

Efficient bicistronic expression of *cre* in mammalian cells

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ABSTRACT

Cre recombinase-mediated DNA recombination is proving to be a powerful technique for the generation of mosaic mutant mice. To develop this technology further, we have altered the *cre* gene to enhance its expression in mammalian cells and have tested its efficiency of expression in a bicistronic message. Using a transient transfection assay, we found that the extension of a eukaryotic translation initiation consensus sequence, the insertion of two N-terminal amino acids, and the mutation of a cryptic splice acceptor site did not detectably alter Cre recombinase activity. The addition of either of two introns resulted in an ~2-fold increase in recombination frequency. We then tested the relative efficacy of Cre-mediated recombination in several bicistronic messages having the encephalomyocarditis virus internal ribosome entry site (IRES). Recombination frequencies were only reduced 2-fold relative to a comparable monocistronic *cre* gene. The latter results indicate that it will be possible to generate transgenic mouse strains having tissue-specific expression of the Cre recombinase through integration of an IRES-*cre* gene without disabling the targeted gene.

The bacteriophage P1 Cre DNA recombinase is proving to be useful for the generation of mosaic mutant mice (1,2). It will often be desirable to ensure that sufficient levels of *cre* are expressed to catalyze loxP recombination in all cells of the *cre*-expressing tissue. Because the Cre recombinase is from a bacteriophage, it must be modified to obtain maximal expression in mammalian cells. Previously, a partial eukaryotic translation initiation consensus sequence was added to the *cre* gene to attempt to enhance translation initiation efficiency (Fig. 1A) (3,4), and an SV40 nuclear localization signal (NLS) was added to ensure nuclear targeting (4). To further optimize *cre* for expression in mammalian cells, we have created several variant *cre* genes and tested their activity using a mammalian cell culture assay.

The use of gene targeting techniques enables tissue-specific expression of *cre* without prior characterization of gene regulatory elements. This strategy has been used successfully (5). Because direct insertion of a foreign gene typically results in a loss-of-function mutation of the targeted gene, we created and tested a bicistronic expression cassette incorporating the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (6). Integration of this cassette into the 3'-untranslated region of a gene could result in expression of the Cre recombinase without disrupting the expression of the targeted gene.

We have performed a systematic comparison between several newly generated Cre expression plasmids and an existing plasmid, pMC-Cre (4) (Fig. 2). All used the same promoter, a hybrid polyoma virus enhancer/HSVtk promoter (MC1; 7). pMC-Cre contains a partial translation initiation consensus sequence, the SV40 T-antigen NLS, and an HSVtk polyadenylation signal (4). pMC/Cre* is identical to pMC-Cre except the entire eukaryotic consensus translation initiation sequence (8) was substituted (Fig. 1A) and a splice acceptor consensus (Fig. 1B) was mutated by PCR amplification of the 5' portion of the *cre* gene using the following primers: 5'-CCATCGATACGCGTGCG-GCCGCCACCATGGGCCGCCCCAAGAAGAAGAGGAAG-GTG-3' and 5'-CAGACCGCGCGCTTGAAGATATAG-3' and subcloning of the resulting PCR fragment into the *Mlu*I and *Bss*HIII sites of pMC-Cre. This manipulation also inserted glycine and arginine residues as the second and third amino acids, generating an *Nae*I restriction site necessary to allow the use of the complete IRES translation initiation sequence in the plasmids described below. The pMC/Intron/Cre* construct was derived from pMC/Cre* by adding a chimeric intron (9) and SV40 late polyadenylation signal, both from pCAT3-Basic (Promega Corp., Madison, WI). The pMC/Cre*/Rβgl.pA construct is identical to pMC/Cre* except the rabbit β-globin 3'-intron and polyadenylation signal replaces the HSVtk polyadenylation signal. The pMC/Ad.lidr/Cre* construct is identical to pMC/Cre*/Rβgl.pA except for the insertion of the adenovirus 5' tripartite leader (10). To generate pMC/IRES/Cre* the EMCV IRES from pCITE-1 (Novagen Inc., Madison, WI) was combined with Cre*/Rβgl.pA. pMC/Emx-1/IRES/Cre* was generated by inserting an *Emx-1* cDNA between the IRES and the MC1 promoter. Similarly, a murine *engrailed-1* (*En-1*) cDNA (11) was used to generate pMC/En-1/IRES/Cre*, a rat *insulin-like growth factor-2* (*IGF-2*) cDNA (12) was used to generate pMC/IGF-2/IRES/Cre*, a murine *sonic hedgehog* (*Shh*) cDNA (13) was used to generate pMC/Shh/IRES/Cre*. Each of the cDNA fragments included an initiation codon but not a polyadenylation signal.

