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# Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination

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## ABSTRACT

**Genomic manipulations using site-specific recombinases rely on their applied characteristics in living systems. To understand their applied properties so that they can be optimally deployed, we compared the recombinases FLP and Cre in two assays. In both *Escherichia coli* and *in vitro*, FLP shows a different temperature optimum than Cre. FLP is more thermolabile, having an optimum near 30°C and little detectable activity above 39°C. Cre is optimally efficient at 37°C and above. Consistent with FLP thermolability, recombination in a mammalian cell line mediated by a ligand- regulated FLP–androgen receptor fusion protein is more efficient at 35°C than at higher temperatures. We also document a mutation in a commercially available FLP plasmid (FLP-F70L) which renders this recombinase even more thermolabile. The different temperature optima of FLP, FLP-F70L and Cre influence their strategies of usage. Our results recommend the use of Cre for applications in mice that require efficient recombination. The thermolabilities of FLP and FLP-F70L can be usefully exploited for gain of function and cell culture applications.**

## INTRODUCTION

Stable integration of DNA into living genomes is central to organism engineering and experimental biology. Random, viral and homologous integration provide the means to stably introduce DNA. Further manipulations of the introduced DNA are possible when targets for site-specific recombinases are included in the integration strategy (1–3). Currently, three site-specific recombinases have been used in this regard: FLP from the yeast *Saccharomyces cerevisiae*, Cre from the bacteriophage P1, and the R recombinase from the yeast *Zygosacchromyces rouxii* (reviewed in 4). These enzymes are all members of the integrase class of site-specific recombinases and show the desirable properties of (i) conservative recombination, (ii) 34 bp recognition targets that are short enough for convenience but long enough for specificity, and (iii) all functions necessary for recombination are contained within each of the single polypeptides (reviewed in 5).

The utility of FLP and Cre rests on their ability to mediate recombination in living systems. They have been used in a broad range of organisms including bacteria (6), plants (7), flies (8) and mammals (9). A variety of applications are tenable, ranging from quantitative ablations to rare event inductions. A substantial amount of work with both recombinases has focused on the steps that are involved in the recombination reaction (reviewed in 10). However, little has been done to analyse the applied properties of these recombinases in living systems (6,11–13). We are investigating FLP and Cre with respect to their applied usage as tools in genome engineering. We report that FLP recombinase is thermolabile in *Escherichia coli* as well as *in vitro* and in a mammalian cell line. These findings are consistent with earlier observations of the instability of FLP during protein purification (14) and the instability of FLP DNA binding activity at high temperatures (15). Since the growth temperature of the host cell imposes an important condition on the recombinase employed, our results present a rationale for recombinase deployment in genomic manipulation strategies.

## MATERIALS AND METHODS

### DNA techniques

Small scale plasmid DNA preparation was performed using the Wizard™ Miniprep System (Promega). Large scale preparation of plasmid DNA was done with the Qiagen plasmid kit (Qiagen). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. PCR reactions were performed using *Taq* polymerase from Perkin Elmer.

### Plasmids

The low copy plasmid 1921-cIFLP was constructed by cloning a *Pst*I–*Xba*I fragment of plasmid pMJ1 (16) containing FLP under the control of the temperature-sensitive λ-repressor cI857 into the vector pCL1921 (17). The Cre gene was PCR amplified from plasmid pcltsCRE (Invitrogen) and cloned into 1921-cIFLP replacing the FLP-gene with the Cre coding region.

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pSVpaZ11 and pSVpaX1 are based on plasmid pBluescript II SK+ (Stratagene) and are identical except for the two recombinase recognition targets (FRT-sites for pSVpaZ11 and *loxP*-sites for pSVpaX1) which are separated by 1.1 kb. Further details of these plasmids are available on request. pSVpaZ11 and pSVpaX1 have been deposited in the EMBL/GenBank/DDBJ databases with the accession numbers Y07573 and Y07631, respectively.

### *Escherichia coli* strains

294-Cre and 294-FLP are described in (18).
294-Cre: *F*$^-$, λ$^-$, *supE44, endA1, thi-1, hsdR17, lacZ::cI857-Cre.*
294-FLP: *F*$^-$, λ$^-$, *supE44, endA1, thi-1, hsdR17, lacZ::cI857-FLP.*

### Western analysis

FLP antibodies were raised in rabbits against the peptide N-terminus-VGNWSDKRASAVART-C-terminus. Western blot analysis was performed using the alkaline phosphatase conjugate obtained from Sigma and NBT and BCIP from Promega.

### Cell culture

Stable transfection of human cells and Southern analysis were performed as previously described (12), except that stable colonies were selected with 1 μg/ml puromycin (Sigma). Cells were induced with $10^{-7}$ M mibolerone (New England Nuclear).

