Research Report

loxP-Directed Cloning: Use of Cre Recombinase as a Universal Restriction Enzyme

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ABSTRACT

We have developed a novel way to use the Cre/loxP system for in vitro manipulation of DNA and a technique to clone DNA into circular episomes. The method is fast, reliable, and allows flexible cloning of DNA fragments into episomes containing a loxP site. We show that a loxP site can serve as a universal target site to clone a DNA fragment digested with any restriction enzyme(s). This technique abolishes the need for compatible restriction sites in cloning vectors and targets by generating custom-designed 5′, 3′, or blunt ends in the desired orientation and reading frame in the vector. Therefore, this method eliminates the limitations encountered when DNA fragments are cloned into vectors with a confined number of cloning sites. The 34-bp loxP sequence assures uniqueness, even when large episomes are manipulated. We present three examples, including the manipulation of a bacterial artificial chromosome. Because DNA manipulation takes place at a loxP site, we refer to this technique as loxP-directed cloning.

INTRODUCTION

Many of the revolutionary changes that have occurred in the biological sciences over the past 20 years can be directly attributed to the manipulation of DNA in defined ways using recombinant DNA technology. Central to this technology is the ability to insert defined fragments of DNA into bacterial or viral vectors that can then be propagated in large quantities.

Cloning vectors commonly contain a stretch of DNA sequence recognized by a series of restriction endonucleases, called multiple cloning sites or polylinkers. Multiple cloning sites are usually positioned at a prominent region within the vectors, often between a promoter and a termination sequence, and allow the use of various restriction enzymes to clone the DNA of interest. Unfortunately, the transfer of a cloned DNA fragment from one vector to another is often difficult because the two vectors do not contain mutually compatible cloning sites. Moreover, many recognition sites for restriction enzymes occur more than once in the vector, making the cloning exercise more difficult. For these reasons, the utility of most restriction enzymes for the manipulation of large constructs is generally limited.

An alternative to DNA manipulation with restriction enzymes is the use of site-specific recombinases. In particular, Cre recombinase has proven to be a valuable enzyme to manipulate DNA. It is easy to purify (5) and is commercially available. Cre recombinase can linearize circular DNA episomes at its 34-bp recognition target sequence (loxP) in the presence of a linear DNA segment that also harbors a loxP site, typically in the form of two annealed oligonucleotides (6,12). Cre recombines the short linear segment into the circular DNA episomes, thereby linearizing the episome (Figure 1). Hence, Cre can act like a restriction enzyme that recognizes a 34-bp recognition sequence. However, in contrast to most classical restriction enzymes, the protruding ends generated are not predetermined. Instead, Cre recombines the circular episome and the linear DNA at the loxP sites, independent of the composition of the DNA ends presented by the short linear DNA. We therefore reasoned that Cre can be used as a universal restriction enzyme to generate any 5′, 3′, or blunt end by the careful design of loxP-containing oligonucleotides (Figure 1). A DNA fragment that presents compatible ends with the overhangs of the annealed loxP oligonucleotides can be ligated into the vector. The procedure described here provides a versatile and highly efficient strategy by which DNA fragments can be cloned into vectors without references to the presence or location of restriction sites.

MATERIALS AND METHODS

DNA Techniques

Plasmid DNA was purified using plasmid preparation kits (Qiagen, Valencia, CA, USA). Restriction endonucleases were purchased from Roche Molecular Biochemicals (Indianapolis,

IN, USA) and used according to the manufacturer's instructions. DNA fragments were excised from 0.7% agarose gels, purified using the QIAquick™ gel-extraction kit (Qiagen), and incubated overnight for ligation at 20°C using T4 ligase (Roche Molecular Biochemicals). The primers used in the colony-PCR assay on the bacterial artificial clones (BACs) were: BAC-1, 5′-CGATACCTGCGTCATAAT-3′ and BAC-2, 5′-GCAGCACATCCCCCT-TTC-3′. The PCR program was as follows: 30× (94°C for 1 min, 58°C for 1 min, and 72°C for 2 min) and 72°C for 7 min. Annealing of loxP oligonucleotides were performed at 10 pmol concentration in annealing buffer (100 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM EDTA) by heating to 95°C for 10 min, followed by slow cooling to room temperature over a period of 3 h.

