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Enhanced efficiency through nuclear localization signal fusion on phage ϕC31-integrase: activity comparison with Cre and FLPe recombinase in mammalian cells

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ABSTRACT

The integrase of the phage ΦC31 recombines an attP site in the phage genome with a chromosomal attB site of its *Streptomyces* host. We have utilized the integrase-mediated reaction to achieve episomal and genomic deletion of a reporter gene in mammalian cells, and provide the first comparison of its efficiency with other recombinases in a new assay system. This assay demonstrated that the efficiency of ΦC31-integrase is significantly enhanced by the C-terminal, but not the N-terminal, addition of a nuclear localization signal and becomes comparable with that of the widely used Cre/loxP system. Furthermore, we found that the improved FLP recombinase, FLPe, exhibits only 10% recombination activity on chromosomal targets as compared with Cre, whereas the *Anabaena* derived XisA recombinase is essentially inactive in mammalian cells. These results provide the first demonstration that a nuclear localisation signal and its position within a recombinase can be important for its efficiency in mammalian cells and establish the improved ΦC31-integrase as a new tool for genome engineering.

INTRODUCTION

During the last few years, site-specific recombinases (SSRs) have become an increasingly used powerful tool to analyze gene function in genetically modified cell lines and mice. This approach depends on the insertion of recognition sites of a SSR into intronic sequences of the target gene, leaving the gene functionally unaffected (1,2). Gene inactivation can then be achieved in a spatially and/or temporally controlled manner through tissue-specific and/or inducible expression of the corresponding SSR. The bacteriophage-derived Cre/loxP system has turned out to be very efficient in transgenic mice and has become the most commonly used SSR system for conditional gene targeting (3). LoxP flanked (floxed) alleles of around 30 genes and more than 50 tissue-specific or inducible Cre mice have been generated (for review see 1,2). However, recently it has been shown that high-level expression of Cre during mouse spermatogenesis or in embryonic fibroblasts can lead to severe chromosomal damage (4,5; N.Faust, unpublished observations). Thus, Cre might not be the optimal site-specific recombinase for certain applications.

A second recombinase system, which has been used to introduce genetic modifications into mice, is the yeast derived FLP/FRT system (6,7). An improved, temperature stable version of the FLP recombinase, FLPe (8), has been successfully employed to generate efficient deleter mice, removing an FRT-flanked DNA segment from all cells during embryogenesis (9,10). No deleterious effects of FLP on the mouse genome have been reported so far. On the other hand, the efficiency of FLPe in comparison with Cre in a mammalian system has not yet been exactly determined.

More sophisticated conditional targeting strategies involve the use of recognition sites for two different SSRs: one pair to flank the exons to be deleted from the target gene; the other pair to flank the selection marker (11). It would therefore be desirable to have another SSR working with high efficiency in mammalian cells. This third recombinase could be used for experiments in which two different tissue-specific KOs are to be combined and could replace Cre in experiments requiring expression during spermatogenesis, such as the protamine-Cre approach (4,12).

The bacteriophage-derived ϕC31-integrase (ϕC31-Int) (13) has recently been shown to be capable of mediating site-specific recombination in human cells (14). However, the efficiency of this recombinase as compared with other SSRs has not been determined. To assess the potential of ϕC31-Int for conditional gene targeting experiments and to further examine the efficiency of the FLPe/FRT system, we developed an assay that allows us to measure the efficacy of a SSR in mammalian cells in relation to Cre.

The use of SSRs of prokaryotic origin to recombine chromosomal targets in mammalian cells raises the question of whether their efficiency may be limited by the lack of nuclear import signals. This question has been studied in detail for Cre recombinase showing that its recombination efficiency in mammalian cells is not enhanced upon the addition of an N-terminal nuclear localization sequence (NLS) (15). In this study it was found that Cre contains an intrinsic, bipartite basic determinant which functions as a NLS, possibly an incidential property of the very basic Cre protein. Thus, Cre may be an exception rather than the rule for the action of prokaryotic recombinases in eukaryotic cells. We were therefore interested to study the effect of a NLS on the performance of other recombinases, specifically ϕC31-Int and XisA, derived from the cyanobacterium

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Anabaena sp. (16). A NLS may be of particular relevance for the efficacy of the 67 kDa ϕC31-Int which belongs to a subgroup of the resolvase/invertase family with molecular masses that do not allow passive entry through nuclear pores. In our quantitative comparison of recombinase efficiencies, FLPe, including an N-terminal NLS, showed 25% of Cre activity on transiently transfected reporter plasmids, whereas the wild-type ϕC31-Int exhibited 50% of Cre activity. The activity of C31 was improved to nearly 100% of Cre activity through the addition of a C-terminal NLS to the protein while the NLS had no effect at the N-terminal position. On targets stably integrated into the chromatin, the efficiency of FLPe and ϕC31-Int were reduced to 10% of Cre activity while the C-terminal C31-NLS fusion retained 50% of Cre activity. XisA recombinase was found to be very inefficient and could not be improved by the addition of a NLS to the protein. Our results show that a NLS can be an important determinant for the efficiency of a prokaryotic recombinase in mammalian cells, and that its position within the protein can also be of critical importance.

MATERIALS AND METHODS

Expression vectors

For the construction of the mammalian expression vectors, we first generated plasmid pIpA. pIpA was built by insertion into pNEB193 (New England Biolabs Inc., Beverly, MA) of a 700 bp Cytomegalovirus immediate early gene (CMV-IE) promoter from plasmid pIREShyg (Clontech, CA), a synthetic 270 bp hybrid intron, consisting of an adenovirus derived splice donor and an IgG derived splice acceptor sequence (17), and a 189 bp synthetic polyadenylation sequence built from the polyadenylation consensus sequence and 4 MAZ polymerase pause sites (18,19).

