

A rapid and efficient method to generate multiple gene disruptions in *Dictyostelium discoideum* using a single selectable marker and the Cre-loxP system

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ABSTRACT

Dictyostelium discoideum has proven an exceptionally powerful system for studying numerous aspects of cellular and developmental functions. The relatively small (~34 Mb) chromosomal genome of *Dictyostelium* and high efficiency of targeted gene disruption have enabled researchers to characterize many specific gene functions. However, the number of selectable markers in *Dictyostelium* is restricted, as is the ability to perform effective genetic crosses between strains. Thus, it has been difficult to create multiple mutations within an individual cell to study epistatic relationships among genes or potential redundancies between various pathways. We now describe a robust system for the production of multiple gene mutations in *Dictyostelium* by recycling a single selectable marker, Blastidicin S resistance, using the Cre-loxP system. We confirm the effectiveness of the system by generating a single cell carrying four separate gene disruptions. Furthermore, the cells remain sensitive to transformation for additional targeted or random mutagenesis requiring Blastidicin selection and for functional expression studies of mutated or tagged proteins using other selectable markers.

INTRODUCTION

The eukaryote *Dictyostelium discoideum* has many advantages that make it amenable to molecular manipulation for the analyses of gene function (1,2). The chromosomal genome of *Dictyostelium* is haploid and relatively small (~34 Mb), although its transcriptome contains ~11 000 genes (2). Targeting mutations in non-essential genes by homologous recombination is often at a relatively high frequency (>20%) and methods for generating genome-wide mutations by insertional (REMI) mutagenesis and for recovering the

targeted genes are also well developed (3). In addition, many transformation vectors exist for the temporal- or spatial-specific expression of mutated or tagged proteins. *Dictyostelium* grow as individual cells, but initiate a multicellular developmental program upon starvation (1,2). Thus, growth and development are distinct and separable events, facilitating the isolation and maintenance of strains carrying gene mutations that are required for the development but not for the growth.

Dictyostelium transformants can be selected using auxotrophy (uracil, thymidine) or antibiotic [neomycin (Neo, G418), Blastidicin S and hygromycin] resistance (4). Auxotrophic selection requires cells with specific mutations and growth in specialized media. The G418 selection often yields cells carrying concatamerized, transforming DNA fragments and sometimes multiple insertions (5). In contrast, Blastidicin-resistance (Bsr) selection is very efficient, tolerates no background growth in standard medium and generally results in cells carrying only single-copy integrants (5,6). Consequently, most targeted and REMI mutageneses in *Dictyostelium* now utilize Bsr. However, since genetic crossing in *Dictyostelium* is inefficient, it is not a simple procedure to generate strains carrying multiple mutations (7). Yet, such multiple mutated strains are critical for studying the interaction among various genes or for examining functional genetic redundancies. Furthermore, strains carrying multiple mutations must still be sensitive to further transformation for expression studies of tagged (e.g. GFP and RFP) or mutated proteins for the analyses of complex and dynamic processes. Finally, in the absence of additional selection procedures, it is not possible to utilize a REMI screen for genetic suppression of Bsr-generated gene disruptants.

To overcome these genetic limitations, we significantly simplified strategies developed for directed recombination/deletion between specific DNA sequences to facilitate the targeted disruption of multiple genes in a single cell. We have adapted the Cre-loxP recombination system (8,9) for use in *Dictyostelium* to permit the recycling of Bsr and the continuous generation of new targeted mutations. Briefly, a gene targeting construct was created that contains a

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Bsr-cassette flanked (floxed) by *loxP* recombination sites; translational stop codons in all six reading frames were also added outside the *loxP* sites. Transient expression of the Cre recombinase removes the Bsr expression cassette through intramolecular recombination but does not remove the translational stops. This event creates a nonsense mutation within the targeted gene in a Blasticidin-sensitive cell line. We have confirmed the molecular recombination events by sequence analyses and proved the robustness of the system by creating cell lines with multiple targeted mutations.

MATERIALS AND METHODS

The floxed-*Bsr* targeting vector pLRBLP

The *loxP* recombination site (Figure 1A) consists of two highly conserved inverted repeats separated by a variable spacer region (10). The spacer region determines the specificity of *loxP* recombination (11). Only *loxP* sites separated by identical spacer sequences can recombine.

