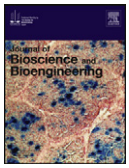

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Accumulative gene integration into a pre-determined site using Cre/*loxP*

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Site-specific gene recombination systems, such as Cre/*loxP*, have been used for genetic modification of cells and organisms in both basic and applied research. We previously developed an accumulative gene integration system (AGIS), in which target gene cassettes could be repeatedly integrated into a pre-determined site on a plasmid or cellular genome by recombinase-mediated cassette exchange (RMCE), using Cre and mutated *loxP*s. In the present study, we designed a simplified AGIS. For gene integration into a target site, the previous system used two *loxP* sites in the acceptor DNA, whereas the new system uses a single *loxP* site. The gene integration reactions were repeated four times *in vitro* using Cre protein and specific plasmids. The expected integration reactions mediated by Cre occurred at the *loxP* sites, resulting in integration of four target genes. The system was also used for genomic integration of reporter genes using Chinese hamster ovary (CHO) cells. The reporter genes were efficiently introduced into the CHO genome in a Cre-dependent manner, and transgene expression was detected after the integration reaction. The expression levels of the reporter genes were enhanced, corresponding to the increase of transgene copy number. Recombinase-mediated AGIS provides a useful tool for the modification of cellular genomes.

[**Key words:** Accumulative site-specific gene integration; Cre/*loxP*; Gene amplification; Chinese hamster ovary (CHO) cells; Transgene expression]

The generation of recombinant animal cells possessing a gene of interest with stable and high expression is an ongoing requirement for biomedicine and biotechnology (1), including use of the cells as tools for biopharmaceutical protein production, virus production, and the generation of transgenic animals. DNA transfection and retroviral vectors have been used to obtain recombinant animal cells with transgenes integrated into their genome. In such gene integration procedures, however, transgenes are randomly integrated into the genome, and the chromosomal position of a transgene cannot be controlled. This uncontrollable integration is likely to cause positional effects in transgene expression (2,3). Furthermore, it is prone to integration at sites where transgene expression is unstable and suppressed during culture, differentiation, and development (4,5). Therefore, targeted integration into a reliable and/or hot spot site for transgene expression is desired for stable and high levels of transgene expression.

Homologous recombination has been used to introduce a transgene into a specific chromosomal locus, if the integration site is pre-determined. Gene-targeted transgenic mice have been generated using embryonic stem cells (ESCs) genetically modified using homologous recombination (6). However, the frequency of homologous recombination, especially in differentiated cells, is extremely low, because the integration event depends on the endogenous repair system (4). Thus, the establishment of gene-targeted cells with homologous recombination is an inefficient and time-consuming process. Therefore, homologous recombination is not a suitable procedure for generating producer cells for recombinant protein production.

One versatile approach for site-specific gene modification is the use of recombinase enzymes such as Cre, Flp, and ϕC31 (7). These enzymes recognize specific DNA sequence motifs (*loxP*, *FRT*, and *attB*/*attP*, respectively) and catalyze efficient DNA rearrangement. Recombinase-based gene modification has been used for genome engineering into a pre-determined chromosomal locus in mammalian cells (7), although the recombination target has to be pre-incorporated into a desired chromosomal locus. Among the recombinases, Cre, derived from bacteriophage P1, is reported to function efficiently in mammalian cells (8). The Cre/*loxP* system has been frequently used as a tool for genetic deletion, because the excision reaction is kinetically favorable compared with the insertion reaction. The reaction characteristics can be changed by using mutated *loxP* sites with one or more base mutations. The *loxP* site is a 34 bp consensus sequence, comprising an 8 bp core spacer sequence and two 13 bp palindromic flanking sequences. When a reaction between *loxP*s with mutations in the arm regions, such as *lox71* (left arm) and *lox66* (right arm) occurs, a double arm-mutated *loxP*, which is seldom recognized by Cre, and a wild-type *loxP* are generated, resulting in the stabilization of the inserted gene (9,10). In addition, when two incompatible *loxP* sites, represented by *lox2272*

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and *lox511* (spacer-mutated *loxP*s), are located adjacent to each other, an exogenous sequence is transferred with another sequence flanked by their *loxP* sites, which is referred to as recombinase-mediated cassette exchange (RMCE) (11–13).