The cyanobacterium *Anabaena* strain PCC7120 (CNCM-Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris, France) was cultured in BG11 medium according to the supplier's recommendations at room temperature. Cells were harvested by centrifugation and resuspended in a buffer containing 50 mM glucose, 25 mM Tris–HCl pH 7.6, 10 mM EDTA and 1 mg/ml lysozyme. After incubation for 15 min at room temperature an equal amount of lysis buffer (50 mM Tris–HCl pH 8.0, 100 mM EDTA, 100 mM NaCl and 1% SDS, 800 µg/ml proteinase K) was added. Lysis was performed at 55°C overnight. After phenol–chloroform extraction, genomic DNA was precipitated with isopropanol. This DNA was used for the cloning of the XisA recombinase gene as follows. First the XisA gene was amplified by PCR using primers XisA1 (5′-ATAAGAATGCGGCCGCCCGATATGCAAAATCAGGGTCAAGACAAATATCAA-3′) and XisA3 (5′-ATAAGAATGCGGCCGCTCAACTATTCTTATAAGCTATTTCCATC-3′). The PCR product was digested with *Not*I and ligated into the *Not*I site of pIpA, giving rise to the XisA expression vector pCMV-XisA.

The XisA gene with the N-terminal addition of the SV40 T-antigen NLS (20) was PCR-amplified by exchanging the primer XisA1 for primer XisA2 (5′-ATAAGAATGCGGCCGCACCATGCCCAAGAAGAAGAGGAAGGTGCAAAATCAGGGTCAAGACAAATATCAACAAGCC-3′) containing the NLS sequence. After *Not*I restriction digest, the PCR product was ligated into the *Not*I site of pIpA, giving rise to the plasmid pCMV-NLS-XisA.

The enhanced FLP (FLPe) gene with an N-terminal NLS fusion was recovered from the plasmid pCAGGS-FLPe (a kind gift of F. Stewart, EMBL, Heidelberg) as a 1.4 kb *Xba*I–*Pac*I fragment and inserted into the *Not*I site of pIpA, creating pCMV-NLS-FLPe.

DNA from phage ϕC31 was isolated from the lysate (DSM-49156) received from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) as follows: lysis of phage was performed by incubation of 1 vol of phage lysate and 1 vol of lysis buffer (50 mM Tris–HCl pH 8.0, 100 mM EDTA, 100 mM NaCl and 1% SDS, 800 µg/ml proteinase K) at 55°C overnight. After phenol–chloroform extraction, genomic DNA was precipitated with isopropanol. From this DNA the ϕC31-Int gene was PCR amplified with primers C31-1 (5′-ATAAGAATGCGGCCGCCCGATATGACACAAGGGGTTGTGACCGGG-3′) and C31-3 (5′-ATAAGAATGCGGCCGCATCCGCCGTACGTCTTCCGTGCC-3′), ligated into the *Not*I site of pBluescript II KS, resulting in plasmid pbs-C31-Int. A sequence error in the stop codon was corrected by PCR amplification of a 300 bp fragment from pbs-C31-Int using primers C31-8 (5′-CCCGTTGGCAGGAAGCACTTCCGG-3′) and C31-9 (5′-GGATCCTCGAGCCGCGGGCGGCCGCCTACGCCGCTACGTCTTCCGTGCCGTCCTG-3′), the latter providing a corrected stop codon. The ends of this PCR fragment were digested with *Eco*47III and *Xho*I and the fragment was ligated into pbs-C31-Int, linearized with *Eco*47III and *Xho*I to release the mutant fragment. The resulting plasmid, pbs-C31-Int(wt), contains the correct ϕC31-Int gene as confirmed by DNA sequence analysis. The ϕC31-Int gene was subsequently excised from pbs-C31-Int(wt) with *Not*I–*Xho*I and ligated into pIpA giving rise to the ϕC31-Int expression vector pCMV-ϕC31.

For the construction of the ϕC31 expression vector with N-terminal NLS, pCMV-NLS-ϕC31, a 1.1 kb *Nco*I–*Not*I fragment was amplified by PCR with primers C31-2 (5′-ATAAGAATGCGGCCGACCATGCCCAAGAAGAAGAGGGTG ACACAAGGGGTTGTGACCGGG-3′) and C31-3 and used to exchange the N-terminal part of the C31 gene in the expression vector.

For the construction of the ϕC31 expression vector with C-terminal NLS, pCMV-ϕC31-NLS, the 300 bp 3′ end of the ϕC31-Int gene was amplified from the C31(wt) expression vector with primers C31-8 and C31-2-2 (5′-TAGAATTCCGCTCGAGAGTCTAAACCTTCCTCTTCTTCTTAGGCGCCGCTACGTCTTCCGTGCCGTCC-3′). Primer C31-2-2 modifies the 3′ end of the wild-type ϕC31-Int gene such that the stop codon is replaced by a sequence of 21 bp coding for the SV40 T-antigen NLS (Proline-Lysine-Lysine-Lysine-Arginine-Lysine-Valine) (20), followed by a new stop codon. This 300 bp PCR fragment was digested with *Eco*47III and *Xho*I and the fragment was used to replace the 3′ end of the wild-type ϕC31-Int gene, resulting in the expression vector pCMV-ϕC31-NLS containing the C-terminal NLS. The identity of the new gene segment was verified by DNA sequence analysis.