The relative orientation of the spacer between two *loxP* sites determines the type of recombination event (11). Intramolecular recombination between two *loxP* sites in the same orientation will circularize and delete the intervening sequences, leaving a single *loxP* site (8,9,11).

Two *loxP* sites were engineered in the same orientation within the pBluescriptKS plasmid and six stop codons in all six reading frames were placed outside of the *loxP* sites (Figure 1B). Finally, the *Dictyostelium Bsr* expression cassette (*act15/Bsr*) was inserted between the two *loxP* sites to create the floxed-*Bsr* gene disruption vector pLRBLP (Figure 1B). Transcriptional regulation of *Bsr* is under control of the *Dictyostelium actin 15* promoter and the *Dictyostelium actin 8* terminator (6). The entire fragment is 1.5 kb and is flanked by *SmaI* sites, permitting the simple cloning into any gene of interest (Figure 1B). Unique cloning sites are also present: *KpnI-SalI-AccI-ClaI-HindIII-SmaI-act15/Bsr-PstI-SmaI-BamHI-SpeI-NotI*

The pLRBLP vector has been deposited with the Dicty Stock Center for distribution (see <http://dictybase.org/StockCenter/StockCenter.html>).

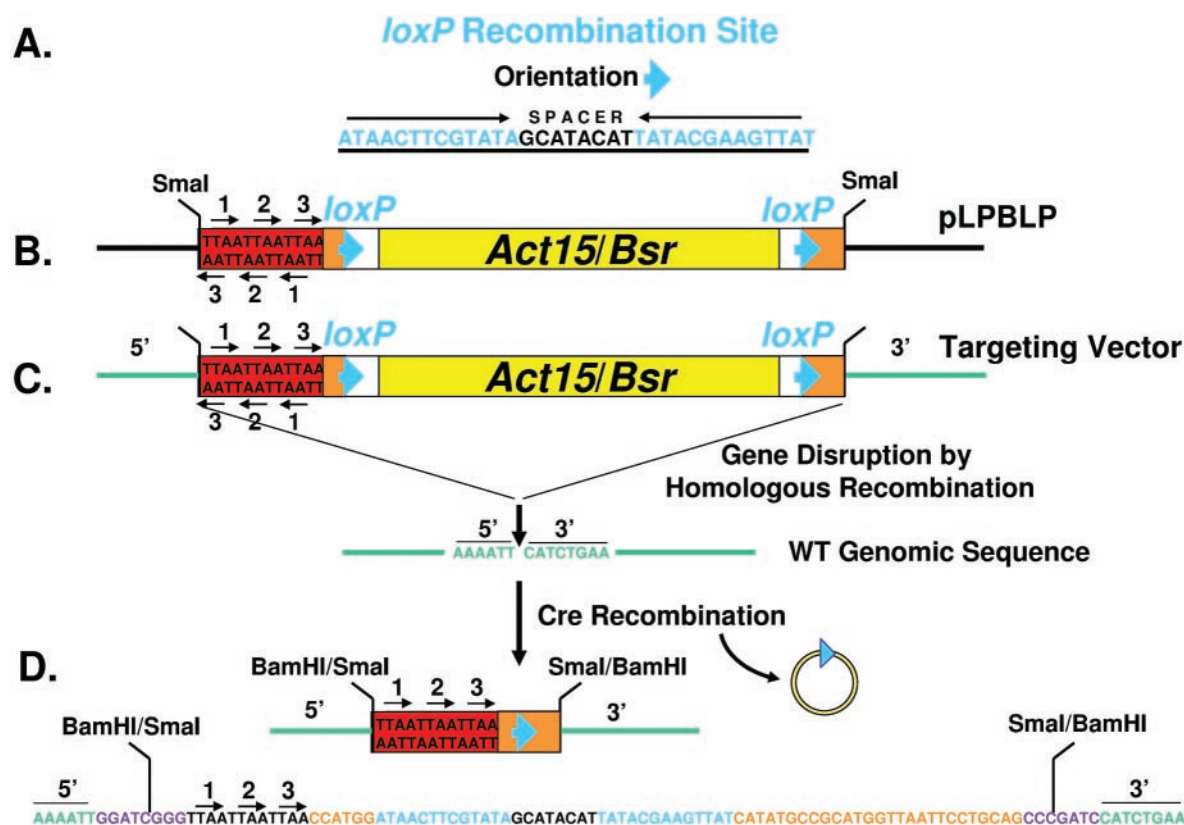


Figure 1. A strategy for Cre-*loxP* recycling of the Bsr selectable marker. (A) The *loxP* recombination site includes an inverted repeat separated by a spacer sequence. (B) The gene targeting vector pLRBLP was constructed with *loxP* sites in the same orientation flanking both sides of the Bsr expression cassette *act15/Bsr*. An oligonucleotide cassette was also added that had translational stop codons in all six reading frames. The restriction enzyme sites outside of the floxed-*Bsr* cassette, such as *SmaI* (see Materials and Methods), permits the cloning of 5' and 3' gene sequences for targeted disruption. (C) The floxed-*Bsr* cassette of pLRBLP was inserted into the gene DDB0183838 sequence as indicated (see also Figure 2A). A *BamHI* site was engineered into the targeted gene sequence. This was filled and blunt-end ligated to the *SmaI* sites flanking the floxed-*Bsr* cassette to generate the gene-targeting vector. WT cells were transformed for gene disruption by homologous recombination and selected for resistance to Blasticidin S. (D) Transient expression of Cre promotes recombination and deletion of sequences between the two *loxP* sites in the disrupted gene DDB0183838. A 73 nt sequence remains that includes the translational stop cassette and a single *loxP* site. The sequence presented was determined directly from a PCR-amplified fragment of the region (see Figure 2B) and matches the predicted recombination event. The disrupted target gene sequences are in green, the *loxP* site is underlined and the stop codons are indicated by arrows. The site of the *BamHI/SmaI* fusion is also indicated.