The CV-1 5B reporter cell line created by Kellendonk *et al.* (14) harbors a stable integration of the pHSVtk/loxNeolox/NLS-lacZ reporter construct. Expression of the *NLS-lacZ* gene is only detected in cells that have undergone Cre-mediated deletion of the loxP flanked neomycin phosphotransferase gene (14). For transfection, 2×10^4 CV-1 5B cells were grown on coverslips using previously described conditions (14). Cells were co-transfected with 0.5 μg DNA of each *cre* plasmid and 0.25 μg of secreted alkaline phosphatase plasmid (SEAP; 15) using the @lipofectin reagent according to the manufacturer's protocol (Life Technologies Inc., Grand Island, NY). After transfection, cells were grown for 48 h prior to quantitation of relative Cre recombinase activity.

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A	B
Consensus	Consensus
CGGCC ^A CCATGG	$\left(\begin{smallmatrix} T \\ C \end{smallmatrix}\right) N^T C \underline{AG}/G$
pMC-Cre	pMC-Cre
CCTCG ^A CCATGG	CTTCTATATCTCAG/G
pMC/Cre*	pMC/Cre*
CGGCC ^A CCATGG	CTTCTATATCTCAA/G

Figure 1. (A) The translation initiation sequence of *cre* was modified at positions +4, -4 and -6 to coincide with the consensus translation initiation sequence (8). Positions -3 and +4 (underlined) are most critical for translation efficiency. The presence of a G at position +4 can increase the rate of translation initiation by up to 2-fold when there is an A at position -3 (8). (B) Position -670 of *cre* (24) was mutated from the conserved AGG (underlined) to AAG in pMC/Cre*, mutating a good match to the highly conserved splice acceptor consensus sequence (25). The pyrimidine-rich tract beginning at position -5 contains 80% pyrimidines in both the consensus and pMC-Cre.