To generate the Cre expression plasmid pCMV-NLS-Cre, the coding sequence of Cre recombinase (21) with an N-terminal fusion of the seven amino acid SV40 T-antigen NLS (see above) was recovered from plasmid pgk-Cre (kind gift of

K. Rajewsky, University of Cologne) and cloned into the *Not*I and *Xho*I sites of plasmid pIpA.

Reporter plasmids

The Cre/ϕC31 reporter vector was constructed as follows. First an attB site (13), generated by the annealing of the two complementary synthetic oligonucleotides C31-4attB (5′-CGTGACGGTCTCGAAGCCGCGGTGCGGGTGCCAG-GGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCCACC-TCACCCATCTGGTCCA-3′) and C31-5attB (3′-ACTGCCA-GAGCTTCGGCGCCACGCCCACGGTCCCGCACGGGA-ACCCGAGGGGCCCGCGCATGAGGTGGAGTGGGTAG-ACCAGGTGC-5′), was ligated into the *Bst*BI restriction site of the vector pSV-PaX1 (22). Next, an attP site (13), generated by the annealing of oligonucleotides C31-6 (5′-GATCAGAA-GCGGTTTTCGGGAGTAGTGCCCCAACTGGGGTAACCT-TTGAGTTCTCTCAGTTGGGGGCGTAGGGTCGCCGAC-ATGACAC-3′) and C31-7 (3′-TCTTCGCCAAAAGCCCT-CATCACGGGGTTGACCCCATTGGAAACTCAAGAGA-GTCAACCCCCGCATCCCAGCGGCTGTACTGTGCTAG-5′), was ligated into the *Bam*HI restriction site of this plasmid, giving rise to the dual Cre/ϕC31 reporter plasmid containing the attB and attP sites in the same orientation.

For the construction of the dual Cre/XisA reporter plasmid, a nifD site (23) was generated by the annealing of two pairs of synthetic oligonucleotides nifD3 (5′-GATCAGCTGTTGAA-AGCTATTAAACCACAAAAAGGATTACTCCGGCCCT-TATCACGGTTACGACGGATTTGCTA-3′)/nifD4 (5′-GAT-CTAGCAAATCCGTCGTAACCGTGATAAGGGCCGGAG-TAATCCTTTTTGTGGTTTAATAGCTTTCAACAGCT-3′) and nifD1 (5′-CGATGGCTCTTCCCTTCCGTCAAATGCA-CTCTTGGGATTACTCCGAACCTAGCGATGGGGTGCA-AATGTCAGATCAGATAAG-3′)/nifD2 (5′-CGCTTATCT-GATCTGACATTTGCACCCCATCGCTAGGTTCGGAGT-AATCCCAAGAGTGCATTTGACGGAAGGGAAGAGCC-AT-3′) creating *Bam*HI- and *Bst*BI-compatible DNA ends. The *Bam*HI-compatible fragment was ligated into the *Bam*HI site of the vector pPGK-paX1, subsequently the *Bst*BI-compatible fragment into the *Bst*BI site of this plasmid giving rise to the Cre/XisA reporter vector with two nifD sites in the same orientation.

For the construction of the dual Cre/FLP reporter, a 54 bp fragment containing the FRT site (24,25) was ligated into both the *Bam*HI and *Bst*BI restriction sites of pPGKpaX1.

Plasmid DNA concentrations were determined by absorption at 260 and 280 nm and the plasmids were diluted to the same concentration; finally the concentrations were confirmed on an ethidium bromide-stained agarose gel.

Cell culture and transfections

Chinese hamster ovary (CHO) cells adapted to growth in DMEM were used for the transfections. The cells were grown in DMEM/Glutamax medium (Life Technologies) supplemented with 10% fetal calf serum at 37°C, 10% CO_2 in a humid atmosphere. One day before transfection, 10^6 cells were plated into a 48-well plate (Falcon). For the transient transfection of cells with plasmids each well received into 250 µl of medium, a total amount of 300 ng supercoiled plasmid DNA pre-complexed with the FuGene6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Each 300 ng DNA preparation contained 50 ng of the Luciferase expression vector pUHC13-1 (26), 50 ng of the respective dual reporter vector, different amounts of the recombinase expression vectors pCMV-FLPe, pCMV-XisA, pCMV-NLS-XisA, pCMV-ϕC31, pCMV-ϕC31-NLS, pCMV-NLS-ϕC31 or pCMV-NLS-Cre and pUC19 plasmid to a total amount of 300 ng DNA, except for the controls which received 50 ng of pUHC13-1 together with 50 ng of reporter vector only. For each sample to be tested, four individual wells were transfected. One day after the addition of the DNA preparations, each well received an additional 250 µl of growth medium. The cells were lysed 48 h after transfection with 100 µl/well of lysate reagent supplemented with protease inhibitors [β-galactosidase (β-gal) reporter gene assay kit; Roche Diagnostics]. The lysates were centrifuged and 20 µl was used to determine the β-gal activities using the β-gal reporter gene assay (Roche Diagnostics) according to the manufacturer's protocol in a Lumat LB 9507 luminometer (Berthold). To measure Luciferase activity, 20 µl of lysate was diluted into 250 µl assay buffer (50 mM glycylglycin, 5 mM $MgCl_2$, 5 mM ATP) and the relative light units were counted in a Lumat LB 9507 luminometer after addition of 100 µl of a 1 mM Luciferin (Roche Diagnostics) solution.

To generate a stably transfected Cre/ϕC31 reporter cell line, 2.5×10^6 NIH 3T3 cells were electroporated with 5 µg of *Sca*I-linearized Cre/ϕC31 reporter plasmid and plated into 10-cm Petri dishes. The cells were grown in DMEM/Glutamax medium (Life Technologies) supplemented with 10% fetal calf serum at 37°C, 10% CO_2 in a humid atmosphere. Two days after transfection the medium was supplemented with 1.5 µg/ml of puromycin (Calbiochem) for the selection of stable integrants. Puromycin-resistant colonies were isolated and individually expanded in the absence of puromycin. To analyze for stable integration of the reporter vector, genomic DNA was prepared according to standard methods and 5–10 µg was digested with *Eco*RV and analyzed by Southern blot using a 1.25 kb *Not*I–*Eco*RV fragment of the LacZ gene as probe.