Transformation for homologous recombination

The floxed-*Bsr* cassette of pLPBLP was used to engineer various gene-targeting disruption constructs by standard cloning procedures. The DNA-mediated transformation was with linear vectors (4) and clonal selection (10–14 days) used 10 µg/ml Blasticidin S (MP Biomedicals) (6). Genomic DNA was purified (High Pure PCR Template Kit; Roche), and targeted gene disruptions were preliminarily identified by PCR. Several PCRs were performed. One PCR design used a primer from within the *Bsr* cassette and another primer outside of the targeting fragment. Another PCR examined the presence of the wild-type or disrupted gene using primers that flanked the floxed-*Bsr* insertion site. Southern blot hybridizations were used for absolute confirmation of gene disruption.

The *Dictyostelium* Cre expression vector pDEX-NLS-cre

We used a Cre protein (NLS-cre) engineered with a nuclear localization signal to promote high efficiency *in vivo* recombination between *loxP* sites. The coding region of *NLS-cre* was amplified from plasmid pTZ-Cre (12) with a 5'-flanking sequence that had been optimized for *Dictyostelium* A+T bias. The *NLS-cre* fragment was cloned into the EcoRI site that lies downstream of the *Dictyostelium actin 15* (*act15*) promoter in the *Dictyostelium* expression vector pDEXRH (13), which also carries a G418-resistance cassette. The resulting Cre-expressing plasmid was termed pDEX-NLS-cre.

The pDEX-NLS-cre vector has been deposited with the Dicty Stock Center for distribution (see <http://dictybase.org/StockCenter/StockCenter.html>).

Cre transformation/expression and selection

Bsr knockout mutants were electroporated (4) with pDEX-NLS-cre. Approximately 10⁷ growth-phase cells were washed at 0°C, once in 17 mM K-phosphate buffer, pH 6.0 and once in electroporation buffer (EP; 50 mM sucrose, 10 mM Na-K-phosphate buffer, pH 6.1). The cell pellet was then resuspended in 700 µl EP and the suspension gently mixed with ~35 µg pDEX-NLS-cre DNA at 0°C in a 4 mm electroporation cuvette. Electroporation was for 1 ms, at 1 kV and 10 µF with the Gene Pulser Xcell (Biorad); two pulses were applied at 5 s intervals. After electroporation, the cells were transferred to a 9 cm plastic petri dish and shaken at ~40 r.p.m. for 15 min at 20°C. The suspension was adjusted to 2 mM CaCl₂ and 2 mM MgCl₂ and shaking was continued for another 15 min. Finally, 12 ml axenic growth medium (14) was added, and the cells were allowed to recover for 24 h.