Plasmids and Strains

Vector pSV-XZ was generated by growing the plasmid pSVpaX1 (5) in the *E. coli* strain 294-Cre (3) and is therefore the recombined version of pSVpaX1, which contains only one loxP site. Plasmid pEGFP-X was generated by cloning a loxP oligonucleotide in frame into the enhanced green fluorescent protein (EGFP) of

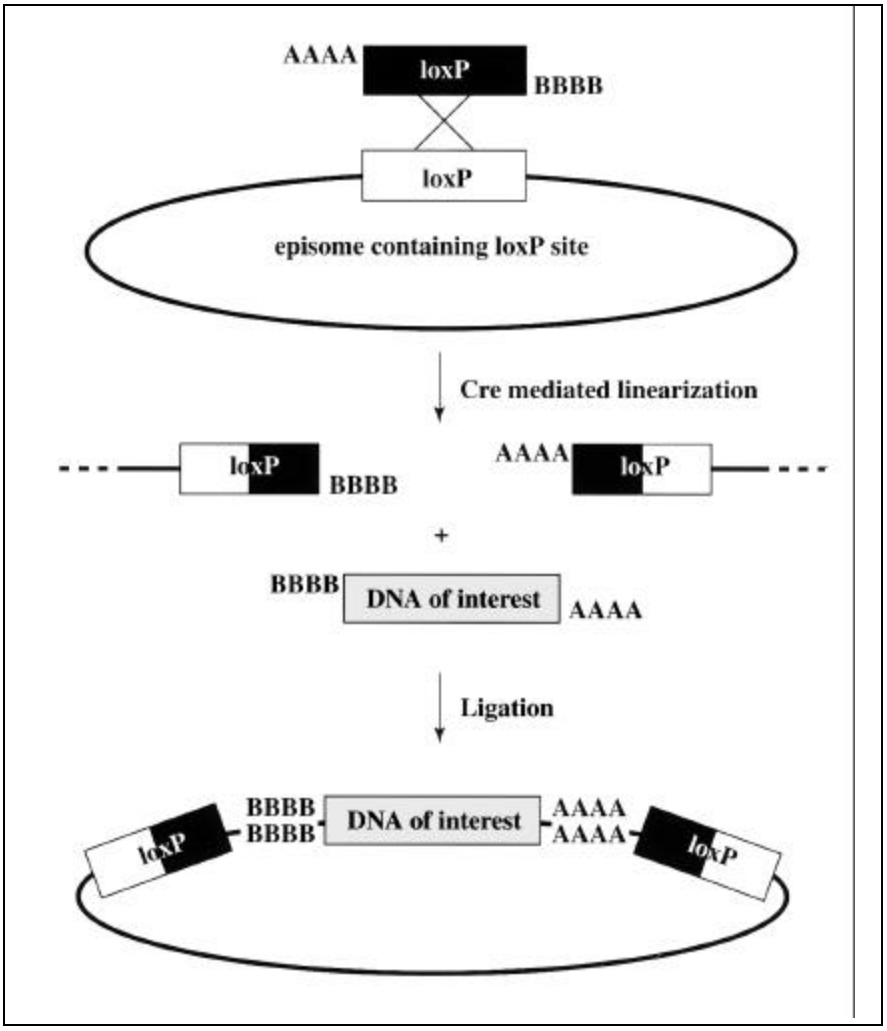


Figure 1. Manifestation of LPD cloning. The black box depicts a linear DNA fragment harboring a loxP site and single-stranded overhangs presented by AAAA and BBBB. The incubation of a circular episome harboring a *lox*P site and Cre recombinase with this fragment results in the linearization of the circular episome, which now carries one loxP site at each end and the single-stranded "sticky ends" (see Figure 2B, lane 4). A DNA fragment cut with restriction enzymes compatible with the sticky ends (DNA of interest) can be inserted by ligation and is flanked by loxP sites.

plasmid pEGFP. Plasmid DNA was introduced by heat shock into the *E. coli* strain DH5α for high-copy plasmids and into DH10B (both from Invitrogen, Carlsbad, CA, USA) by electroporation after drop dialysis on MF VS 0.025-mm pore-size nitrocellulose filters (Millipore, Bedford, MA, USA) for the BAC vector. DNA fragments were isolated from agarose gels using the QIAquick gel extraction kit (Qiagen).