To generate a stably transfected FLP reporter cell line, 2.5×10^6 NIH 3T3 cells were electroporated with 5 µg of *Not*I-linearized FLP reporter plasmid and plated into 10-cm Petri dishes. Growth and selection conditions were the same as described above. To select for clones expressing β-gal, cells were transiently transfected with 50 ng Cre- and FLP-expression vector and stained with X-Gal 48 h later. Blue staining of cells showed expression of β-gal due to deletion of the stop cassette. Subsequently, only the positive clones were analyzed for stable integration of the reporter vector by Southern analysis which was performed as described above. For the following transfections and measurements of β-gal activities in lysates we chose one clone with a single integration of the reporter vector.

Transfections of the selected reporter clones with plasmid DNAs and the measurement of β-gal activities in lysates were essentially performed as described before for CHO cells, except that the reporter vector was omitted from all samples.

Histochemical detection of β-galactosidase activity

For histochemical detection of β-gal activity, the culture medium was removed, the cells were washed once with phosphate buffered saline (PBS), and fixed for 5 min at room temperature in a solution of 2% formaldehyde and 1% glutaraldehyde in PBS. Next, the cells were washed twice with PBS and finally incubated in X-Gal staining solution {5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 2 mM $MgCl_2$, 1 mg/ml

X-Gal [BioMol] in PBS} for 24 h at 37°C until images were taken using an Hitachi HVC20M camera and the Diskus imaging program (C. Hilgers, Königswinter, Germany).

RESULTS

Cloning of the SSRs

To test the performance of φC31-Int in eukaryotic cells, we amplified the coding sequence of the φC31-Int from purified φC31 phage DNA by PCR, using sequence-specific primers. The amplified sequence was identical to the published sequence (27).

Since recombinases have to enter the nucleus in order to perform DNA recombination in eukaryotic cells, it has been attempted to increase their efficiency through the addition of a NLS to the protein. In the cases of Cre or the γδ resolvase this had no significant effect on recombination activity (15,28). However, given the relatively large size of the φC31-Int (69 kDa) we suspected that, in this case, a NLS might be beneficial. We therefore constructed a modified version of the φC31-Int gene resulting in a protein with a NLS. As the catalytic center of the φC31-Int is very close to the N-terminus of the protein (Serine at position 20) (13), we generated two different NLS fusions: one version with an N-terminal NLS (NLS-φC31) and another version with a C-terminal NLS (φC31-NLS) in order to examine whether the position of the NLS would influence the activity of the recombinase.

The XisA coding sequence was amplified from *Anabaena* sp. DNA. The sequence of the amplified fragment was identical to the published sequence (29) apart from four silent point mutations. Additionally, a XisA-recombinase with an N-terminal NLS was generated. All four open reading frames as well as the coding sequences for NLS-FLPe and NLS-Cre (30) were inserted downstream of the CMV promoter of a eukaryotic expression plasmid to generate the recombinase expression plasmids.

Analysis of recombinase efficiency in transient transfections

For direct comparison of SSR activities, a set of dual reporter vectors, shown in Figure 1, was constructed. In these vectors the lacZ gene is under the control of a eukaryotic promoter but its expression is inhibited by an intervening stop cassette, consisting of a selectable marker followed by a polyadenylation signal. The selection marker is flanked by loxP sites and, adjacent to each loxP site, the recognition motifs for the recombinase to be tested. Recombination through either Cre or the respective recombinase will remove the stop cassette allowing expression of the LacZ-encoded β-gal.

In order to quantify the efficacies of FLPe, Xis-A and φC31-Int, all three recombinases were directly compared with Cre thus allowing an indirect comparison between them.

The results from transient transfections in which a dual reporter plasmid was co-transfected with either Cre or the respective SSR into the mammalian CHO cell line are displayed in Figure 1. We found that FLPe, although five times more active than wild-type FLP (8), exhibits only ~25% of the efficiency of Cre. The Xis-A recombinase displayed an efficiency of <1% of Cre activity. RT–PCR analysis demonstrated that the correct Xis-A transcript is generated (data not shown), thus confirming that the low recombinase activity observed is

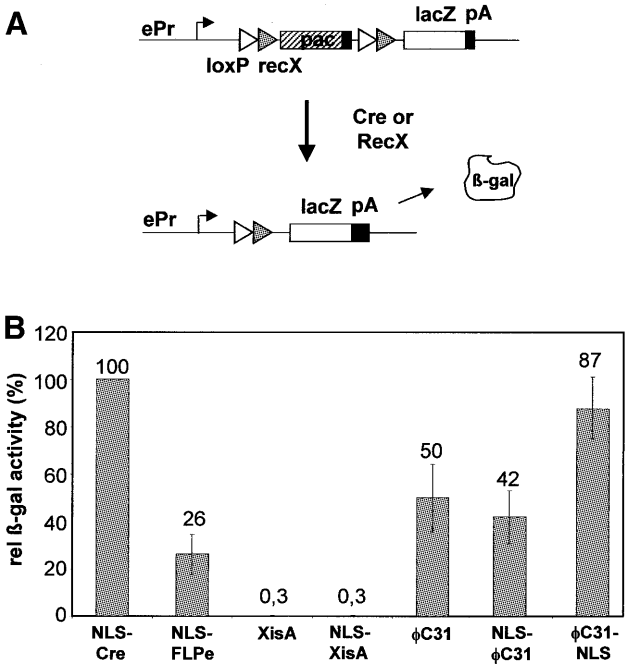

Figure 1. Comparison of Cre recombination activity with other site-specific recombinases. (**A**) Schematic diagram of the dual reporter plasmid facilitating direct comparison of Cre and RecX activities. ePr, eukaryotic promoter; pac, puromycin resistance gene; recX, recognition site for second recombinase (RecX). (**B**) CHO cells were transiently co-transfected with 50 ng of luciferase standard, 50 ng of the respective dual reporter plasmid and either between 0.5 and 100 ng of pCMV-NLS-Cre or the corresponding amount of RecX expression plasmid (pCMV-NLS-FLPe, pCMV-XisA, pCMV-NLS-XisA, pCMV-φC31, pCMV-NLS-φC31 or pCMV-φC31-NLS). Cells were harvested 48 h after transfection and β-gal and luciferase activities were measured. For each recombinase, at least two independent experiments (with quadruplicate values each) were carried out. After normalization to luciferase activity, the β-gal activity was calculated as percentage of the Cre activity, whereby only datapoints that were within the linear range of the assay were taken into account. The average and the standard deviations (error bars) of these values are displayed.