After the 24 h recovery period, the cells were placed in axenic medium (14) containing 10–20 µg/ml of G418 (Invitrogen Life Technologies) to select for pDEX-NLS-cre transformants. Selection in G418 continued for 3–10 days. The cells were then plated for clonal isolation by standard dilution. The clonal cell lines were subsequently picked in replica onto two different plates. The first was a standard SM agar plate (14) using growing *Klebsiella aerogenes* (*K.a.*) as the nutrient source. The second was a non-nutrient phosphate (30 ml) agar plate (17 mM Na/K-phosphate, pH 6.0 in 1.5% agar) layered with 500 µl of a concentrated *K.a.* suspension in Na/K-phosphate buffer and 120 µl of Blasticidin S at 10 mg/ml; the final concentration of Blasticidin S in the agar plate was 40 µg/ml. All *Dictyostelium* cells grow on SM *K.a.* plates, but

only *Dictyostelium* that have retained the *Bsr* cassette grow rapidly in the presence of Blasticidin S. A WT parental cell was always used for growth control. This selection procedure allows for the rapid screening of hundreds of Cre transformants.

Cells not growing in Blasticidin S were replica plated into 24 well microtitre plates containing either axenic media, axenic media with Blasticidin S or axenic media with G418. Each transformation gave different frequencies for selective and optimized growth. Genomic DNA from Blasticidin- and G418-sensitive cell lines was analyzed by PCR, Southern blot hybridization and/or sequencing of PCR-amplified fragments to confirm Cre recombination and also the absence of both the *Bsr* and *Neo* expression cassettes. Usually, >95% of cells selected by growth criteria showed appropriate Cre recombination. This method was used to multiply target four diaphanous-related formin (*drf*) genes: *drf1* on chromosome 5, accession no. J812237; *drf2* on chromosome 4, accession no. AJ748258; *drf3* on chromosome 1, accession no. AJ812236; and *drf4* on chromosome 3, accession no. AJ812734.

As an alternative, one can bypass the entire G418 and Blasticidin S selections, and directly analyze clonal populations of cells after transient Cre expression. Following electroporation, the cells were clonally diluted into microtitre dishes at 0.5 cells/well and grown in the absence of Blasticidin S or G418 selection. While this strategy requires less time, it is also less efficient; in general, <5% of the colonies isolated may be pure Cre recombinants. This approach was used to multiply target DDB0183838 and DDB0184318 (see <http://dictybase.org/>), separated by 1.2 Mb on chromosome 6.

Finally, it should be noted that we were unable to promote *in vivo* recombination between *loxP* sites by direct electroporation of Cre protein. Our approach was modeled after the success observed with restriction enzymes during REMI mutagenesis (3), but we did not exploit all possible experimental variations.

RESULTS

Validation of Cre-mediated recombination

We created a disrupting gene construct of DDB0183838 (see Figure 1), transformed it into *Dictyostelium*, selected cells for *Bsr* growth and isolated individual colonies. Genomic DNA was prepared from the clonal isolates and homologous recombination monitored by PCR using primers within the gene sequence that lay outside of the floxed-*Bsr* insertion (Figure 1C). The endogenous, WT control band was ~450 bp, while the gene containing the floxed-*Bsr* insert was ~2000 bp (Figure 2A). Southern blot hybridization was used to confirm gene disruptions and the absence of secondary sites of insertion. The frequency for this targeted disruption was ~80%.

A *Bsr* knockout cell line was chosen and transformed transiently to express NLS-cre. The cells were then clonally diluted into microtitre dishes at 0.5 cells/well and grown in the absence of Blasticidin S or G418 selection. PCR was used to assess the loss of *Bsr* sequences via recombination between *loxP* sites (Figure 1D). The Cre-mediated recombinant fragment is ~520 bp, 73 bp larger than the endogenous, WT 450 bp band (Figures 1D and 2B). To confirm the