### Temperature ramp

A temperature gradient incubator was constructed similar to the one described by Landman *et al.* (19). The incubator was made of a sheet of aluminium (89 × 32 × 1 cm). One end was heated by thermofoil heaters (MINCO) regulated by an AC-voltage regulator (FG-Elektronik). Heat dissipates along the length of the metal producing a temperature gradient. The surface temperature of the agar was measured with a GTH 1200 A digital thermometer (Greisinger Electronic).

### *In vitro* recombination assay

Recombination reactions were carried out on excision substrates, consisting of a $^{32}$P-end-filled *Bsu*36I–*Not*I 4.0 kb fragment of pSVpaz11 (FLP-reporter) or pSVpax1 (Cre-reporter). Recombination generates a 2.9 kb labelled linear product and a 1.1 kb unlabelled circle. FLP or Cre proteins were translated *in vitro* in rabbit reticulocyte lysate (Promega) and expression levels were determined by [$^{35}$S]methionine incorporation. The reaction buffer contained 2 mM $MgCl_2$, 70 mM NaCl, 50 mM Tris–HCl pH 7.5 and 0.1 mg/ml BSA. For time course experiments, reactions were carried out in 100 μl total volume. Reaction mixtures were pre-incubated at various temperatures, and reactions were started by the addition of protein. Aliquots of 10 μl were removed at various time points and reactions were terminated by addition of SDS to 0.05%, EDTA to 10 mM and protease K to 0.5 mg/ml, followed by incubation at 37°C for 15 min. Reactions were then phenol extracted and the aqueous phase was loaded onto a 0.6% agarose gel. Recombination products were visualised by autoradiography and quantified by PhosphorImager analysis.

## RESULTS

### FLP recombinase is thermolabile in *E.coli*

To compare the properties of FLP and Cre recombinases in *E.coli*, equivalent expression and recombination reporter vectors were constructed. The expression vectors (Fig. 1C) were based on the low copy plasmid pCL1921 (17), modified so that FLP or Cre expression could be regulated by the cI857 temperature-sensitive λ-repressor. The recombination reporters, pSVpaZ11 and pSVpaX1 (Fig. 1A), were based on pBluescript (Stratagene) and differ only in the presence of either two 34 bp FLP recombination targets (FRTs; pSVpaZ11) or two 34 bp Cre recombination targets (loxPs; pSVpaX1). These plasmids allow easy detection of recombination by monitoring lacZ activity. Before recombination, pSVpaZ11 and pSVpaX1 express the lacZ gene, thus presenting blue *E.coli* colonies when cultured on plates containing X-Gal. Recombination between the FRTs or loxPs excises the lacZ gene promoter, thus ablating lacZ expression. White colonies carrying pSVpa plasmids grown on X-Gal medium therefore indicate complete excision recombination.

We transformed pSVpaZ11 or pSVpaX1 into the lacZ$^-$ strain XL1-Blue (Stratagene) harbouring either 1921-cIFLP or 1921-cICre and plated the cells immediately onto a temperature gradient incubator set to generate a gradient from 28 to 46°C. The culture medium contained X-Gal and antibiotic selection for both expression and reporter plasmids. Blue colonies could be seen over the whole temperature range when either the FLP expression plasmid and Cre reporter (Fig. 1B; lane FLP + pSVpaX1) or Cre expression plasmid and FLP reporter (lane Cre + pSVpaZ11) were combined. Thus β-galactosidase expression and activity were not substantially affected by the temperature gradient and neither recombinase appeared to recognize the substrate for the other. As expected, combining either the FLP expression vector and FLP-reporter (lane FLP + pSVpaZ11) or the Cre expression vector and Cre reporter (lane Cre + pSVpaX1) produced white colonies, indicative of complete recombination. However temperature limits to apparent recombination were seen. Below 34°C, there was some apparent Cre activity, but no apparent FLP activity. This may reflect differences in basal expression levels, protein stabilities or the possibility that Cre is active at much lower levels of expressed protein in *E.coli*. Between 34 and 39°C, both recombinases appeared to be fully active, however, above 39°C FLP appeared to be inactive, possibly reflecting thermolability. Cre was active up to 46°C. An equivalent effect of high temperature on FLP activity, and no effect on Cre activity, was observed when FLP and Cre were expressed using an arabinose inducible expression system (20) (data not shown).