not caused by aberrant splicing or premature polyadenylation of the transcript. In contrast, we found that the φC31-Int is about half as efficient as Cre. Moreover, φC31-Int efficacy could be increased to >80% of Cre activity by adding a C-terminal NLS to the protein. Interestingly, addition of an N-terminal NLS had no positive effect on recombination efficiency of the φC31-Int. The specificity of the recombination reaction was confirmed by sequencing the recombined plasmid upon recovery from the transiently transfected cells (data not shown). Unlike the φC31-Int, the XisA recombinase showed no improved performance when an NLS was added to the protein (Fig. 1B).

Analysis of φC31-Int efficiency on chromatin targets

Having found that φC31-Int efficiently catalyzes recombination between its attB and attP target sites located on extrachromosomal DNA in eukaryotic cells, we were interested to determine its efficiency on targets stably integrated into the chromatin. To this end, we generated reporter cells by stably transfecting NIH 3T3 cells with the linearized Cre/C31 reporter plasmid. In Southern blot experiments, 4 out of 20 puromycin-resistant clones were shown to carry the transgene. In three of these clones LacZ expression could be detected after Cre mediated excision of the stop cassette. Two of these

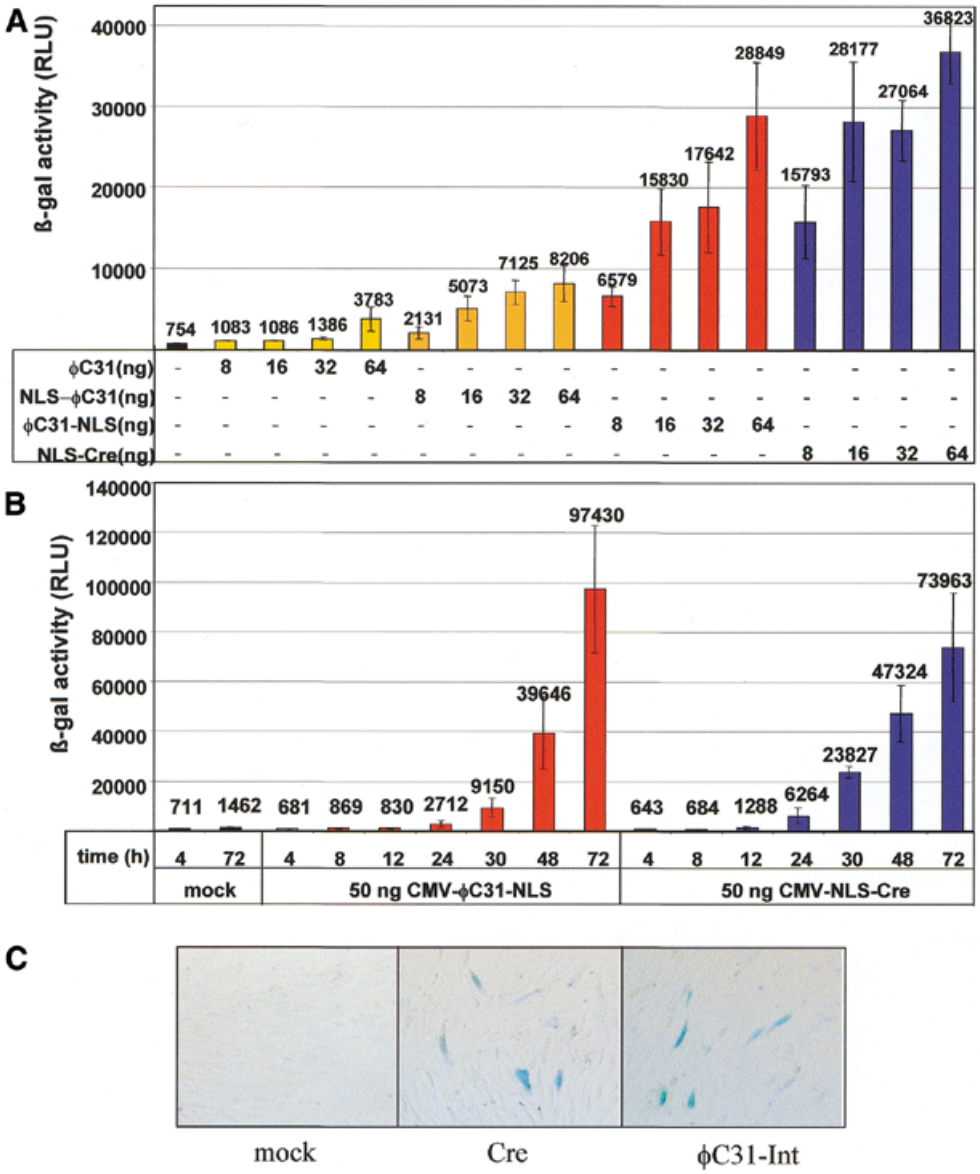

Figure 2. Quantitative analysis of φC31-Int activity on stably integrated substrate. (**A**) An NIH 3T3 cell clone carrying a single copy integration of the Cre/φC31-Int reporter (clone 3) was transfected with the indicated amounts of pCMV-NLS-Cre or φC31-Int expression vectors (pCMV-φC31, pCMV-NLS-φC31 or pCMV-φC31-NLS). Forty-eight hours after transfection the cells were harvested and β-gal activity was measured. Results show the averages from two experiments with duplicate values. Error bars represent standard deviations. (**B**) NIH 3T3 clone 3 was transfected with either 50 ng of pCMV-NLS-Cre expression plasmid or 50 ng of pCMV-φC31-NLS expression plasmid. Cells were harvested 4, 8, 12, 24, 30, 48 or 72 h after transfection and β-gal activities were measured. (**C**) X-Gal staining of mock-, Cre- or φC31-NLS-transfected NIH 3T3 cells. Representative areas are shown.

