Efficient bicistronic expression of cre in mammalian cells

Jessica A. Gorski and Kevin R. Jones*

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309, USA

Received April 14, 1998; Revised and Accepted March 11, 1999

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 a 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. 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 polio 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′-CCATCATAGCGTGCGGCCGCCACCATTGGCGGCCCAAGAAGAAGAGGAAG-3′ and 5′-CAGACCGCGCCGCTTGAAGATAG-3′ and subcloning of the resulting PCR fragment into the MluI and BssHIII sites of pMC-Cre. This manipulation also inserted glycine and arginine residues as the second and third amino acids, generating an Nael 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*/RβglpA construct is identical to pMC/Cre* except the rabbit β-globin 3′-intron and polyadenylation signal replaces the HSVtk polyadenylation signal. The pMC/Ad.ldr/Cre* construct is identical to pMC/Cre*/RβglpA 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*/RβglpA. 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 × 10⁶ 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.

*To whom correspondence should be addressed. Tel: +1 303 492 1363; Fax: +1 303 492 7744; Email: krjones@stripe.colorado.edu
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 sequence and the mutation of a cryptic splice acceptor site, both denoted as vertical bars through the cre coding sequence (gray box). 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 and an SV40 late polyadenylation signal, or with a hybrid intron (9) and an SV40 late polyadenylation signal increased relative Cre recombinase activity significantly from pMC-Cre or pMC/Cre* (data not shown). Because our constructs are intended for expression in transgenic mice, use of the cre gene and an SV40 late polyadenylation signal, or with a hybrid intron (9) and an SV40 late polyadenylation signal increased relative Cre recombinase activity significantly from pMC-Cre or pMC/Cre* (data not shown).

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

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

Expression of sufficient Cre recombinase to catalyze loxP recombination was determined by 5-bromo-4-chloro-3-indoly-β-D-galactosidase staining (X-gal) 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.
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.lde/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 Arakii 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*/Rfβg.pA reduced Cre activity to 14% of the most comparable monocistronic construct, pMC/Cre*/Rfβg.pA (Table 1). However, the relative Cre recombinase activity increased to 44, 41, 42 and 46%, respectively, of the pMC/Cre*/Rfβg.pA Cre activity when each of four mammalian cDNAs, Emy-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 no more 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.

ACKNOWLEDGEMENTS

We thank Michael Klymkowsky, Steven Langer, Jacqueline Lee, Bradley Olwin, Leslie Leinwand, Peter Sarnow, Brian Parr, Joel Sevinsky and Lauren Sompayrac for helpful suggestions and/or reagents. We gratefully acknowledge Christoph Kellendonk and Klaus Rajewsky for providing key reagents. Zachary Baquet, Deborah Sapienza and Frank Bozyan provided valuable technical assistance. This work was supported by the University of Colorado Council on Research and Creative Work and a Burroughs-Wellcome Fund New Investigator in Pharmacology Award to K.R.J.

REFERENCES


NOTE ADDED IN PROOF

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