The RMCE procedure enables the integration of target gene sequences into a pre-determined chromosomal locus. RMCE has been used for gene trapping and targeted knock-in of a specific gene in ESCs (14,15). Although multiple genes could be integrated at the same time via RMCE, the integration could not be repeated (16). Repeated use of characterized genomic sites with known expression properties is expected to be highly useful for exogenous gene expression in ESCs. This particularly applies to circumventing influences on integrated gene expression by chromosomal surroundings during differentiation (5), and for gene amplification to achieve a high productivity yield (17,18).

In our previous study, we developed an accumulative gene integration system (AGIS) for integration into a pre-determined site by RMCE using Cre and mutated *loxP*s, which are composed of arm and spacer mutations (19), and demonstrated its use in the production of recombinant antibodies (20). Here, we report the design of an AGIS using a single *loxP* target as the integration site to simplify and facilitate serial gene integration. We evaluated the new system both *in vitro*, using plasmids, and in the genome of animal cells. Upon serial gene integration into the genome, the expression level of the transgenes and the integration efficiency of each reaction were investigated.

MATERIALS AND METHODS

Cells and medium Chinese hamster ovary (CHO-K1) cells (Riken BioResource Center, Tsukuba, Japan) and recombinant CHO cells generated in this study were cultured in Ham's F12 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G potassium, and 0.1 mg/ml streptomycin sulfate (Wako Pure Chemical Industries, Osaka, Japan) at 37°C in a 5% (v/v) CO_2 incubator.

Plasmid construction Schematic representations of plasmid constructs used as the gene acceptor and donors and the *loxP* sequences used in this study are shown in Figs. 1A, 2, and Table 1, respectively.

The construction of the acceptor plasmid pcDNA4/*loxP1* (R1) was described in our previous report (19). R1 contained a zeocin resistant gene (*Zeo*^{r}) as a selection marker.

pBlue/*loxP4*-IRES/EGFP-*loxP2* (19) included an internal ribosomal entry site (IRES) sequence and an enhanced green fluorescent protein (EGFP) gene flanked by *loxP* sites. To remove the gene fragment except for the gene of interest in pBlue/*loxP4*-IRES/EGFP-*loxP2* after integration, the following plasmid was constructed. Chemically synthesized oligonucleotides corresponding to the *loxP7* sites, 5′-TCG AGA TAA CTT CGT ATA GGC TAT AGT ATA CGA AGT TAT GGT AC-3′ and 5′-CAT AAC TTC GTA TAC TAT AGC CTA TAC GAA GTT ATC-3′, were annealed to provide *Xho*I and *Kpn*I sites. The *loxP7* sequence was ligated into the *Xho*I- and *Kpn*I-digested pBlue/*loxP4*-IRES/EGFP-*loxP2* to generate pBlue/*loxP4*-IRES/EGFP-*loxP2*-*loxP7* (R2).

A chloramphenicol resistance gene (*Chl*^{r}) expression unit was amplified by PCR from the plasmid 705-Cre (Gene Bridges, Heidelberg, Germany), using the primers, 5′-CCG CTC GAG CCG AAA AGT GCC ACC TG-3′ and 5′-CCG CTC GAG GGC GTT TAA GGG CAC C-3′, to append *Xho*I sites (underlined) onto the both ends of the PCR product. The PCR was initiated using KOD plus DNA polymerase (Toyobo, Osaka, Japan) at 94°C for 2 min; followed by 30 cycles of amplification at 94°C for 15 s, 51°C for 30 s, and 68°C for 60 s. The amplified PCR product was digested with *Xho*I and ligated into *Xho*I-digested pBlue/*loxP4*-IRES/EGFP-*loxP2*-*loxP7* (R2) to generate pBlue/*loxP4*-IRES/EGFP-*loxP2*-Chl^r-*loxP7* (R2_Chl), which included a *Chl*^{r} expression unit together with an IRES sequence and an EGFP gene.

A blasticidin resistance gene (*Bla*^{r}) fragment prepared from pCEP4/Bla (20) by digestion with *Xba*I and *Mfl*I was ligated into *Xba*I- and *Mfl*I-digested pBlue/*loxP4*-IRES/EGFP-*loxP2* to generate pBlue/*loxP4*-Bla^r/IRES/EGFP-*loxP2* (R2_Bla), which included a *Bla*^{r}, an IRES sequence, and an EGFP gene flanked by *loxP* sites.