Linearization of Circular Episomes with Cre Recombinase

Reactions (20 µL) containing 500 ng supercoiled circular episomes were incubated with 500 ng purified Cre protein (4) in the presence of 0.5 pmol annealed loxP oligonucleotides including: SpeloxP, 5′-CTAGTATAACTTCGTATAGCATAC ATTATACGAAGTTATG-3′; RIloxP, 5′-AATTCATAACTTCGTATAATGTATGCTATACGAAGTTATA-3′; XbaloxP, 5′-CTAGAATAACTTCGTATAGCATACATTATACGAAGTTATACC-3′; XholoxP, 5′-TCGAGGTATAACTTCGTATAATGTAT-GCTATACGAAGTTATT-3′; SalloxP, 5′-TCGACAATTGATAACTTCGTATAATGTATGCTATACGAAGTTATCTGCAG-3′; and H3loxP, 5′-AGCTCTGCAGATAACTTCGTATAGCATACATTATACGAAGTTATCAATTG-3′ in 1× ligase buffer (Roche Molecular Biochemicals) at 25°C for 1 h. The reaction was terminated by a 5-min incubation at 70°C and loaded directly onto 0.7% agarose gels to extract the linearized fragment. Cre recombinase is commercially available (Invitrogen).

Cell Culture

Hela cells were grown in DMEM (10% FCS) and transfected with FuGENE™ (Roche Molecular Biochemicals), following the instructions of the manufacturer. Dexamethasone (Sigma, St. Louis, MO, USA) was added to the culture medium at a final concentration of 10^{-6} M. Cells were analyzed for GFP expression using an HBO50 microscope (Carl Zeiss, Thornwood, NY, USA).

RESULTS

To test the feasibility of loxP-directed (LPD) cloning, we attempted to replace the puromycin resistance gene in pSVpaX1 (5) with the neomycin resistance gene, resulting in the plasmid pSVneoX1. As a starting point, we used plasmid pSV-XZ (Figure 2A). pSV-XZ contains a loxP site that is 3′ of the simian virus 40 (SV40) promoter, followed by the *lacZ* gene, and allows strong expression of *lacZ* in many mammalian cell lines (5) (data not shown). Therefore, the loxP site does not compromise expression from the SV40 promoter.

The plasmid pGK-neo contains the neomycin gene that can be excised from this plasmid as a 1415-bp fragment with the restriction enzymes *Spe*I and *Eco*RI (Figure 2B, lane 3). We designed the oligonucleotides SpeloxP and RIloxP (see Materials and Methods) and annealed them to form a double-stranded loxP site. The oligonucleotides were then mixed with plasmid pSV-XZ in the presence of Cre protein. The loxP oligonucleotides recombined with the plasmid, resulting in its linearization (Figure 2B, lane 4). In addition to the linearized band, a slower, migrating band was observed. This band migrates at the expected size of relaxed circular pSV-XZ. The linearized vector alone (control) and the vector plus insert (mixed at a ratio of 1:5) were ligated overnight at 20°C. DH5α cells were transfected with the

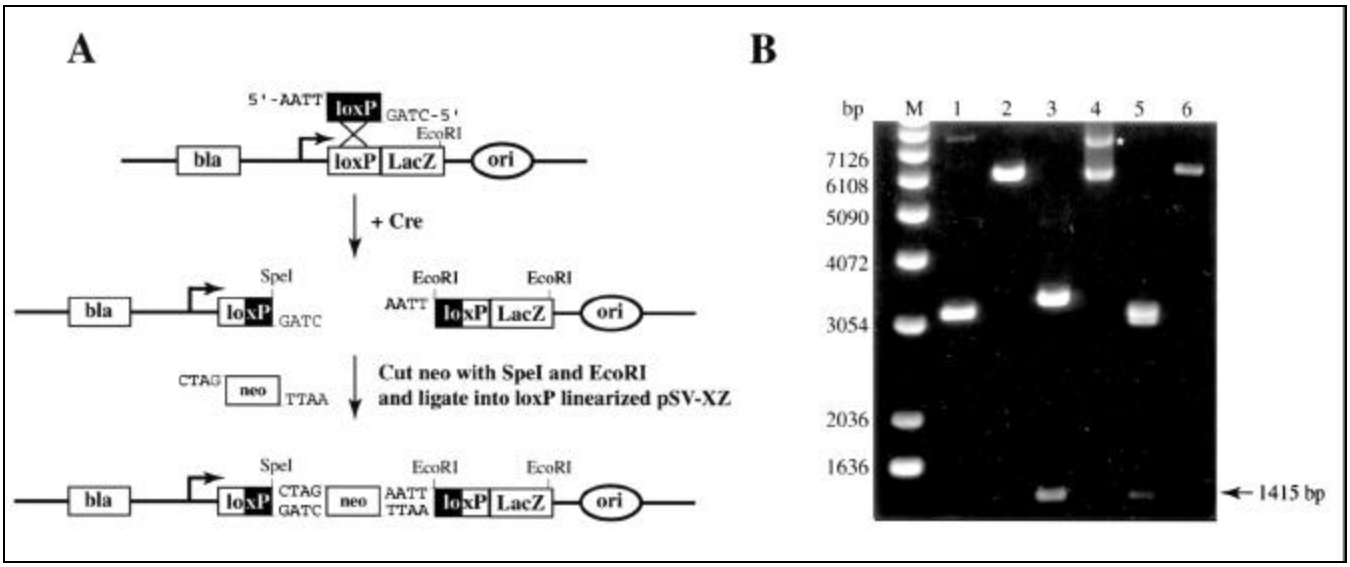
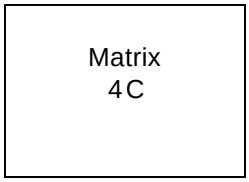