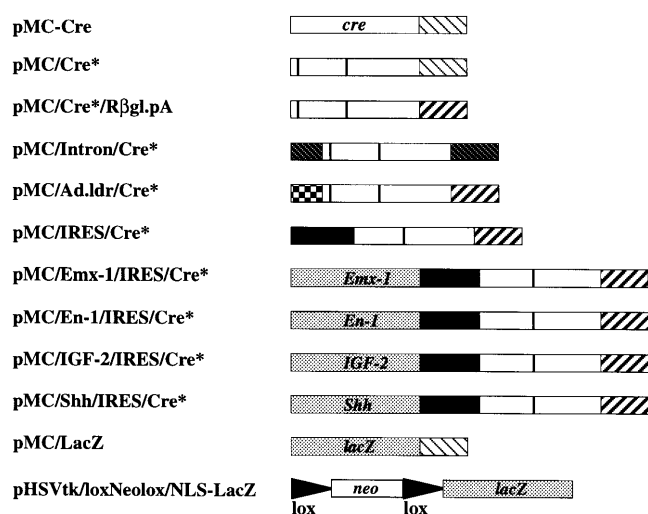


Figure 2. Schematic representation of Cre recombinase constructs. The vector backbone is not shown but was identical in all of the *cre* plasmids. All inserts are drawn beginning at the transcription start site of the MC1 promoter, except pHSVtk/lox Neo lox/NLS-LacZ which is drawn at the beginning of the HSVtk transcription start site. pMC-Cre (4) was the starting plasmid from which the new *cre* plasmids were derived. The *cre* coding sequence (open box) and HSVtk polyadenylation signal (light hatch box) are represented schematically. pMC/Cre* contains a more complete eukaryotic translation initiation consensus sequence and the mutation of a cryptic splice acceptor site, both denoted as vertical bars through the *cre* coding region. pMC/Cre*/Rβgl.pA contains the rabbit β-globin 3'-intron and polyadenylation signal (dark hatched bars). In pMC/Ad.lnr/Cre*, the adenovirus tripartite leader (checkerboard box) is inserted. pMC/Intron/Cre* contains a chimeric intron 5' of the *cre* gene and an SV40 polyadenylation signal (dark hatched bars). pMC/IRES/Cre* contains the EMCV IRES sequence (filled box) upstream of Cre*/Rβgl.pA. pMC/Emx-1/IRES/Cre* contains the *Emx-1* coding sequence (gray box) upstream of the IRES to create a bicistronic message. Similarly, pMC/En-1/IRES/Cre* contains the *engrailed-1* coding sequence, pMC/IGF-2/IRES/Cre* contains the *insulin-like growth factor-2* coding sequence and pMC/Shh/IRES/Cre* contains the *sonic hedgehog* coding sequence. pMC/LacZ contains the *E. coli lacZ* gene and HSVtk polyadenylation signal. pHSVtk/lox Neo/lox/NLS-LacZ was stably transfected into CV-1 cells by Kellendonk *et al.* (14).

Expression of sufficient Cre recombinase to catalyze loxP recombination was determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining as described (16). For each experiment, 100 random microscope fields (area = 0.636 mm²) were examined per coverslip and β-galactosidase-positive cells (β-gal+, those stained blue by the X-gal reaction product) were

counted. The counts were normalized for transfection efficiency using a colorimetric SEAP assay (15). Relative Cre recombinase activity (RCRA) for a given plasmid was calculated by dividing the normalized counts obtained with that plasmid by those obtained with pMC-Cre (Table 1). β-gal+ cells relative to total cells averaged 8% for pMC-Cre transfections. No β-gal+ cells were observed when CV1-5B cells were transfected with either SEAP alone or with no plasmid.

Table 1. Results from transient transfection of the Cre expression constructs

Plasmid	RCRA	n	P
pMC-Cre	1.0	18	n.m.
pMC/Cre*	0.92 ± 0.17	18	n.s.
pMC/Cre*/Rβgl.pA	1.8 ± 0.20	18	<0.01
pMC/Intron/Cre*	1.8 ± 0.57	18	<0.05
pMC/Ad.lnr/Cre*	0.42 ± 0.13	14	<0.01
pMC/IRES/Cre*	0.26 ± 0.087	18	<0.01
pMC/Emx-1/IRES/Cre*	0.79 ± 0.12	18	<0.01
pMC/En-1/IRES/Cre*	0.73 ± 0.11	6	<0.05
pMC/IGF-2/IRES/Cre*	0.75 ± 0.066	6	<0.05
pMC/Shh/IRES/Cre*	0.83 ± 0.029	4	n.s.
none	0	18	<0.01

After normalization using a co-transfected internal standard, the relative Cre recombinase activity (RCRA) was determined as described in the text and the mean ± standard deviation for all transfections is reported. The number of transfections analyzed is indicated (n). A student's *t*-test was used to test for significant differences between the RCRA of each new Cre expression construct and pMC-Cre (*P* values shown: n.m., not meaningful; n.s., *P* > 0.05).

Extending the eukaryotic translation initiation consensus sequence, inserting two N-terminal amino acids, and mutation of a splice acceptor consensus sequence present in *cre* did not significantly alter the relative Cre recombinase activity (Table 1). In independent experiments, a pMC/Cre plasmid, not described here, lacking the two inserted amino acids but having an extended translation initiation consensus sequence and splice acceptor mutation was found to not differ significantly in relative Cre recombinase activity from pMC-Cre or pMC/Cre* (data not shown). Because our constructs are intended for expression in transgenic mice, use of the *cre* gene having the mutated splice acceptor site will reduce the risk of undesired mRNA splicing events. Function of this splice acceptor would result in transcripts coding for only a portion of the Cre enzyme, and could interfere with the normal splicing of an exon into which an IRES-*cre* gene was targeted.

