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Highly efficient generation of recombinant baculoviruses by enzymatically mediated site-specific *in vitro* recombination

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ABSTRACT

We have used the Cre-lox system of bacteriophage P1 to develop a highly efficient *in vitro*system for construction of recombinant baculoviruses. A positive visual selection has been included to make identification of recombinant viral progeny rapid and straightforward. We report recombination frequencies as high as 5×10^7 recombinants/μg starting plasmid DNA and under certain conditions, up to 50% of the viral progeny are recombinants. Genes inserted into the baculovirus genome can be readily recovered in a simple one step process and re-inserted after manipulation if required. We have confirmed the structure of recovered plasmids by diagnostic restriction endonuclease digestion and the structure of recombinant viral genomes by Southern analysis. Possible uses and the significance of the system are discussed and experiments currently being done to improve it are described.

INTRODUCTION

Since the discovery and characterisation of baculoviruses, considerable use has been made of infection of *Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus (AcNPV) for the expression of foreign genes. The advantages of this system lie in the high levels of expression which can be achieved, the ability of cells to modify recombinant proteins correctly and to assemble subunits into functional proteins (for review see (1)). The system remains inconvenient mainly because of the laborious process required to identify and isolate the recombinant virus. Foreign coding sequences are usually cloned into transfer vectors under the transcriptional control of very late viral promoters such as the promoter for the polyhedrin gene or the P10 gene. These genes are naturally expressed at very high levels and are non-essential for viral propagation in continuous culture. After insertion of the foreign gene into the transfer vector, it is then transferred into the virus by a process of homologous recombination between sequences flanking the polyhedrin gene in the virus and the modified polyhedrin gene in the transfer vector. The resulting recombinant has a deleted polyhedrin gene and is unable to synthesise polyhedrin occlusion bodies. This phenotype can be visually detected on a standard agarose overlay assay (2).

Significant progress has been made in the refinement of baculovirus vectors in order to optimise expression levels of foreign proteins. In particular, the sequences required for full activity of the polyhedrin promoter have been determined (3 and 4). Examples of vectors currently used for high level expression of non-fused proteins are p36C (5), pVL941 (6) and pAcYM1 (3). A major problem associated with the baculovirus system is the identification of recombinant viruses. This has been considerably enhanced by the construction of dual expression vectors. In most cases, one transcription unit directs expression of the β-galactosidase gene and recombinants are identified on a standard agarose overlay assay by the blue plaque phenotype in the presence of the chromogenic indicator X-gal. An example of such a vector, pJVNheI, has been described previously (7). In our hands, however, the use of such vectors has led to the identification of a large number of 'false' recombinants, exhibiting the blue phenotype whilst remaining polyhedrin positive on an overlay assay (TP, DG–unpublished data).These viral progeny may have arisen by the integration of the transfer vector by a single homologous cross-over event resulting in tandemly repeated viral sequences. Our experience suggests that these are unstable in long term propagation. In addition, although dual expression vectors aid the identification of recombinant viruses, they do not improve the frequency of recombination between virus and transfer vector. Indeed, because the addition of a second transcription unit increases the size of the transfer vector, the frequency of transplacement may conceivably be reduced. Another limitation to the wider application of the baculovirus/insect cell system for the expression of foreign proteins is the low efficiency of the homologous recombination (typically $1:10^2$ to $1:10^3$) resulting in small total numbers of recombinant progeny virus. In the absence of a positive colour selection, identification of recombinant viruses can be very difficult. A significant step in improving the frequency of recombinants among viral progeny has been the observation that the use of linear viral DNA in the co-transfection procedure considerably reduces the background of non-recombinants (8). However, although the actual percentage of recombinants increases, the total number of viral progeny dramatically decreases. This is an efficient method of producing baculovirus recombinants for 'one-off' users although there is as yet no report in the literature of a colour selection being incorporated. Furthermore, it relies on two double stranded homologous exchange events so that the efficiency of recombination could be expected to decrease as the size of the foreign gene increases. We have previously described a method

to increase the frequency of recombination by irradiating the cells prior to transfection with short wave ultra-violet light (9). Although this only increases the frequency of recombination by about 3-fold, it is a useful method for obtaining recombinants where the foreign gene is very large (10).