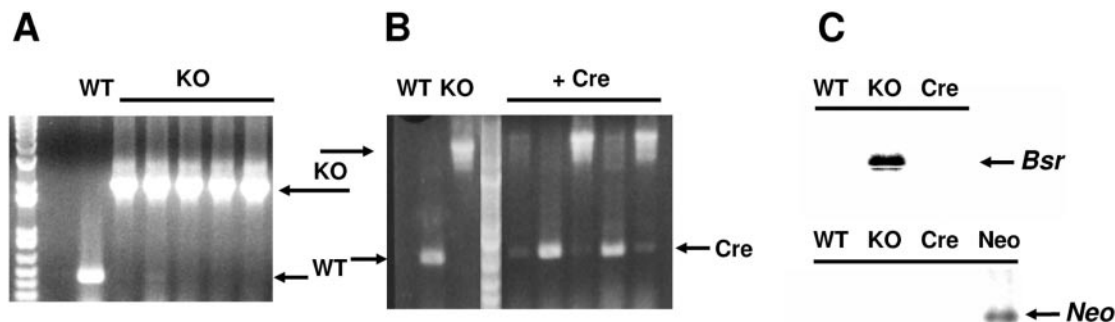


Figure 2. Cre recombination deletes the *Bsr* selectable marker. (A) Genomic sequences flanking the insertion site of gene DDB0183838 depicted in Figure 1C were amplified by PCR and analyzed by agarose gel electrophoresis. WT is the endogenous wild-type gene sequence of ~450 bp. Knockout mutants (KO) are clonal isolates of cells transformed and selected for resistance to Blasticidin S; insertion of the floxed-*Bsr* cassette yields a fragment of ~2 kb. (B) A KO isolate from Figure 2A was transformed for transient expression of Cre, and several clonal isolates were randomly screened for functional recombination by PCR as in Figure 1A. Wild-type is the endogenous WT gene sequence of ~450 bp. KO is the gene sequence carrying the insertion of the floxed-*Bsr* cassette (~2 kb). Recombination between *loxP* sites generates a fragment that is ~520 bp. The fragment was sequenced for confirmation (see Figure 1D). (C) Genomic DNA from WT, KO and Cre cells were probed by southern blot hybridization for the presence of *Bsr* and *Neo* gene sequences. DNA from a *Neo*-expressing transgenic cell line was included as a control.

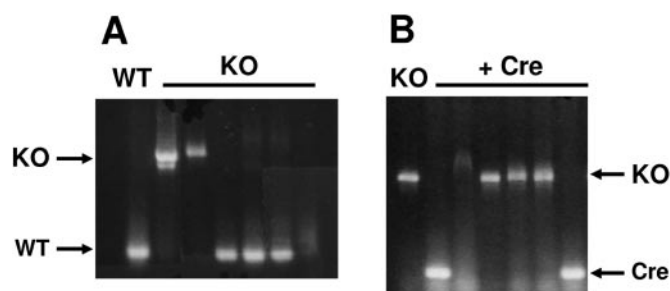


Figure 3. Cre-recombined cells are susceptible to additional *Bsr* recycling. (A) A Cre-recombinant isolate from Figure 2B was transformed for a secondary gene disruption within DDB0184318 and selected for resistance to Blasticidin S. Genomic sequences flanking the insertion site of gene DDB0184318 were examined by PCR amplification. WT is the endogenous wild-type gene sequence; KO mutants are selected clonal isolates with the insertion of the floxed-*Bsr* cassette. (B) A KO isolate from Figure 3A was transformed for transient expression of Cre, and several clonal isolates were randomly screened for functional recombination by PCR as in Figure 2A. KO is the gene sequence carrying the insertion of the floxed-*Bsr* cassette. Recombination between *loxP* sites generates a fragment that is only ~70 bp larger than the WT sequence.

specificity of the recombination event, we sub-cloned and sequenced the WT and Cre-generated fragments. As seen in Figures 1C and 1D, there was an exact recombination between the *loxP* sites. The *Bsr* cassette was removed but the in-frame stop codons and a single *loxP* site remained. This created a nonsense mutation in the gene. The resulting cell line was unable to grow in either Blasticidin S or G418, indicating that neither *Bsr* of pLPBLP nor *Neo* of the Cre plasmids were integrated into the genome. This conclusion was confirmed by Southern blot hybridization (Figure 2C). This simple Cre recombination and selection approach had an efficiency of ~3% for the isolation of pure populations of Cre-recombined cells. An alternative approach using G418 selection (see Materials and Methods) and described below for *drf* gene targeting can increase this frequency to >95%.