Replacement of the HSVtk polyadenylation signal with either the rabbit β-globin 3'-intron and polyadenylation signal, or with a hybrid intron (9) and an SV40 late polyadenylation signal increased relative Cre recombinase activity significantly (1.8-fold; Table 1). Given these results and prior observations that addition of introns can improve gene expression in transgenic mice (16), we expect that both Cre*/Rβgl.pA and Intron/Cre* are well suited for the generation of transgenic mice. Insertion of the adenovirus tripartite leader upstream of *cre* resulted in a 4.3-fold decrease of recombinase activity. This contrasts with reports in which the 5' adenovirus tripartite leader has been shown to

enhance expression up to 18-fold (e.g. 17). Perhaps an unfavorable RNA structure interfered with translation of the Cre messenger RNA transcribed from pMC/Ad.Idr/Cre*.

The observation that two of the new plasmids gave a 1.8-fold increase in recombination frequency compared to pMC-Cre indicates that <55% of the CV-1 5B cells underwent recombination following take up of the pMC-Cre plasmid. It is concluded that addition of either of the two introns and polyadenylation signals resulted in an increase of Cre expression beyond the threshold necessary for recombination in a larger percentage of the transfected cells. Because the assay measures a threshold level of Cre activity, it is possible that the increase in Cre protein expression was actually >1.8-fold.

Transfection with pMC/LacZ resulted in 3-fold fewer β -gal+ cells than observed following transfection with pMC-Cre (corresponding to an RCRA of 0.332 ± 0.35 , $P < 0.01$). pMC/LacZ directly expresses β -galactosidase and is identical to pMC-Cre with the exception that the *Escherichia coli lacZ* gene replaces the *cre* gene. Therefore, it can be argued that in our transfections Cre is active at relatively low expression levels, below the level of detection of β -galactosidase by X-gal histochemical stain. An identical conclusion was reached by Araki *et al.* (18) when they interpreted the results of transfecting embryonic stem cells with Cre and *lacZ* expression plasmids. These observations are also consistent with the results of Gagneten *et al.* (19), who found that the frequency of loxP recombination in an NIH 3T3-derived cell line transfected with an expression plasmid encoding a GFP-Cre fusion was ~2.5-fold higher than the apparent frequency of transfected cells as detected by fluorescence microscopy. Therefore, it is clear that while the threshold of Cre expression necessary for recombination is low, it may not be achieved when weak promoters and suboptimal *cre* genes are combined.

Bicistronic expression offers the advantage of placing two protein-coding sequences under the control of the same transcriptional regulation. Though IRES-mediated bicistronic expression has been reported to occur in transgenic mice, its efficiency has not been well studied (20). We believed it to be important to determine the relative efficacy of Cre expression from monocistronic and a series of bicistronic messages as a prelude to creating similar messages in transgenic mice. The EMCV IRES was chosen because it had been shown to function in all cell types studied in transgenic mice (20). More recently, the EMCV IRES was found to function better than other viral IRES sequences in mouse derived cell lines (21). Placement of the IRES between the MC1 promoter and Cre*/R β gl.pA reduced Cre activity to 14% of the most comparable monocistronic construct, pMC/Cre*/R β gl.pA (Table 1). However, the relative Cre recombinase activity increased to 44, 41, 42 and 46%, respectively, of the pMC/Cre*/R β gl.pA Cre activity when each of four mammalian cDNAs, *Emx-1*, *En-1*, *IGF-2* and *Shh*, were inserted upstream of the IRES to yield a bicistronic gene. Insertion of additional RNA sequences 5' of the IRES has previously been reported to enhance translation (22,23).

Because the relative Cre recombinase activity expressed from these four different bicistronic plasmids was comparable to that of pMC-Cre and approximately half that of the improved monocistronic *cre* gene described here, we suggest that bicistronic expression of the Cre recombinase is very likely to be effective in transgenic mice. We also propose that the use of an IRES-*cre* gene having a mutation in the cryptic splice acceptor site described here will help avoid splicing events that result in partially

functional or non-functional messenger RNAs. Homologous recombination of an IRES-*cre* gene into specific loci of the mouse genome is an attractive means of obtaining tissue-specific Cre expression without prior characterization of gene regulatory elements and without interfering with the function of the targeted genes.

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NOTE ADDED IN PROOF

During the revision of this manuscript, Lindeberg and Ebendal reported that Cre protein can be expressed from a bicistronic gene transfected into COS cells [Lindeberg,J. and Ebendal,T. (1999) *Nucleic Acids Res.*, **27**, 1552–1554].