clones were used for the quantitative analysis of φC31-Int versus Cre. Figure 2 shows the result of the experiments with one of the clones. Transfecting increasing amounts of recombinase expression plasmid resulted in a proportional increase in β-gal activity (Fig. 2A), indicating that recombinase concentrations were within the dynamic range of the assay.

Comparing the values for Cre and φC31-Int demonstrates that the φC31-NLS also performs highly efficient recombination on chromatin targets with at least 50% of Cre's activity. In this assay the φC31-Int without NLS showed little activity over background. Only at high plasmid concentrations could significant recombination be detected reaching 10% of the corresponding Cre value. On the stably integrated target sequence the NLS-φC31 was clearly more efficient than the wild-type form, but still three times less active than the φC31-NLS protein. An experiment with the second φC31 reporter clone showed similar results (data not shown).

A timecourse experiment (Fig. 2B) shows that β-gal activity can be detected for both recombinases after 24 h. In both cases β-gal activity increases over the next 48 h. Consistent with the 2-fold higher activity of Cre, Cre shows faster reaction kinetics than φC31-NLS during the first 48 h. However, after 72 h the same proportion of reporter cells have undergone recombination for both recombinases.

The efficiency of φC3-Int-mediated recombination was also demonstrated by visualizing β-gal activity at the single cell level. Forty-eight hours after transfection with either Cre- or φC31-NLS-expression plasmid the reporter cells were stained

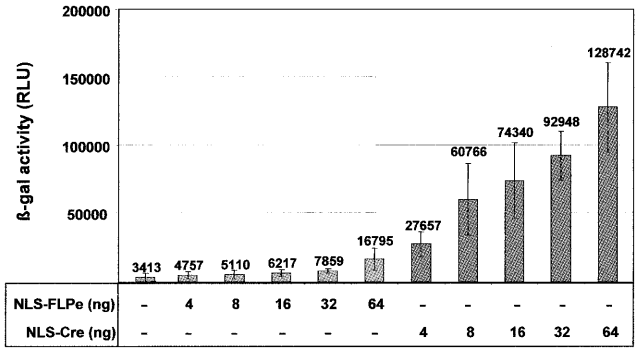

Figure 3. Quantitative analysis of FLPe activity on stably integrated substrate. An NIH 3T3 cell clone carrying a single copy integration of the Cre/FLP reporter was transfected with the indicated amounts of pCMV-NLS-Cre or pCMV-NLS-FLPe. Forty-eight hours after transfection the cells were harvested and β-gal activity was measured. Results show the averages from two experiments with duplicate values. Error bars represent standard deviations.

with X-Gal (Fig. 2C). With both recombinases comparable numbers of blue cells could be detected, whereas no blue cells could be seen in a mock-transfected population.

Since for ϕC31 and NLS-ϕC31 we observed a significant reduction in activity on chromosomal targets as compared with transiently transfected plasmids, we were interested to determine the activity of FLPe on FRT sites that are stably integrated into the chromatin. We generated stable 3T3 clones carrying the Cre/FLP reporter construct. The comparison of Cre and FLPe on one of these clones is shown in Figure 3. The data show very clearly that the efficiency of FLPe on chromatin targets is also reduced and reaches maximally 10% of the recombination activity obtained with Cre.

In summary our results demonstrate that ϕC31-Int, modified by the addition of a C-terminal NLS, is capable to catalyze site-specific recombination in eukaryotic cells with an efficacy significantly higher than that of FLPe and comparable with that of Cre recombinase.

DISCUSSION

With the growing importance of conditional gene targeting for functional genetics, an increasing number of mice expressing SSRs are being generated. Up until now this technology has been based on two systems: the bacteriophage P1-derived Cre/loxP system and, to a lesser extent, the yeast FLP/FRT system. Although initially FLP was found to be relatively inefficient, we and others have successfully generated constitutively expressing tissue-specific FLP mice (9,10,31,32 and N.Faust, unpublished data) using either the wild-type FLP recombinase or the more efficient thermostable FLPe recombinase. However, one important aspect of conditional gene targeting is inducibility of recombination. From the experience with Cre it is clear that achieving complete recombination upon induction is still a challenge in inducible gene targeting (33–35). A highly efficient recombinase is therefore absolutely critical to all inducible applications. To assess the capability of FLPe to perform well in inducible systems we have compared its activity in mammalian cells directly with Cre. In parallel we have analyzed for the first time the efficiency of other known SSRs, ϕC31-Int and XisA side by side to Cre in order to find another effective system that can be employed in mammalian genome engineering.

We were further interested to assess the effect of a nuclear import signal on the performance of ϕC31-Int since this recombinase belongs to an unusual resolvase/invertase subfamily of high molecular weight (13) which should not be able to transverse nuclear pores by passive diffusion. In our experiments FLPe shows only 25% of Cre activity on a transiently transfected target. In assays on chromatin targets we found FLPe 10 times less active than Cre. This observation is consistent with the fact that the deletion of an FRT-flanked neomycin gene from ES cells requires the transfection of about 10 times the amount of expression vector that is required for Cre-mediated neo-deletion (R.Kühn, unpublished results). We conclude that FLPe is not well suited for genomic applications involving inducible recombinase expression since a considerably longer induction period would be required as compared with Cre. The reduced efficiency of FLPe, but also ϕC31-Int on chromatin targets in comparison with an extrachromosomal substrate also show that the latter assay type is not fully appropriate to extrapolate on the utility of SSR for the engineering of genomic DNA.