Validation of *Bsr* recycling

We next tested the efficacy of Cre-*loxP* for recycling of Blasticidin S selection. A second gene, DDB0184318, disruption fragment was prepared using pLPBLP. This was used to transform the Cre recombination, Blasticidin-sensitive cell line of Figure 2B. The frequency for the second site disruption was ~20% (Figure 3A). The same frequency

was obtained using a WT, parental cell line. Secondary, Cre transformation of the double knockout cell line was able to drive recombination within the new floxed-*Bsr* insertion (Figure 3B).

Highly efficient generation of multiple gene disruptions in *Dictyostelium*

The *Dictyostelium* genome contains six *drf* genes (2,15). To test the robustness of the Cre-*loxP* system, we chose four *drf* genes (*drf1*, *drf2*, *drf3* and *drf4*) for targeted disruption through four alternating rounds of gene inactivation followed by *Bsr* deletion through transient expression of NLS-cre. As depicted in Figure 4A and exemplified for *drf1*, each targeting vector contained the floxed-*Bsr* cassette inserted between 5' and 3' sequences that were specific for each *drf* gene. After transformation with linearized constructs, Blasticidin-resistant, *drf* knockout mutants were identified by PCR using sequence-specific primers and genomic DNA of clonal isolates (see Figure 4B). The floxed-*Bsr* cassette of *drf*-null cells was deleted by transient transfection using pDEX-NLS-cre, initial growth in the presence of G418, and final screening on selective *K.a.* plates (see Materials and Methods). Genomic