Figure 2. LPD cloning into a mammalian expression vector. (A) A map of plasmid pSV-neoX1. The ends generated by Cre-mediated linearization used to insert the neomycin gene are presented in detail. Relevant restriction sites are shown. Note that *Eco*RI is present in the vector backbone and, therefore, would have been difficult to use in a conventional cloning strategy. (B) Agarose gel showing the steps involved in LPD cloning. M, 1-kb marker (Invitrogen, Carlsbad, CA, USA); lane 1, pSV-XZ uncut; lane 2, pSV-XZ linearized with *Eco*RI; lane 3, pGK-neo cut with *Spe*I and *Eco*RI; lane 4, pSV-XZ linearized with the SpeIloxPRI oligonucleotide in the presence of Cre recombinase. A slower migrating band, probably relaxed circular plasmid DNA, is marked by an asterix; lane 5, pSV-neoX1 cut with *Spe*I and *Eco*RI; and lane 6, pSVneoX1 grown in 294-Cre at 37°C and digested with *Spe*I and *Eco*RI. The 1415-bp fragment containing the neomycin gene is indicated.



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ligation mixtures by heat shock and plated on ampicillin plates.

We observed no colonies on the control plate (data not shown). We found that the background in LPD cloning was essentially zero when we used loxP oligonucleotides that did not carry a 5′-phosphate because DNA ligase requires 5′-phosphate for its enzymatic reaction. In contrast, thousands of colonies were obtained from the ligation that included the 1415-bp insert, indicating that LPD cloning had worked at high efficiency and that plasmid pSVneoX1 had been generated. Plasmid DNA isolated from 10 different colonies was analyzed by restriction digest, and all 10 clones displayed the expected fragments (Figure 2B, lane 5, and data not shown), suggesting that LPD cloning is reliable.

To confirm that the neomycin gene in pSV-neoX1 was flanked by loxP sites, the plasmid was grown in the *E. coli* strain 294-Cre (3), which expresses Cre at 37°C and leads to the deletion of DNA flanked by loxP sites. Restriction analysis demonstrated that the neomycin gene had been removed from plasmid DNA (Figure 2B, lane 6). pSVneoX1 has subsequently been used to generate a neomycin-resistant Cre recombination reporter cell line using CHO cells and 5 µg/mL G418 selection (data not shown).