To verify that the colony colour assay faithfully reflected recombination, 294-FLP and 294-Cre strains (18) were transformed with pSVpaZ11 or pSVpaX1 and cultured at 30, 37 or 42°C. These two strains contain stably integrated forms of the cI857 regulated FLP or Cre genes and thus, on plasmid extraction, only the recombination reporter plasmids are recovered. 294-FLP and 294-Cre transformed with pSVpaX1 or pSVpaZ11 showed similar temperature profiles as shown for the double plasmid experiment of Figure 1B (data not shown). pSVpa plasmids were extracted from 294-FLP or 294-Cre grown at 30, 37 or 42°C and linearised by *Not*I restriction digestion (Fig. 1D). Consistent with the blue–white temperature gradient assay, pSVpaZ11 was fully recombined only in 294-FLP cells cultured at 37°C (lane 2). Culture at 30 or 42°C resulted in very low levels of pSVpaZ11

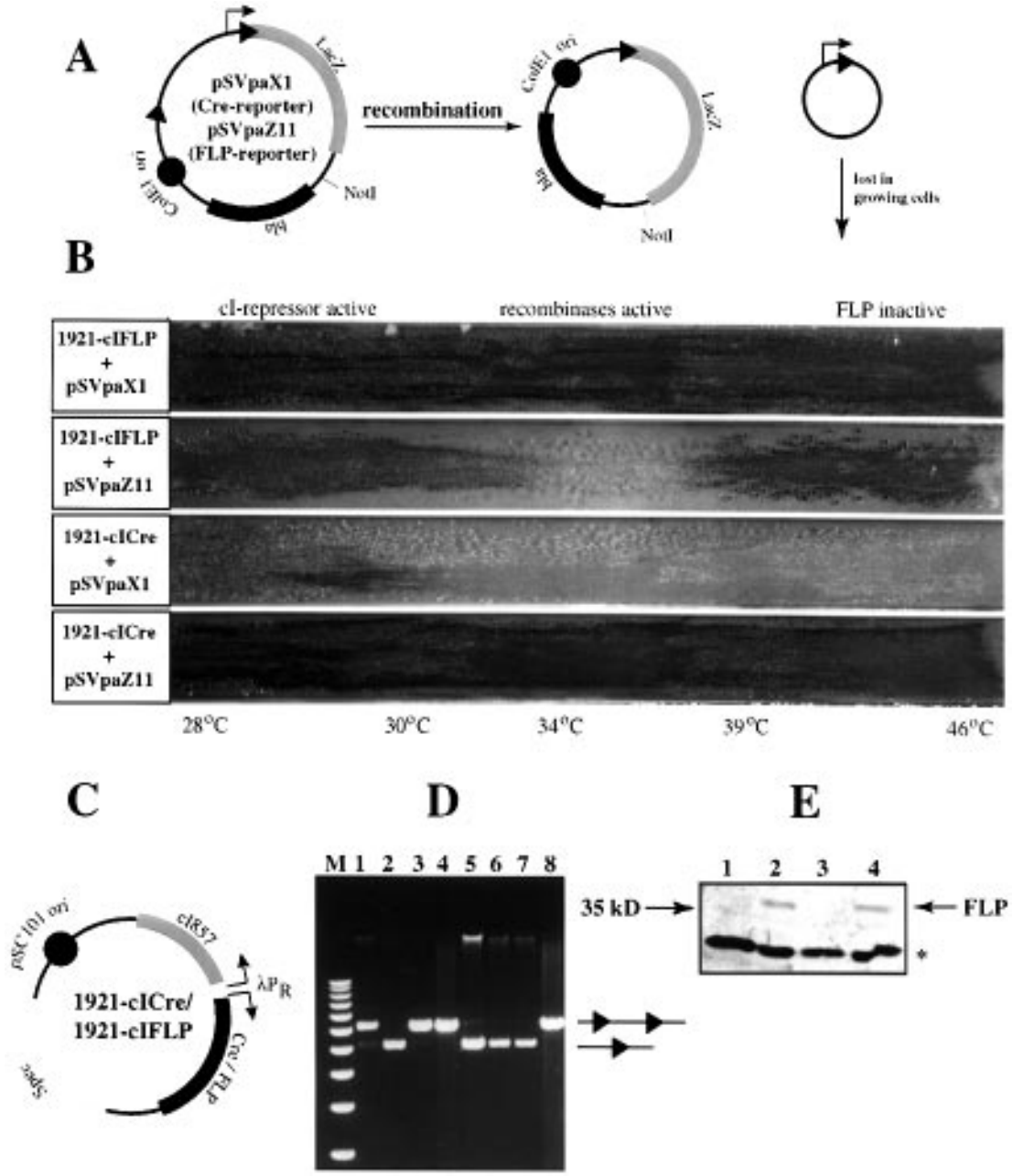


**Figure 1.** Temperature effect on Cre- or FLP-mediated recombination in *E.coli.* (**A**) Recombinase reporter plasmids pSVpaX1/pSVpaZ11. Recombination excises 1.1 kb including the *E.coli* promoter (arrow) of the LacZ gene, thus giving a white phenotype on X-Gal medium. The excised circle does not carry an origin of replication and therefore is lost in replicating cells. The coding regions of the LacZ gene and the β-lactamase gene are indicated as thick lines. Filled triangles depict *loxP*- (pSVpaX1) or FRT- (pSVpaZ11) sites, respectively. The ColE1 origin of replication is shown as a filled circle. The restriction