The construction of the donor plasmid pBlue/*loxP1*-IRES/DsRed-*loxP5* (R3) was described in our previous paper (19). R3 included an IRES sequence and a red fluorescent protein (DsRed) gene flanked by *loxP* sites.

A kanamycin resistance gene (*Kan*^{r}) expression unit was amplified from pMW219 (Nippon Gene, Tokyo, Japan) using the primers, 5′-CCC AAG CTT AGA ACG CTC ATG TTT GAC AG-3′ and 5′-CGG GAT CCG TTC GGT GTA GGT CGT TCG-3′, to append *Hin*dIII and *Bam*HI sites (underlined) onto the ends of the PCR product. The PCR was initiated using KOD plus DNA polymerase at 94°C for 2 min; followed by 30 cycles of amplification at 94°C for 15 s, 52.5°C for 30 s, and 68°C for 60 s. The amplified PCR product was digested with the corresponding restriction enzymes and ligated into *Hin*dIII- and *Bam*HI-digested pBlue/*loxP1*-IRES/DsRed-*loxP5*, from which a DNA fragment of IRES/DsRed was released, to generate pBlue/*loxP1*-Kan^r-*loxP5* (R3_Kan). The *Bla*^{r} expression unit derived from pLenti6/V5/GW/lacZ (Invitrogen, Carlsbad, CA, USA) was ligated into blunt-ended pBlue/*loxP1*-IRES/DsRed-*loxP5* after digestion with *Bam*HI and *Hin*dIII to generate pBlue/*loxP1*-Bla^r-*loxP5* (R3_Bla). R3_Kan and R3_Bla plasmids contain *Bla*^{r} and *Kan*^{r} expression units flanked by *loxP* sites, respectively.

A neomycin resistant gene (*Neo*^{r}) fragment was amplified from pBlue/*loxP*-Neo^r-*loxP1*-IRES/DsRed-*loxP5* (19) using the primers 5′-CGG GAT CCA TGA TTG AAC AAG ATG GAT TGC-3′ and 5′-CGG GAT CCT CAG AAG AAC TCG TCA AGA AGG-3′, to append *Bam*HI digestion sites (underlined) onto each end of the PCR product. The PCR was initiated using KOD plus DNA polymerase at 94°C for 2 min; followed by 30 cycles of amplification at 94°C for 15 s, 54°C for 30 s, and 68°C for 60 s. The amplified PCR product was ligated into *Bam*HI-digested pBlue/*loxP1*-IRES/DsRed-*loxP5* to generate pBlue/*loxP1*-Neo^r/IRES/DsRed-*loxP5* (R3_Neo). A hygromycin resistance gene (*Hyg*^{r}) fragment was amplified from pCEP4 (Invitrogen) using the primers, 5′-CGG GAT CCA TGA AAA AGC CTG AAC TCA CCG C-3′ and 5′-CGG GAT CCT CAG TTA GCC TCC CCC ATC T-3′ to append *Bam*HI digestion sites (underlined) onto each end of the PCR product. The PCR was initiated using KOD plus DNA polymerase at 94°C for 2 min; followed by 30 cycles of amplification at 94°C for 15 s, 54°C for 30 s, and 68°C for 60 s. The amplified PCR product was digested with *Bam*HI and ligated into *Bam*HI-digested pBlue/*loxP1*-IRES/DsRed-*loxP5* to generate pBlue/*loxP1*-Hyg^r/IRES/DsRed-*loxP5* (R3_Hyg). R3_Neo and R3_Hyg plasmids contain *Neo*^{r} and *Hyg*^{r} upstream of an IRES sequence and a DsRed gene, respectively, flanked by *loxP* sites.

A Cre gene fragment containing a nuclear localization signal derived from SV40 was amplified from pxCANCre (21) using the primers, 5'-AAG GTA CCA TGG CTA GCA GCG GCC CT-3' and 5'-GAA GAT CTC TAA TCG CCA TCT TCC AGC-3' to append *Kpn*I and *Bgl*II digestion sites (underlined) onto the ends of the PCR product. The PCR was initiated using KOD plus DNA polymerase at 94°C for 2 min; followed by 30 cycles of amplification at 94°C for 15 s, 54°C for 30 s, and 68°C for 60 s. The amplified PCR product was digested with corresponding restriction enzymes and ligated into *Kpn*I- and *Bgl*II-digested pCEP4 to generate pCEP4/NCre. pCEP4/NCre was used as a Cre expression plasmid.