The creation of hybrid proteins has greatly facilitated the ability to investi-

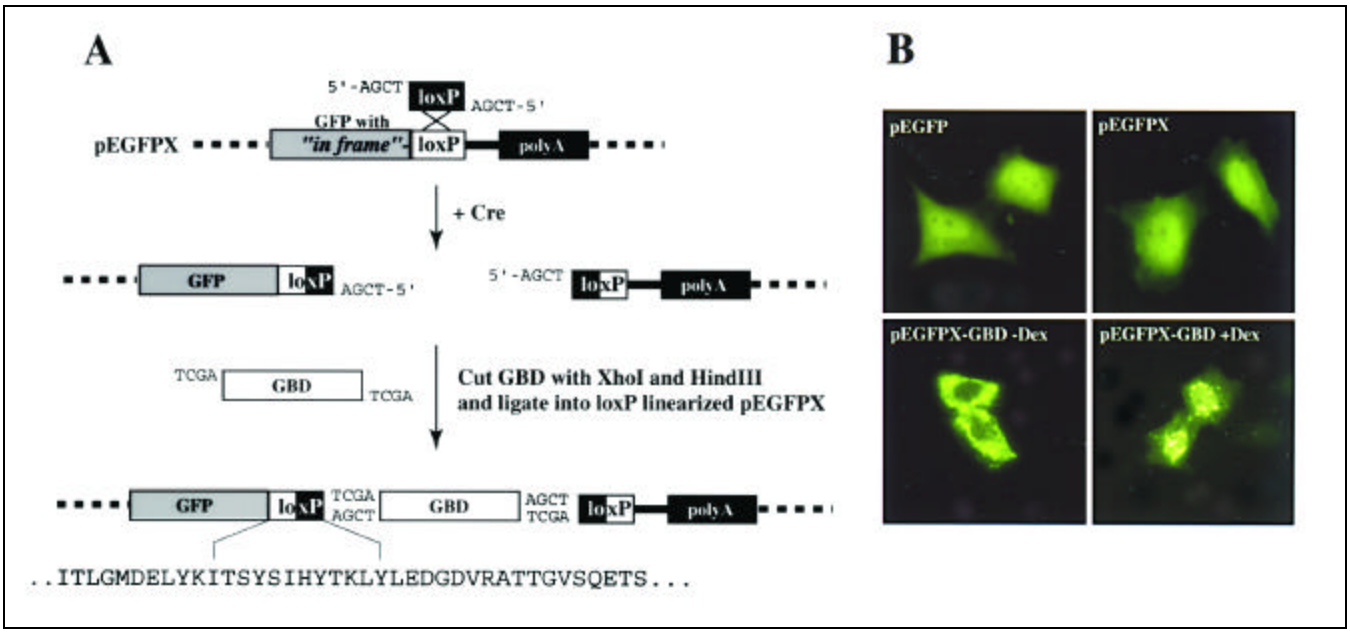


Figure 3. Generation of GFP-fusions by LPD cloning. (A) LPD cloning scheme to insert the human glucocorticoid ligand-binding domain into the vector pEGFP-X. The amino acid sequence of translation through the loxP site is shown. (B) Hela cells transiently transfected with the indicated plasmids analyzed for GFP expression. The addition of dexamethasone to the culture medium is indicated by +Dex.

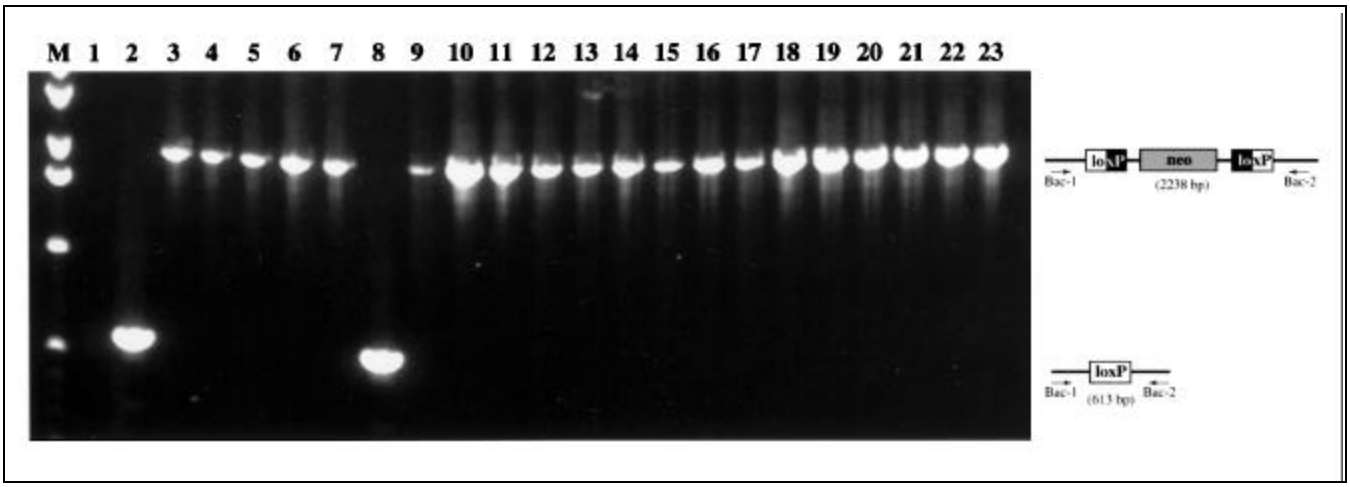


Figure 4. Colony PCR analysis on BACs manipulated by LPD cloning. M, 1-kb ladder; lane 1, negative control (water); lane 2, colony harboring the parent BAC (pBAC no. 7) analyzed by PCR; and lanes 3–23 represent 21 independent kanamycin-resistant clones analyzed with the same primers. A schematic presentation of the PCR products and the primers (Bac-1 and Bac-2) is shown.

gate gene products. Many vectors allow fusion of the protein of interest to a well-defined tag through a limited number of restriction sites. Because of the limited options that these cloning sites offer, it is often necessary to alter the DNA fragment to be cloned by PCR or other means. Placing a loxP site in frame with the desired tags circumvents the need to alter the DNA to be cloned and provides complete flexibility in the cloning process.