site for*Not*I is indicated. (**B**) XL1-Blue cells harbouring either the low copy plasmid 1921-cICre or 1921-cIFLP transformed with either pSVpaX1 or pSVpaZ11, as indicated. The cells were plated on a temperature ramp producing a temperature gradient from 28 to 46°C with measured temperatures noted below. (**C**) Expression plasmids 1921-cICre/1921-cIFLP. The coding regions for Cre-/FLP-recombinase, the cI857 λ-repressor and the spectinomycin resistance gene are indicated as thick lines. The pSC101 origin of replication is depicted as a filled circle. The $\lambda P_R$-promoter driving the recombinase and the cI857 repressor is indicated as arrows. (**D**) *Not*I digestions of pSVpaX1 or pSVpaZ11 plasmids isolated out of strain 294-Cre or 294-FLP after culture at different temperatures. Lanes: M, 1 kb ladder (BRL); 1, pSVpaZ11 grown in 294-FLP at 30°C; 2, pSVpaZ11 grown in 294-FLP at 37°C; 3, pSVpaZ11 grown in 294-FLP at 42°C; 4, pSVpaX1 grown in 294-FLP at 37°C; 5, pSVpaX1 grown in 294-Cre at 30°C; 6, pSVpaX1 grown in 294-Cre at 37°C; 7, pSVpaX1 grown in 294-Cre at 42°C; 8, pSVpaZ11 grown in 294-Cre at 37°C. (**E**) Western blot using an FLP antibody on protein extracts from cells grown at different temperatures. The asterix marks a background band that is picked up in all*E.coli* extracts. FLP protein and the 35 kDa molecular weight marker are indicated. Lane 1, strain MM294 grown at 37°C; lane 2, strain 294-FLP grown at 37°C; lane 3, strain 294-FLP grown at 25°C; lane 4, strain 294-FLP grown at 42°C.