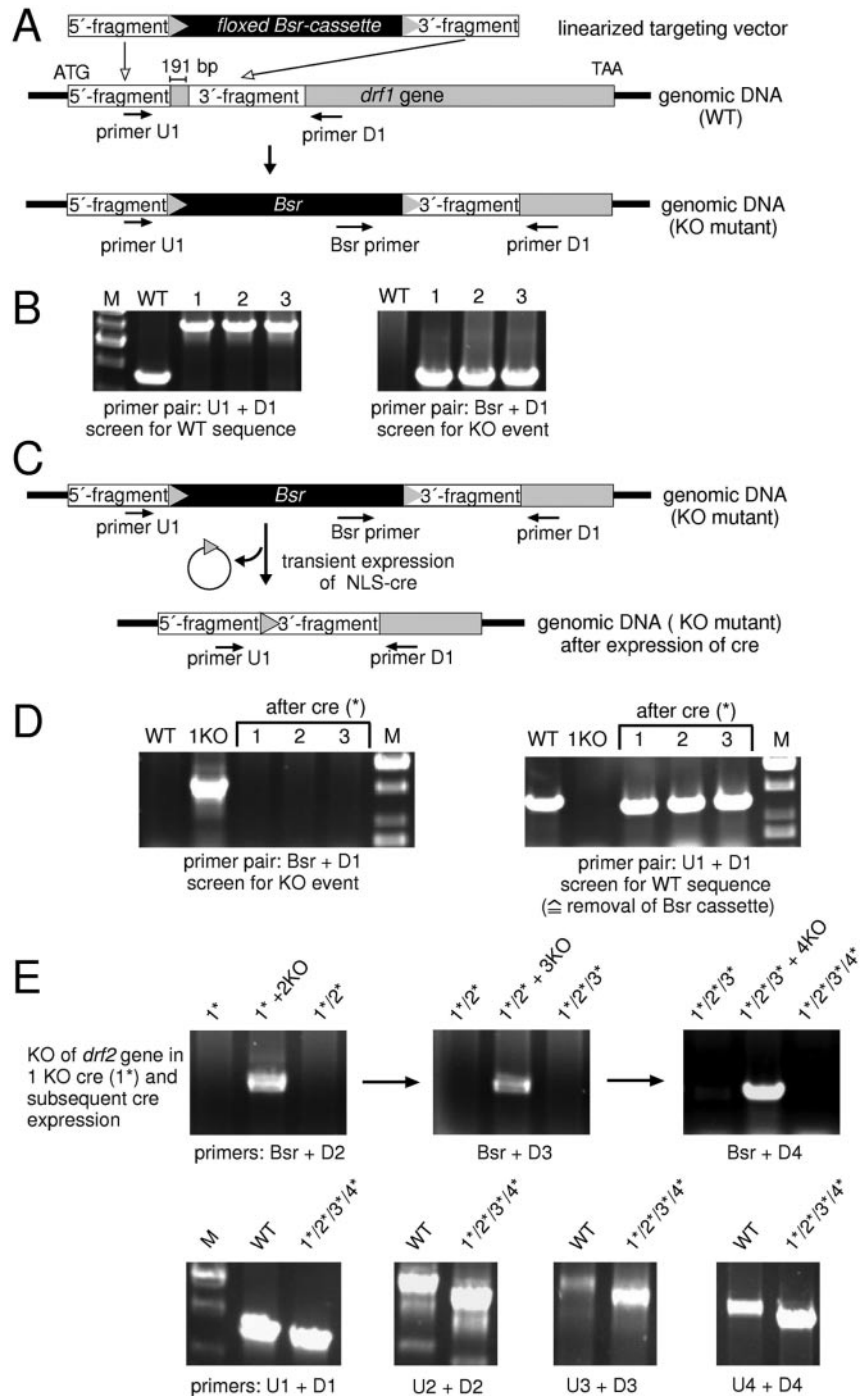


Figure 4. Strategy for the generation of a quadruple *drf*-null cell line. (A) Generation of a *drf1*-null cell line. The 5' and 3' specific sequences of the *drf1* gene were cloned into pLPBLP. The linear targeting vector was then used to disrupt the *drf1* gene by homologous recombination. Targeted integration also caused a small deletion in the gene. A similar approach was used to construct targeting vectors for *drf* genes 2, 3 and 4. (B) WT cells and three independent *Bsr* mutants were examined by PCR amplification employing two different sets of *drf1* primers. *Left panel*: the primer combination of U1 and D1 (see A) identifies WT and homologously recombined *drf1* sequences. Upon homologous recombination, the *drf1* PCR product is ~1.3 kb larger than that of WT for all three cell lines shown. *Right panel*: the primer combination of *Bsr* and D1 (see A) specifically identifies only the homologous recombination event. (C) Strategy for deletion of *Bsr* by transient expression of NLS-cre. Deletion of the floxed-*Bsr* cassette leaves a sequence of ~70 nt. (D) PCR analysis of *drf1*-null derived cells following transient expression of NLS-cre (*). All three clonal cell lines that were sensitive to Blasticidin and G418 for growth and were also deleted of the floxed-*Bsr* cassette. (E) Elimination of three additional *drf* genes. *Top panel*: three additional alternating rounds of gene inactivation followed by the subsequent elimination of the floxed/*Bsr*-cassette through transient expression of NLS-cre were performed to obtain a single mutant cell line lacking four *drf* genes. Each PCR screen used a common *Bsr* upstream primer and a downstream primer that was specific to *drf* genes 2, 3 or 4 (see A). *Bottom panel*: DNA from WT cells and the quadruple mutant $1^{*}/2^{*}/3^{*}/4^{*}$ was amplified by PCR using U and D primers specific to each *drf* gene to illustrate the stability of the four single *loxP* sites in the genome of the quadruple mutant. Each of the original homologous recombination events was designed to delete sequences from the endogenous gene (see A). Following the Cre-mediated recombination, an additional ~70 bp remains that includes the translational stop cassette and a single *loxP* site (see Figures 1 and 4C), but still, the PCR products amplified from each gene of the $1^{*}/2^{*}/3^{*}/4^{*}$ mutant are always smaller than that of the WT gene fragment.

DNA from transformants that were sensitive to both Blasticidin S and G418 was assayed by PCR in the absence of the *Bsr* (Figure 4C and D) and *Neo* cassettes (see also Figure 2C).

This strategy proved to be successful for the elimination of three additional *drf* genes in the same cell line (Figure 4E). As a final step of confirmation, we analyzed the rearrangements of the four *drf* genes in the quadruple mutant. Each locus was amplified with U and D specific primers (see Figure 4A), and their PCR products were compared with those obtained from WT genomic DNA. Since *drf* gene fragments were deleted during each of the targeted homologous recombination events (see Figure 4A), the PCR products amplified from the mutant DNA were always smaller than those from WT.