We generated vector pEGFP-X to test whether an in-frame loxP site would alter the function of GFP (Figure 3A). This vector contains a loxP site that was inserted at the C-terminus of GFP so that transcription and translation proceed through the loxP site and terminate shortly after it. The additional amino acids added by the loxP site did not have a significant effect on protein function, as judged by similar fluorescence patterns and intensities of Hela cells transfected with constructs pEGFP or pEGFP-X (Figure 3B).

To test whether the bridging loxP site affected either the production or function of a fusion protein, we cloned the ligand-binding domain of the human glucocorticoid receptor α gene (GBD) into pEGFP-X by LPD cloning, as outlined in Figure 3A. Transfection of pEGFP-X-GBD resulted in the production of cytoplasmic fusion protein, and the addition of the glucocorticoid analogue dexamethasone resulted in the translocation of the fusion protein into the nucleus (Figure 3B), as seen with many nuclear receptor-GFP fusion proteins (8,9,15,18). It can be concluded that the bridging loxP site did not significantly affect either the production or function of the fusion protein. Plasmid pEGFP-X, in combination with designed loxP oligonucleotides, therefore serves as a flexible vector to generate GFP-fusion constructs. The generation of vectors containing other useful tags with an in-frame loxP site should translate this flexibility to different fusion partners.

Large episomes such as BACs are becoming more popular in molecular biology and have proven to be of great value in the generation of transgenic animals. Small transgenes are often subject to position effects, leading to the lack of consistent and tissue-specific expression in animals carrying the transgene (7). In contrast, many scientists have reported faithful expression of transgenes when larger constructs such as BACs or P1 clones were used to generate transgenic animals (2,11,14,16). In many cases, it is desirable to introduce modifications into the construct—for instance, to clone a reporter gene downstream of the promoter of the gene of interest. Cloning into large episomes by conventional means can be difficult because of the lack of unique cutting sites for most restriction enzymes.

We tested the utility of LPD cloning for the manipulation of a BAC containing a murine genomic fragment (>120 kb) by inserting a neomycin cassette into the loxP site present in a vector (pBAC no. 7). Twenty-one clones were analyzed by colony PCR. All but one clone displayed the expected increase in fragment size, indicating that the neomycin cassette had integrated into pBAC no. 7 at the loxP site (Figure 4).

We found, depending on the loxP oligonucleotides used, that we obtained varying linearization efficiencies, ranging from approximately 80% with oligonucleotides SpeIloxPRI to about 20% with oligonucleotides XbaloxPXho (Figure 2B, lane 4, and Figure 5). This was the case even though similar concentrations of oligonucleotides, vector, and Cre were used. However, the amount of linearized vector was always sufficient to clone the DNA of interest. It is possible that the purity of the oligonucleotides is critical for optimal recombination efficiency because the most effective oligonucleotides (SpeIloxPRI) were purified using PAGE.

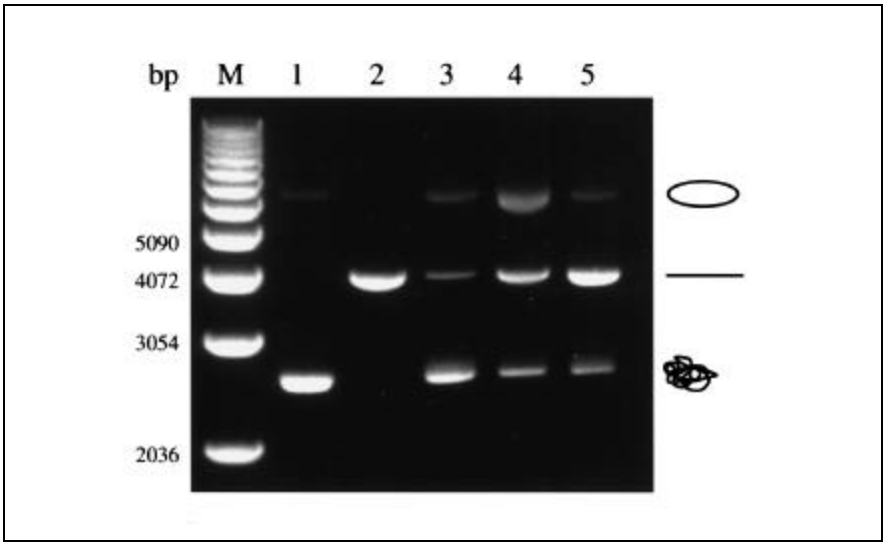
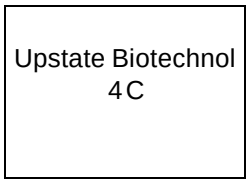