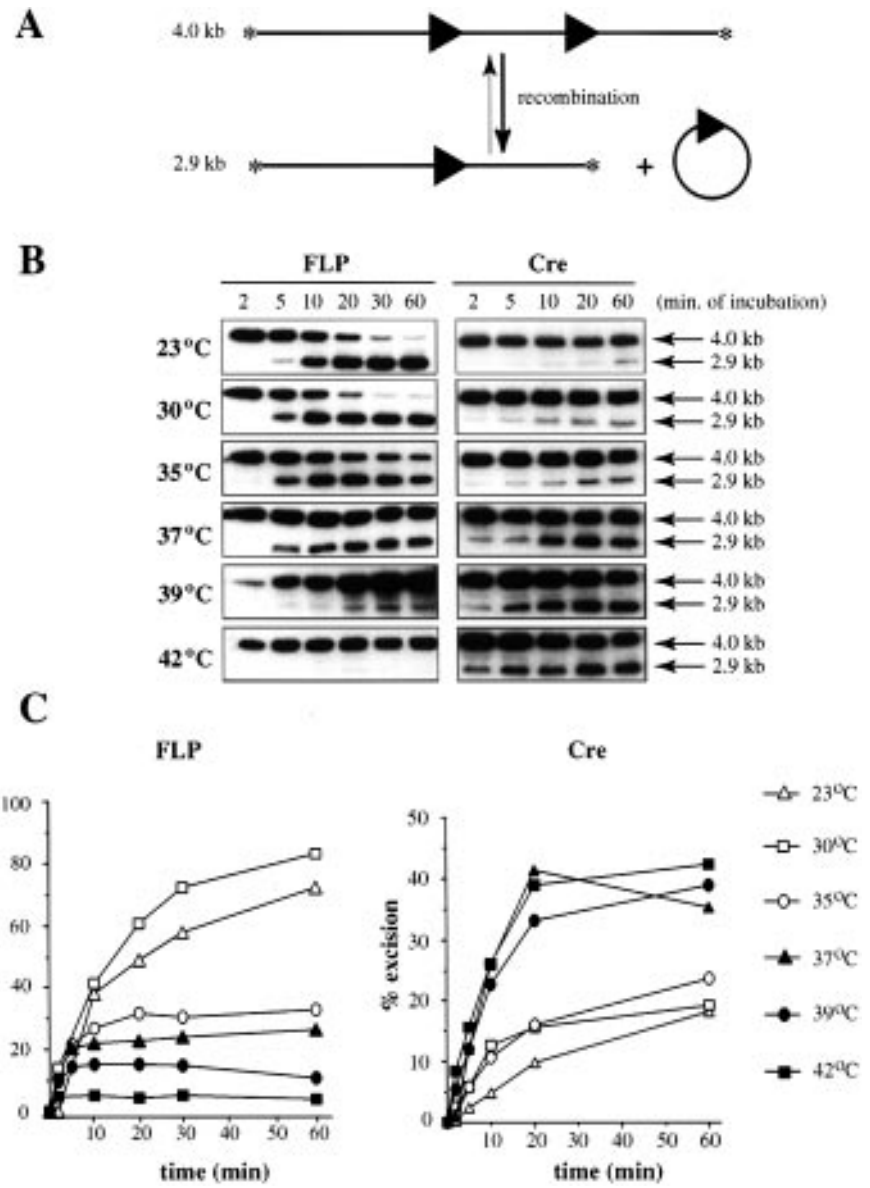


**Figure 2.** Temperature effect on FLP- or Cre-recombination *in vitro*. (**A**) Schematic presentation of the excision recombination reaction employed for *in vitro* recombination. Filled triangles represent recombinase recognition targets. Asterixes indicate radioactive label. (**B**) *In vitro* recombination reactions performed with either FLP or Cre on their corresponding substrates at indicated temperatures. The (initial) 4.0 kb and (recombined) 2.9 kb bands are shown on the right. (**C**) Graphical representation of (B) after quantification by PhosporImager analysis.

recombination (lanes 1 and 3), and culture in 294-Cre at 37°C resulted in no detectable recombination (lane 4). pSVpaX1 was fully recombined when present in 294-Cre cultured at both 37 and 42°C (lanes 6 and 7) and almost completely recombined when cultured at 30°C (lane 5). No pSVpaX1 recombination was detectable in 294-FLP cultured at 37°C (lane 8). Thus the recombined states of the reporter plasmids support the observations made in the blue–white temperature gradient assay. In particular, FLP and Cre showed activity differences at higher temperatures.

To evaluate FLP protein levels in cells grown at different temperatures, a western blot was performed on the *E.coli* strain 294-FLP grown at 25, 37 and 42°C (Fig. 1E). FLP protein was not detected at 25°C, as expected from repression of FLP expression by the cI-repressor. FLP protein levels at 37 and 42°C were virtually identical, indicating that the lack of recombination at 42°C was not due to the lack of FLP protein. Taken together, the results indicate that FLP is thermolabile in *E.coli*.

## FLP is a temperature-sensitive recombinase *in vitro*

To test whether this temperature effect on recombination could also be observed *in vitro*, the Cre- and FLP-genes were cloned into the expression vector pET-22b (Novagen) and Cre and FLP proteins were produced in reticulocyte lysates. *In vitro* excision recombination reactions were performed at different temperatures in time course experiments using linear fragments from pSVpaZ11 or pSVpaX1 as substrates (Fig. 2). Cre recombined the substrate equally well at 37, 39 and 42°C. In contrast, FLP-mediated recombination worked best at ≤30°C. Recombination efficiency dropped with increasing temperature, indicative of the thermolabile