In contrast to FLPe or ϕC31-Int the *Anabaena* derived XisA recombinase exhibited, with or without NLS, almost no activity in our assay system. Presently we cannot distinguish among the various explanations that could account for this failure: XisA may require one or more additional prokaryotic proteins for its action, the XisA protein could be unstable at 37°C, or its enzymatic activity may be intrinsically low.

By demonstrating the high recombination efficiency of ϕC31-Int in eukaryotic cells, we have identified a third recombinase that can be employed in conditional gene targeting. Additionally, ϕC31-Int introduces a novel aspect to conditional gene targeting: unlike Cre or FLP it acts unidirectionally on a pair of different recognition sites (36), making this recombinase extremely useful for applications like integrations (14) or irreversible inversions. Our comparative analysis showed that the wild-type ϕC31 integrase is twice as efficient as FLPe in recombining extrachromosomal target sequences within eukaryotic cells. A factor of two might not make a significant difference in applications in which the recombinase is constitutively expressed. In contrast, for inducible applications, this has a strong impact and will reduce induction times by half. The activity of ϕC31-Int was strongly enhanced by adding a NLS to the C-terminus of the protein rendering it five times as active as FLPe with an N-terminal NLS. This is the first example in which the addition of a NLS improved the efficiency of a SSR. Neither for Cre (15) nor γδ resolvase (28) could such an effect be shown. Possibly these proteins are small enough to passively enter the nucleus or they carry an endogenous NLS (15), whereas the majority of the large wild-type ϕC31-Int protein is excluded from the nucleus. The effect of the addition of a NLS to ϕC31-Int was more pronounced when a stably integrated target was examined. This might be explained by the possibility that in transient transfections some of the target DNA molecules can be recombined in the cytoplasm. It is not quite clear why fusion of the NLS to the N-terminus of the protein was not effective. Possibly additional amino acids at this position interfere with the active center of ϕC31-Int, located close to the N-terminus, or the NLS may not be exposed at the protein surface so that it cannot be recognized

by nuclear import receptors. It is unlikely that a C-terminal NLS fusion is generally more effective for recombinases than an N-terminal one, since in the case of γδ resolvase, a C-terminal NLS fusion did not show enhanced recombination as compared with the wild-type form (28). However, our results indicate that various positions of a NLS within a recombinase protein should be tested to enhance its efficacy in mammalian cells. It therefore remains to be seen if the efficiency of FLPe can be further enhanced by fusing the NLS to the C-terminus of the protein. The recombination efficiency measured in our assay is defined by a combination of protein expression level, stability and catalytic activity. Since there are no antibodies available against the φC31 integrase protein yet, the contribution of each of these components cannot currently be determined. It also remains to be shown whether φC31-Int acts with Cre-like activity *in vivo*. To address this, we are currently generating φC31-Int transgenic mice. It will be highly interesting to analyze recombination efficacy in these mice as well as the effect of φC31-Int expression during spermatogenesis. Theoretically it is possible that all SSRs will cause chromosomal aberrations during mouse spermatogenesis as has been described for Cre (4). On the other hand, the φC31 target sites are substantially longer than loxP sites (85 versus 35 bp). Thus the risk of pseudo-att sites in the genome being recombined through φC31-Int is likely to be smaller than seems to be the case for Cre.

Taken together, our data show that a nuclear import signal and its position within a prokaryotic recombinase can be important determinants of its efficiency in mammalian cells. Besides the implications for the design of other improved recombinases, our work establishes the NLS-modified φC31-Int as an efficient tool to assist or replace Cre in mammalian genome engineering.