All DNA sequences derived from chemically synthesized oligonucleotides and PCR products in this study were confirmed by DNA sequencing using a Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

***In vitro* accumulative gene integration using the Cre protein** An acceptor plasmid (R1, R12_Chl, R123_Kan, or R1232_Chl), a donor plasmid (R2_Chl, R3_Kan, or R3_Bla) and a Cre protein solution (Creator DNA Cloning Kits; Clontech, Palo Alto, CA, USA) were mixed in a microtube and *in vitro* recombination was performed as described previously (19). Briefly, an acceptor plasmid and a donor plasmid were mixed at an equal molar ratio (0.6 pmol) in a microtube. The Cre protein solution (1 μl), 10× Cre reaction buffer, and 10× bovine serum albumin (BSA) were then added. The total volume of the reaction mixture was 10 μl. The integration reaction was performed at 8 cycles of 32°C for 60 min and 37°C for 60 min; the Cre protein was inactivated by heating the tube at 70°C for 5 min.

Competent *Escherichia coli* cells (DH5α strain) transformed with the reaction mixture were pre-incubated at 30°C for 90 min in LB medium (10 g/l polypeptone, 5 g/l yeast extract and 10 g/l NaCl, pH7.2). They were then plated on the LB agar-plates (10 g/l polypeptone, 5 g/l yeast extract, 5 g/l NaCl, and 15 g/l agar, pH7.5) containing 100 μg/ml ampicillin (Sigma-Aldrich), 50 μg/ml zeocin (Invitrogen), and 200 μg/ml chloramphenicol (Wako Pure Chemical Industries) for the first and third integration reactions, or 100 μg/ml kanamycin (Wako Pure Chemical Industries) and 100 μg/ml Blasticidin S (Invitrogen) for the second and fourth integration reactions, respectively. The plated dishes were incubated at 30°C overnight to allow the formation of colonies. The colonies were picked and incubated in 2 ml of LB medium containing corresponding antibiotics at 30°C overnight. The plasmids extracted from each clone were digested with restriction enzymes to confirm the integration reaction. The successful plasmids were used for plasmid size analysis after digestion with *Mlu*I (single-digestion) and for DNA sequencing analysis for target sites.

Accumulative gene integration in the genome of CHO cells The targeted gene integration into the genome of CHO cells using Cre-mediated AGIS was performed as described previously (19,20). First, a recipient founder CHO cell line containing the R1 plasmid sequence in its genome was established (CHO/R1). The R1 plasmid was linearized by digestion with *Pvu*I and was transfected into CHO-K1 cells using a lipofection reagent (Lipofectamine 2000; Invitrogen), according to the manufacturer's protocol. At 48 h post-transfection, the cells were cultured in a selective medium containing 200 μg/ml zeocin to screen for stable transfectants. Genomic integration of the plasmid sequence was confirmed by PCR. A CHO/R1 cell line containing single copy of the R1 sequence was selected by Southern blotting (data not shown). The scheme for accumulative gene integration into CHO/R1 cells is summarized in Fig. 2. For transfection of donor plasmids in each gene integration reaction, the cells were seeded at a density of 1.2×10^5 cells in 0.5 ml medium in the wells of 24-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany). After 24 h of cell seeding, the cells were co-transfected with the Cre expression plasmid (pCEP4/NCre) [5 ng] and the donor plasmid [800 ng], using a lipofection reagent (Lipofectamine 2000). For the first and third reactions in the cycle of gene integration, R2 and R2_Bla were used as the donor plasmids, respectively. For the second and fourth reactions, R3_Neo and R3_Hyg were used as the donor plasmids, respectively. For first integration of R2 into CHO/R1 cells, EGFP-positive colonies were selected as targeted integration cell clones (CHO/R12). For the second integration reaction using R3_Neo plasmid and CHO/R12 cells, the transfected cells (CHO/R123) were screening in medium containing 500 μg/ml G418 (Sigma-Aldrich). In a similar fashion, the third and fourth integration