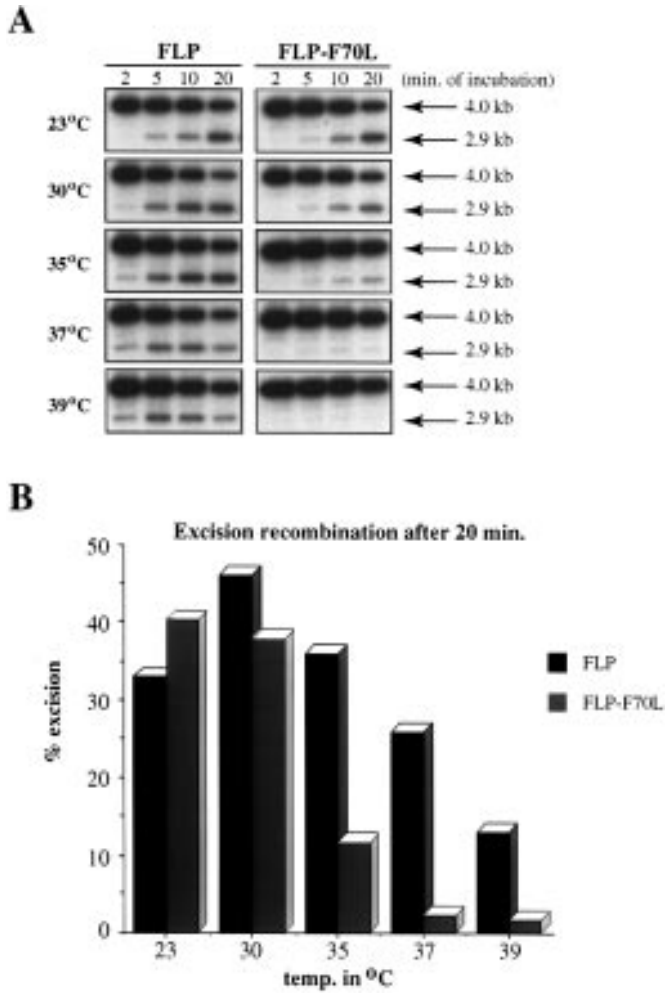


**Figure 3.** Temperature effect on FLP-F70L recombination. (**A**) *In vitro* recombination reactions performed with either FLP or FLP-F70L on the pSVpaZ11 derived substrate at 23, 30, 35, 37 and 39°C at indicated timepoints of incubation. The (initial) 4.0 kb and (recombined) 2.9 kb bands are shown on the right. (**B**) Amount of excision recombination after 20 min at the temperatures indicated quantified by PhosporImager analysis. The black bars represent FLP data, the grey bars represent FLP-F70L data.

character of FLP recombinase (Fig. 2B and C). These results are consistent with the *E.coli* based assays and demonstrate that FLP activity is thermolabile. The protein–substrate ratio employed was based on the FLP–pSVpaZ11 ratio that displayed efficient excision after 1 h. The same Cre/pSVpaX1 molar ratio appears to be less optimal, since in Figure 2B and C the Cre panels show less excision of pSVpaX1 in 1 h (83% FLP-mediated excision of pSVpaZ11 at the optimal temperature versus 42% Cre-mediated excision of pSVpaX1 at optimal temperatures). At different Cre–pSVpaX ratios, near complete excision was achieved (data not shown). Recent experiments with highly purified bacterially expressed Cre and FLP gave virtually identical thermostability results (data not shown).

### FLP-F70L shows increased temperature sensitivity

In the course of experiments with FLP, we found that the commercially available pOG44 plasmid (Stratagene) carries a point mutation in the FLP coding region. This mutation changes FLP amino acid 70 from Phe to Leu. To ascertain the effect of this mutation, FLP-F70L was compared with wt FLP by *in vitro* excision recombination at different temperatures (Fig. 3). Equal amounts of FLP and FLP-F70L recombined the substrate to the same extent at 23 and 30°C. In contrast, FLP-F70L recombined significantly less of the substrate at 35, 37 and 39°C (Fig. 3A and B). Thus F70L has the characteristics of a temperature-sensitive mutation.

### Temperature effect on FLP-recombination in a mammalian cell line

In order to test the effect of temperature on FLP-mediated recombination in mammalian cells, we utilized the inducible recombination system previously described from our laboratory (12). This permitted an experimental design where parallel culture plates of cells containing an unrecombined reporter could be transferred to incubators at 35 or 39°C, or left at 37°C, shortly before commencing a time course of recombination. The recombination reporter was a variation of pSVpaZ11, stably integrated as a single transgene (Fig. 4A). FLP was expressed as a fusion protein with the ligand binding domain of the androgen receptor fused onto the C-terminus. This protein, FLP–ABD, is inactive as a recombinase until provided with an androgen. The time courses were initiated by adding the androgen mibolerone to each of the 35, 37 and 39°C culture plates. Figure 4B shows Southern analysis of these time courses. Quantification by PhosphorImager analysis (Fig. 4C) revealed that the efficiency of FLP-mediated recombination varies with the culture temperature. After 24 h of mibolerone treatment the percentage of recombination was 26.5% at 35°C, 12.2% at 37°C and 6.7% at 39°C. Thus the effect of temperature observed on FLP recombination in a mammalian cell line is consistent with our observations *in vitro* and in *E.coli*.

## DISCUSSION

In the course of experiments with FLP recombinase in *E.coli*, we observed an apparent inactivity of FLP >39°C. The experiments reported here were designed to examine the basis of this temperature effect. In three different systems, *E.coli*, *in vitro* and in a mammalian cell line, we observed a consistent reduction of FLP activity >35°C. Thereby we conclude that FLP is thermolabile over temperatures relevant to the growth temperatures of certain important experimental systems.

In *E.coli*, the effect of temperature on recombination mediated by either FLP or Cre was compared. Whereas Cre recombinase retained activity up to 46°C, FLP recombination was evident only to 39°C. This was not due to selective degradation of FLP. An unexpected outcome of the experimental design was the observation that Cre displayed considerable activity at temperatures below those where its expression should be repressed by the temperature-sensitive cI857 repressor (21). In contrast, FLP activity could be completely repressed at these low temperatures using the same promoter. We do not know whether this difference is due to a difference in the basal expression levels of FLP and Cre from this promoter at temperatures <34°C, to differences in FLP and Cre protein stabilities, or to the possibility that Cre functions more efficiently than FLP in *E.coli* at low expression levels, even at 30°C.