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REFERENCES

1. Metzger,D. and Feil,R. (1999) Engineering the mouse genome by site-specific recombination. *Curr. Opin. Biotechnol.*, **10**, 470–476.
2. Kühn,R. and Schwenk,F. (2002) Conditional knock-out mice. In Hofker,M. and van Deursen,J. (eds), *Transgenic Mouse Methods and Protocols*. Humana Press, Totowa, NJ, Vol. 209, in press.
3. Nagy,A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis*, **26**, 99–109.
4. Schmidt,E.E., Taylor,D.S., Prigge,J.R., Barnett,S. and Capecchi,M.R. (2000) Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc. Natl Acad. Sci. USA*, **97**, 13702–13707.
5. Loonstra,A., Vooijs,M., Beverloo,H.B., Allak,B.A., van Drunen,E., Kanaar,R., Berns,A. and Jonkers,J. (2001) Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc. Natl Acad. Sci. USA*, **98**, 9209–9214.
6. Broach,J.R., Guarascio,V.R. and Jayaram,M. (1982) Recombination within the yeast plasmid 2mu circle is site-specific. *Cell*, **29**, 227–234.
7. Meyer-Leon,L., Senecoff,J.F., Bruckner,R.C. and Cox,M.M. (1984) Site-specific genetic recombination promoted by the FLP protein of the yeast 2-micron plasmid *in vitro*. *Cold Spring Harb. Symp. Quant. Biol.*, **49**, 797–804.
8. Buchholz,F., Angrand,P.O. and Stewart,A.F. (1998) Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat. Biotechnol.*, **16**, 657–662.
9. Rodriguez,C.I., Buchholz,F., Galloway,J., Sequerra,R., Kasper,J., Ayala,R., Stewart,A.F. and Dymecki,S.M. (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nature Genet.*, **25**, 139–140.
10. Roberds,S.L., Anderson,J., Basi,G., Bienkowski,M.J., Branstetter,D.G., Chen,K.S., Freedman,S., Frigon,N.L., Games,D., Hu,K., Johnson-Wood,K., Kappenman,K.E., Kawabe,T.T., Kola,I., Kuehn,R., Lee,M., Liu,W., Motter,R., Nichols,N.F., Power,M., Robertson,D.W., Schenk,D., Schoor,M., Shopp,G.M., Shuck,M.E., Shina,S., Svensson,K.A., Tatsuno,G., Tintrup,H., Wijsman,J., Wright,S. and McConlogue,L. (2001) BACE knockout mice are healthy despite lacking the primary B-secretase activity in brain: implications for Alzheimer's disease therapeutics. *Hum. Mol. Genet.*, **10**, 1317–1324.
11. Farley,F.W., Soriano,P., Steffen,L.S. and Dymecki,S.M. (2000) Widespread recombinase expression using FLPeR (flipper) mice. *Genesis*, **28**, 106–110.
12. O'Gorman,S., Dagenais,N.A., Qian,M. and Marchuk,Y. (1997) Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc. Natl Acad. Sci. USA*, **94**, 14602–14607.
13. Thorpe,H.M. and Smith,M.C. (1998) *In vitro* site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. *Proc. Natl Acad. Sci. USA*, **95**, 5505–5510.
14. Groth,A.C., Olivares,E.C., Thyagarajan,B. and Calos,M.P. (2000) A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl Acad. Sci. USA*, **97**, 5995–6000.
15. Le,Y., Gagneten,S., Tombaccini,D., Bethke,B. and Sauer,B. (1999) Nuclear targeting determinants of the phage P1 cre DNA recombinase. *Nucleic Acids Res.*, **27**, 4703–4709.
16. Brusca,J.S., Hale,M.A., Carrasco,C.D. and Golden,J.W. (1989) Excision of an 11-kilobase-pair DNA element from within the nifD gene in *Anabaena variabilis* heterocysts. *J. Bacteriol.*, **171**, 4138–4145.
17. Choi,T., Huang,M., Gorman,C. and Jaenisch,R. (1991) A generic intron increases gene expression in transgenic mice. *Mol. Cell. Biol.*, **11**, 3070–3074.
18. Levitt,N., Briggs,D., Gil,A. and Proudfoot,N.J. (1989) Definition of an efficient synthetic poly(A) site. *Genes Dev.*, **3**, 1019–1025.
19. Ashfield,R., Patel,A.J., Bossone,S.A., Brown,H., Campbell,R.D., Marcu,K.B. and Proudfoot,N.J. (1994) MAZ-dependent termination between closely spaced human complement genes. *EMBO J.*, **13**, 5656–5667.
20. Kalderon,D., Roberts,B.L., Richardson,W.D. and Smith,A.E. (1984) A short amino acid sequence able to specify nuclear location. *Cell*, **39**, 499–509.
21. Sternberg,N., Sauer,B., Hoess,R. and Abremski,K. (1986) Bacteriophage P1 cre gene and its regulatory region. Evidence for multiple promoters and for regulation by DNA methylation. *J. Mol. Biol.*, **187**, 197–212.
22. Buchholz,F., Angrand,P.O. and Stewart,A.F. (1996) A simple assay to determine the functionality of Cre or FLP recombination targets in genomic manipulation constructs. *Nucleic Acids Res.*, **24**, 3118–3119.
23. Haselkorn,R. (1992) Developmentally regulated gene rearrangements in prokaryotes. *Annu. Rev. Genet.*, **26**, 113–130.
24. Kilby,N.J., Snaith,M.R. and Murray,J.A. (1993) Site-specific recombinases: tools for genome engineering. *Trends Genet.*, **9**, 413–421.
25. Jayaram,M. (1985) Two-micrometer circle site-specific recombination: the minimal substrate and the possible role of flanking sequences. *Proc. Natl Acad. Sci. USA*, **82**, 5875–5879.
26. Gossen,M. and Bujard,H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA*, **89**, 5547–5551.
27. Kuhstoss,S. and Rao,R.N. (1991) Analysis of the integration function of the streptomycete bacteriophage phi C31. *J. Mol. Biol.*, **222**, 897–908.
28. Schwikardi,M. and Dröge,P. (2000) Site-specific recombination in mammalian cells catalyzed by γδ resolvase mutants: implications for the topology of episomal DNA. *FEBS Lett.*, **471**, 147–150.
29. Lammers,P.J., Golden,J.W. and Haselkorn,R. (1986) Identification and sequence of a gene required for a developmentally regulated DNA excision in *Anabaena*. *Cell*, **44**, 905–911.
30. Gu,H., Zou,Y.R. and Rajewsky,K. (1993) Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell*, **73**, 1155–1164.

31. Dymecki,S.M. (1996) Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc. Natl Acad. Sci. USA*, **93**, 6191–6196.
32. Dymecki,S.M. and Tomasiewicz,H. (1998) Using Flp-recombinase to characterize expansion of Wnt1-expressing neural progenitors in the mouse. *Dev. Biol.*, **201**, 57–65.
33. Kühn,R., Schwenk,F., Aguet,M. and Rajewsky,K. (1995) Inducible gene targeting in mice. *Science*, **269**, 1427–1429.
34. Schwenk,F., Kühn,R., Angrand,P.O., Rajewsky,K. and Stewart,A.F. (1998) Temporally and spatially regulated somatic mutagenesis in mice. *Nucleic Acids Res.*, **26**, 1427–1432.
35. Vooijs,M., Jonkers,J. and Berns,A. (2001) A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent. *EMBO Rep.*, **2**, 292–297.
36. Thorpe,H.M., Wilson,S.E. and Smith,M.C. (2000) Control of directionality in the site-specific recombination system of the Streptomyces phage phiC31. *Mol. Microbiol.*, **38**, 232–241.