Figure 5. Linearization efficiencies using different loxP oligonucleotides. LoxP-containing plasmid DNA treated with different annealed loxP oligonucleotides in the presence of Cre protein are shown after gel electrophoresis. M, 1-kb ladder; lane 1, untreated plasmid pEGLP-X (supercoiled); lane 2: pEGFP-X digested with *Eco*RI (linearized); lane 3, pEGFP-X linearized with oligonucleotide XbaloxPXho; lane 4, pEGFP-X linearized with oligonucleotide H3loxPSal; and lane 5, pEGFP-X linearized with oligonucleotide SpeIloxPRI. Supercoiled, linearized, and relaxed plasmid DNA is indicated to the right of the gel.

DISCUSSION

DNA cloning with restriction enzymes and DNA ligases has been the method of choice for the generation of recombinant DNA. However, target vectors carry only a limited number of restriction sites as receptacles for DNA fragments. Therefore, the DNA to be cloned frequently has to be altered to carry the restriction sites present in the target vector. Usually, this task is accomplished in a PCR that integrates the necessary sites within the DNA of interest. This process is laborious and usually requires sequencing to authenticate the integrity of the cloned DNA. In addition, the relatively short recognition sequence (4–8 bp) of most restriction enzymes dictates that a recognition sequence can appear more than once within the vector, making it difficult to clone a DNA fragment at the desired site.

Recently, the Cre/loxP system has



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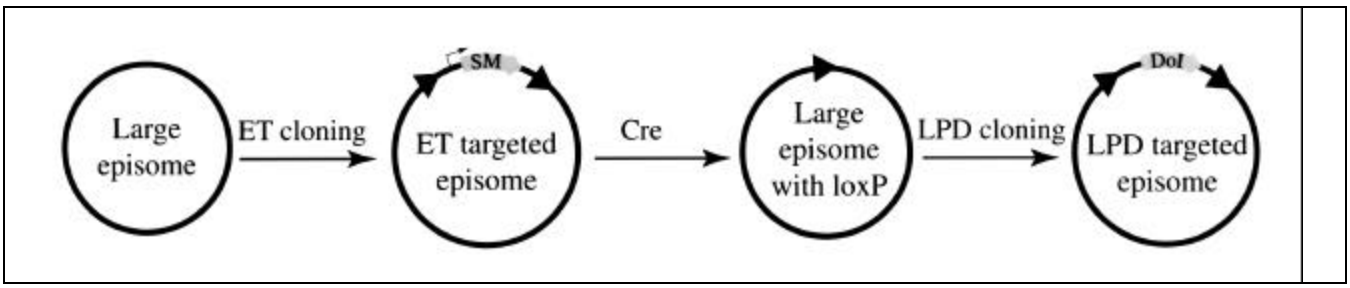

Figure 6. Schematic presentation of flexible manipulation of large episomes using LPD cloning. In the first step, a DNA fragment containing a selectable marker gene (SM1) flanked by loxP sites (filled triangle) and short homology stretches to the large episome (filled and white box) is transfected into cells harboring the large episome. In the correct genetic background, the fragment can integrate into the large episome at the homology regions through ET recombination (17). Upon the identification of the correct recombinants, the selectable marker is removed by transient exposure to Cre recombinase [e.g., by introducing plasmid p705-Cre (5)]. After the isolation of the large episome (now carrying a single loxP site in the desired locus), the DNA of interest (DoI) can be introduced by LPD cloning. The episomes are subsequently transfected back into *E. coli* for amplification.

been developed for DNA cloning (10). The technology was termed the univector plasmid-fusion system and uses Cre-loxP site-specific recombination to catalyze plasmid fusion at loxP sites between the univector—a plasmid containing the gene of interest—and a host vector. This system eliminates the need for restriction enzymes, DNA ligases, and many in vitro manipulations required for subcloning, and allows for the rapid construction of multiple constructs. However, in the initial step, the DNA of interest still has to be cloned into the univector by conventional means.