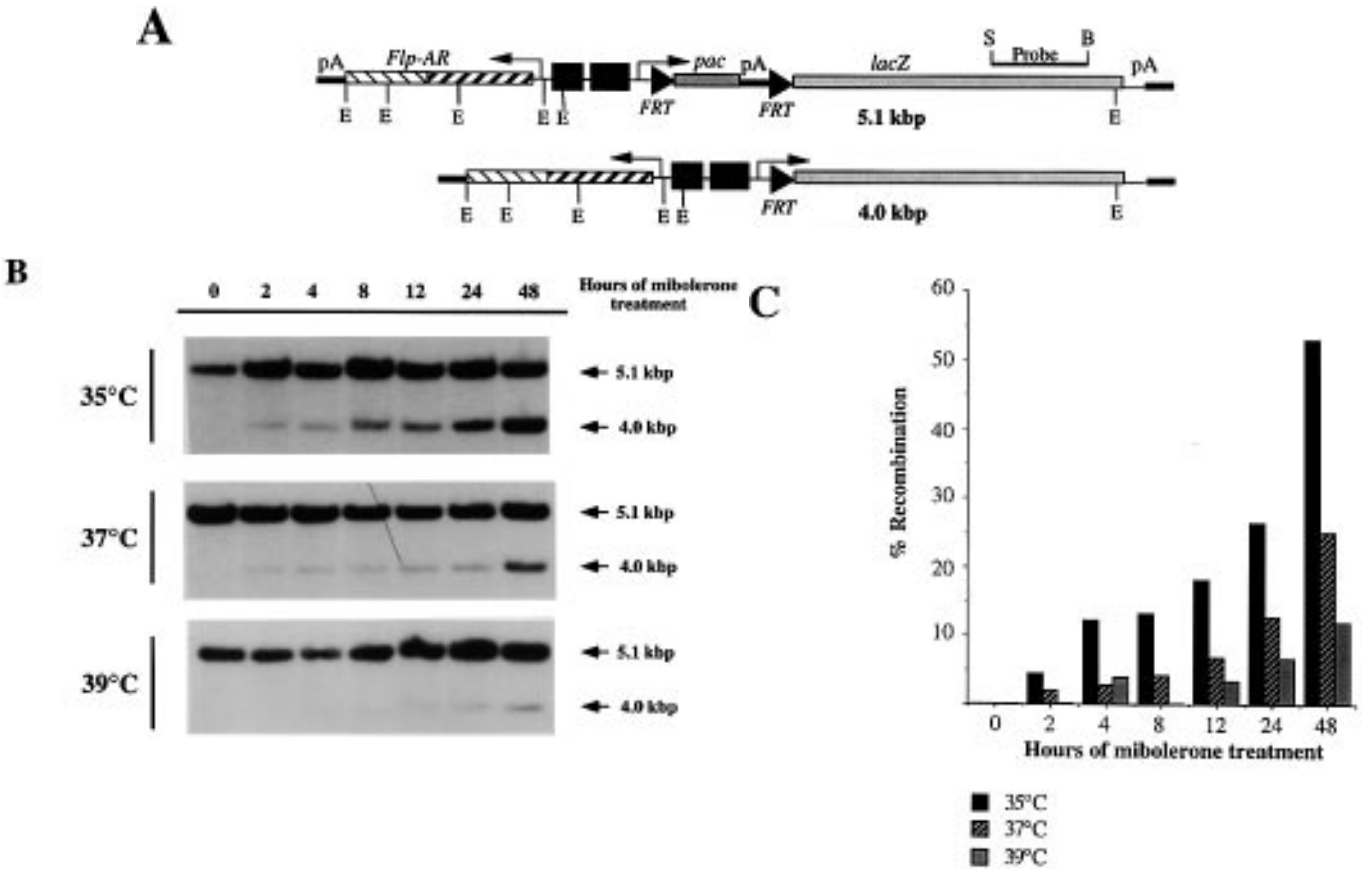


**Figure 4**. Temperature effect on FLP-mediated recombination in mammalian 293 cells. (**A**) The construct used in this assay. A single vector, p22LFA3, contains a bi-directional enhancer promoter that directs expression of both the FLP–ABD fusion protein gene and a puromycin resistance (*pac*) gene/lacZ inducible gene cassette. The *pac* gene cassette was placed between two FRTs (triangles). The SV40 early polyadenylation signal (pA) was included between the FRTs. The entire lacZ gene was placed 3′ of the downstream FRT. Before recombination, *Eco*RI will produce a 5.1 kb fragment. Upon induction of FLP–ABD-mediated recombination by mibolerone treatment, this fragment will be reduced to 4.0 kb. Arrows indicate the location of the transcription start points; pA, poly adenylation signals; E,*Eco*RI sites; S, *Sac*I site; B, *Bsi*WI site. The relevant probe used for Southern analysis is indicated. (**B**) Time course of mibolerone-induced recombination at the temperatures indicated, as assessed by Southern analysis. Genomic DNA was isolated from a representative clone cultured in 100 nM mibolerone-containing medium for the indicated hours at 35°C (top), 37°C (middle) or 39°C (bottom panel) and digested with *Eco*RI. (**C**) Quantification by PhosphoImager analysis of percent recombination observed in the Southern analysis of (B). Percent recombination is calculated as recombined counts (4.0 kb band) divided by the unrecombined (5.1 kb band) plus recombined counts, multiplied by 100.

