrane was incubated for 2 h at room temperature in the presence of an anti-FLP peptide rabbit polyclonal antibody diluted to 0.3 μg/mL with PBS–Tween, followed by incubation with biotin-conjugated goat anti-rabbit IgG (Bio Source) diluted to $1/10^5$ with PBS–Tween for 1 h at 4 °C. The membrane was then incubated for 1 h at 4 °C in the presence of horseradish-peroxidase-conjugated streptavidin (Bio Source) diluted to 1/70,000 with PBS–Tween. To reduce nonspecific binding, the antisera were incubated at 4 °C overnight with a membrane transferred with the lysate of CV-1 cells infected with the control virus AxCA1w1. The recovered supernatant was used as the source of the anti-FLP polyclonal antibodies. An anti-actin mouse monoclonal antibody (Neomarkers) diluted to $1/10^4$ with PBS–Tween was also detected on the same membrane to show equal loading.

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