Fusing the univector with the host plasmid generates a plasmid that now contains two loxP sites. Consequently, subsequent cloning steps by the same method are difficult. Another drawback of this technique is that fusing the univector and the host vector generates plasmids that, in addition to the host-vector sequence, carry the whole sequence of the univector. While the extra sequence from the univector carried into the final construct may not influence all downstream usages, it may affect some applications.

LPD cloning combines conventional cloning using restriction enzymes with DNA manipulation using a site-specific recombinase. As for the univector plasmid-fusion system, LPD cloning shares the limitation that, because of the presence of two loxP sites in the resulting construct, a second cloning step using the same technique is difficult. However, in contrast to the univector plasmid-fusion system, LPD cloning does not add additional sequences from another vector to the final construct. LPD cloning preserves the flexibility of cloning with a site-specific recombinase and allows cloning of DNA fragments without the addition of extra sequence. The length and complexity of the 34-bp recognition sequence for Cre recombinase make it highly unlikely that most DNA sequences will contain the sequence. Because the loxP site serves as the universal polylinker, the presence of internal recognition sequences for restriction enzymes within the vector does not hinder the cloning exercise. In LPD cloning, one can cut the fragment to be cloned with the desired restriction enzymes without taking internal recognition sequences within

the vector into consideration. Therefore, the integrity of the cloned DNA fragment is maintained, and no alteration by PCR is necessary. Moreover, LPD cloning allows complete flexibility in the directionality of cloning because the generation of the presented ends after cutting is determined by the design of the oligonucleotides.

Another advantage of LPD cloning is that the removal of 5′-phosphate groups from the vector DNA with alkaline phosphatase, often performed to prevent the self-ligation of the vector, is unnecessary if the oligonucleotides do not carry a 5′-phosphate group. The background of false positives in LPD cloning is therefore very low.

As larger episomes become more popular in molecular biology, there is an increasing need to develop technologies that allow their manipulation. Recently, a technique based on homologous recombination in *E. coli* (ET cloning) has been developed to address this need (13,17). However, this method requires a selectable marker to identify positive clones and is most efficient when the fragment to be recombined into the vector is generated by PCR, which in turn creates the possibility of introducing mutations into the amplified sequences. Sequences other than the selectable marker are therefore prone to contain errors. This problem can be prevented by an initial ET-cloning step, introducing recognition sites for rare cutting restriction enzymes, followed by a conventional in vitro cloning step (1). However, the episome to be manipulated has to be void of the desired restriction enzyme sites. The likelihood that sequences will contain these sites increases with size.

An alternative to this approach, especially for very large constructs, is LPD cloning in combination with ET cloning (Figure 6). First, the large episome is targeted by ET cloning with a selectable marker flanked by two loxP sites. The selectable marker is subsequently removed by transient exposure to Cre recombinase. Potential errors introduced during the generation of the fragment used for ET cloning can be ignored because this sequence is removed, leaving behind only one loxP site at the desired location. This loxP site then serves as the entry point to clone in the desired DNA fragment generated with restriction enzymes by LPD cloning. Because the cloned DNA is not amplified by PCR, its integrity is preserved and no additional sequences other than the loxP sites remain in the modified construct.

Other recombinase/target-site systems such as FLP/FRT, from the yeast 2-μm plasmid, should work similarly to the Cre/loxP system, with the result that the cloned DNA fragment would be flanked by the corresponding recognition target site. The FLP/FRT system could be used instead of LPD cloning (e.g., when the Cre/loxP system is reserved for conditional mutagenesis in the mouse) or in addition to it, adding even more flexibility. Unfortunately, unlike Cre recombinase, FLP is rela-

tively difficult to purify and is not commercially available. However, the improved FLPe recombinase could prove valuable in these experiments (4).

Modifications of nucleotides during oligonucleotide synthesis could prove useful in the LPD cloning protocol. For instance, the incorporation of biotinylated nucleotides into the oligonucleotides could be used to purify the linearized vector away from the uncut plasmid, which would also eliminate the need to gel purify the vector DNA. This process would simplify LPD cloning, especially when large episomes are used.

In summary, LPD cloning is a versatile way to clone DNA into episomes. A loxP site can substitute for all recognition sites of restriction enzymes because, in principle, DNA fragments that are digested with any restriction enzyme can be ligated into a vector containing a loxP site. Therefore, a loxP site can be used as a universal polylinker. This presents substantial advantages over conventional cloning into vectors with multiple cloning sites. We propose that a loxP site can replace or complement multiple cloning sites in cloning vectors. In addition, LPD cloning presents a valuable method to manipulate large episomes.

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