*In vitro*, FLP shows a temperature optimum near 30°C, whereas Cre shows optimal recombination activity at and above 37°C. This difference in temperature optima is consistent with their origins. FLP is derived from the *S.cerevisiae* 2μ circle and Cre from the *E.coli* phage P1.

We identified a point mutation in a commercially available FLP plasmid that changes FLP amino acid 70 from Phe to Leu. This mutation destabilises FLP activity so that it is even more sensitive to temperature.

In a mammalian cell line, FLP recombination shows thermolability consistent with our results from *E.coli* and *in vitro*. Recombination in these experiments was regulated by expressing FLP as a fusion protein with hormone binding domains from the steroid receptors. These fusion proteins require ligand to derepress recombinase activity (12). It is possible that the temperature effect on FLP–ABD fusions (Fig. 4) reflects sensitivity of the ABD rather than of FLP. Equivalent experiments with FLP–EBD (estrogen binding domain) fusions also showed similar reductions of recombination at 37 and 39°C when compared with 35°C (data not shown). In contrast, equivalent experiments with Cre-PBD (progesterone binding domain) (22) or Cre-EBD constructs did not show increased recombination at 35°C when compared with 37 or 39°C (data not shown). Since our results are consistent with temperature effects observed for FLP in *E.coli* and *in vitro*, we reason that the sensitivity of FLP is dominant over any effects that may bear upon the androgen or estrogen ligand binding domains.

Site-specific recombination offers great potential to the technology of genomic manipulation. The use of site-specific recombinases relies on their ability to mediate recombination in living systems. Therefore the growth temperature of the host cell imposes a condition on the recombinase employed. FLP is most efficient at 23–30°C and loses activity at higher temperatures. Since Cre appears to function most efficiently at and above 37°C, it is suitable for use in hosts that grow in this temperature range. These conclusions are concordant with successful FLP and Cre applications reported so far. FLP and Cre have both been used in plants. In contrast, FLP has found widespread use in flies, whereas Cre is being successfully used in mice (reviewed in 1,2,4).

To optimise the usage of Cre and FLP as tools in applied site-specific recombination we discuss experimental models and suggest the appropriate recombinase. (i) Both FLP and Cre can be used in organisms at temperatures <35°C (e.g. flies, plants, fish, nematodes, frogs). (ii) In mammalian systems our results recommend the use of Cre rather than FLP for those applications that require efficiency. Applications, such as tissue-specific (23) or inducible gene knock-outs in mice (24), that require quantitative recombination in a cell population will be better served by Cre. However, FLP may be particularly useful for applications that do not rely on efficiency but depend on tight regulation. For example, cell lineage studies using recombination depend on a single, rare recombination event. This should be easier to accomplish with FLP rather than Cre. Similarly, in certain gain-of-function applications, such as recombinase induced cell differentiation (25) or those that initiate tumourigenesis by recombination (26,27), FLP may be more suited to the demand for the complete absence of recombination before the chosen induction point. We also note that FLP's thermolability, including the enhanced thermolability mutant FLP-F70L, could prove useful in temperature shift experiments. Temperature shifts coupled to a second mode of regulating FLP, such as ligand regulation of FLP–LBD fusion proteins, can be exploited to enhance regulation. Figure 4 shows an example of this application. (iii) In both flies and plants, FLP has been regulated by expression from heat shock promoters (8,28). Our results suggest that the temperatures chosen to stimulate FLP expression from these promoters should account for FLP's thermolability. Best results might be obtained by an induction temperatures that balances FLP expression levels and FLP thermolability. (iv) The utility of site-specific recombination in genomic manipulations in mice has led to the design of experiments that employ two recombinases. Our results recommend the use of FLP in ES cells (e.g. to delete the selectable marker) and Cre for the experiment in the organism. Furthermore, the efficiency of FLP recombination for this application may be enhanced by shifting down culture temperatures during the transient expression period.

It remains to be established if other differences in the applied properties of these site-specific recombinases, such as DNA binding affinities, will also be important in determining the choice of recombinase used in the experimental design.

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