DISCUSSION

We have demonstrated a highly effective system to recycle a single selectable marker for the generation of multiple gene disruptions within a single cell. The technique described greatly surpasses the efficiencies of approaches that rely on parasexual genetics or fortuitous molecular events dictated by co-transformation for isolating *Dictyostelium* with multiple mutations (7,16).

The difficulty to generate *Dictyostelium* cell lines with multiple gene mutations has restricted the analyses of complex biochemical and genetic pathways and the identification of processes controlled by multigene families or functional redundancy (17). We believe that the described *Bsr* recycling approach largely overcomes this limitation. The cells deleted of *Bsr* are sensitive to additional rounds of gene disruption or to REMI mutagenic screening. In addition, the cells can be engineered for the regulated expression of specific protein variants, for global screening, for complementation expression or for gene interference by RNAi or antisense methodologies (3,18–20).

There are some potential engineering confines that should be considered when designing targeting constructs using the floxed-*Bsr* system. Gene disruptions that carry the entire *Bsr* cassette usually do not produce a stable transcript or protein fragment. However, we frequently detect normal levels of mRNA expression after the floxed-*Bsr* is removed via Cre recombination. Although the mRNA now encodes an in-frame nonsense codon that prevents the synthesis of a full-length protein, there is high probability for the production of truncated protein variants. The position of the engineered nonsense codon within an individual gene dictates the protein variant produced, and different protein truncations can cause distinct phenotypic effects. This feature can be used to an advantage if one wishes to examine the function of proteins with specific C-terminal truncations; otherwise floxed-*Bsr* insertions should be designed near the 5' end of the gene. Parental and Cre-recombined cells must always be compared to ensure that *Bsr* deletion (or Cre-transformation) has not created a dominant (or secondary) phenotype.

After each successful Cre recombination event, one *loxP* site will remain in the genome. If two *loxP*-targeted genes were located on the same chromosome, there is a theoretical potential for Cre-mediated recombination between the *loxP* sites of the two genes and thus, for deletion of the entire

genomic region between them. However, since *Dictyostelium* has a haploid genome, large deletions will be lethal, making such recombinations unlikely to be a common occurrence. Indeed, we were able to successfully disrupt genes DDB0183838 and DDB0184318 that are located on the same chromosome, but separated by ~1.2 Mb. While small deletions are possible, they will be readily identified during genomic mapping by the PCR or southern blot hybridizations that are required to confirm the desired, site-specific recombination event. Nonetheless, there are simple modifications that can be used to prevent recombination between genes. The spacer sequence of the *loxP* site conveys recombination specificity (11). Thus, different floxed-*Bsr* cassettes will not recombine. Alternatively, the Cre-*loxP* system can be coupled with other recombination systems, such as Flp-*FRT* and ϕ C31-*att* (9), for increased specificity and flexibility.

The Cre-*loxP* approach should also be easily adapted for many of the applications proven so effective for mouse genetics (8,9). Gene constructs can be engineered that become disrupted only upon the conditional expression of Cre. Many *Dictyostelium* transformation vectors already exist that direct gene expression with specific developmental patterns. In addition, the expression can also be regulated by a tetracycline-responsive system (21). Thus, it should be possible to study the effects of gene loss at specific developmental stages or in specific cell types. We do not believe that high-level expression of Cre will cause deleterious biological effects in *Dictyostelium* by inducing recombination within WT chromosomes. We have not observed any abnormal phenotypes upon the expression of Cre from a constitutive promoter (data not shown). Furthermore, sequence analysis of the entire *Dictyostelium* genome does not reveal any endogenous cryptic *loxP* sites, as are suggested to occur in genomes of higher complexity (22).

Conditional Cre expression can also be used to provide an additional layer of transcriptional regulation during development. Recombination between *loxP* sites can be used to induce gene expression by directly fusing a promoter and gene target separated by a floxed inactivating sequence, or conversely, to promote gene repression by deleting an element essential for transcription.

The utility of Cre-*loxP* recombination is also not limited to *Bsr* recycling for gene inactivation. Once *loxP* sites have been inserted into a gene of interest, it now becomes a site for recombination with exogenous transforming DNA elements for gene replacement studies (8,9,11). The *loxP* constructs are thus facile targets for the creation of gene knockins for the expression of mutant or tagged protein variants or novel promoter/reporter fusions.

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