The work described here addresses both the problems of the low frequency of recombination inherent in the system and also the difficulty in identifying double crossover recombinants. We have made use of a bacteriophage P1 encoded enzyme Cre recombinase (for 'Causes REcombination') and its substrate *lox*P (for 'Locus Of Crossover P1') to achieve high recombination efficiencies and rapid and staightforward identification of recombinants by a colour selection. The Cre-*lox*P system was first characterised in detail by Sternberg and Hamilton (11). Bacteriophage P1 encodes a site-specific recombination system that consists of a site (*lox*) at which recombination occurs and a gene, *cre*, the protein product of which is essential for the recombination. The Cre protein has been purified and cloned and the sequence and structure of *lox* determined (12,13). The interaction between Cre and it's substrate *lox*, has been studied using nuclease protection techniques. The region protected against DNAse1 attack is a 34bp sequence containing two 13bp perfect inverted repeats separated by an 8bp non-palindromic spacer (13). These sequences have been shown to be necessary and sufficient for Cre mediated recombination. No high energy co-factors are required and the Cre protein binds to *lox* containing DNA to form a complex. The bound Cre converts about 70% of DNA substrate to products in a stoichiometric manner. Significantly, the action of Cre on a super-coiled substrate containing two *lox* sites reversably generates product molecules that are topologically unlinked.

Here we describe the application of this technology to the baculovirus system. Both host virus and transfer vector have been engineered to contain *lox* sites and, in addition, the vector contains the β-galactosidase gene to allow rapid and simple screening of viral progeny after transfection. We report very high recombination frequencies (as high as 5×10^7 recombinants/μg DNA transfected) and describe a number of potential uses for the system beyond the selection of recombinant baculoviruses.

MATERIALS AND METHODS

Viruses and cells

Spodoptera frugiperda (Sf) cells were maintained in spinner flasks in TC100 medium (Flow laboratories) supplemented with 10% foetal calf serum and 0.1mg/ml gentamicin (Sigma). Wild type and recombinant viruses were grown and plaque purified according to the procedures described previously (14).

DNA manipulations

All plasmid DNA manipulations and Southern blotting techniques were done as described in reference (15). Plasmid constructions used in this work are described in the results section. DNA restriction and modification enzymes were purchased from GIBCO-BRL and Boehringer Mannheim and used according to the manufacturers instructions. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim. Plasmid DNA for transfections was purified on caesium chloride gradients.

In vitro recombination reactions and screening of products:- detection of β-galactosidase activity

The plasmid ploxZ and viral vAclox DNA were mixed in varying ratios (see Results) in a buffer containing 50mM Tris.HCl pH7.5, 33mM NaCl, 10mM $MgCl_2$ and 100μg/ml BSA. Reactions were incubated at 37°C for various times (see Results) in the presence or absence of 1 unit Cre recombinase (NEN-Du Pont). The reactions were terminated by incubation at 70°C for 15min. After cooling to room temperature, the reactions were mixed with the Lipofectin reagent and used to transfect a monolayer of 2×10^6 Sf cells as described and progeny virus were screened in an overlay assay. The presence of integrated ploxZ plasmid in the viral progeny was detected by the addition of 40μl of a solution of 40mg/ml (in DMSO) of the chromogenic indicator X-Gal (Boehringer Mannheim) 72hpi followed by incubation at 27°C until vivid blue plaques were visible (typically 2−4 h.). These recombinant viruses were designated vloxZ. Non-recombinant viruses were visualised by the addition of 250μl of neutral red solution (Jensens; BDH) to the overlay 72hpi followed by incubation at 27°C for 1h. The plaques were destained at 4°C overnight. Recombinant viruses were purified to homogeneity (100% blue staining plaques) by 2−3 successive rounds of agarose overlay assay.

Assays of β-galactosidase activity of cells infected with vloxZ

Monolayers of Sf cells (2×10^6) were infected with virus at a multiplicity of infection (MOI) of 1 and incubated for 3 days at 27°C. After infection, the cells were rinsed with ice-cold PBS and scraped from the plates in 0.5ml ice-cold PBS. The cells were lysed by the addition of 30μl of toluene and 30μl of a 1%w/v solution of sodium deoxycholate and the lysates assayed for β-galactosidase activity as previously described (14) using the chromogenic indicator 2-nitrophenyl-β-D galactopyranoside (ONPG, -Boehringer Mannheim). The units of activity were expressed as μmoles ONPG hydrolysed per minute per million virally infected cells.

Recovery of ploxZ plasmid DNA from vloxZ

Monolayer Sf cells were infected with purified vloxZ virus at a MOI of 1. Total DNA, containing viral and host cell DNA, was prepared from the infected cells 72hpi (14). Various amounts of DNA were incubated in Cre buffer at 37°C for 30min. in the presence or absence of 1 unit Cre recombinase. After termination of the reaction, the DNA mixture was used to transform *Escherichia coli* DH5 and plated on L-agar plates containing 100μg/ml ampicillin (Sigma). DNA was prepared from resulting bacterial colonies and analysed by agarose gel electrophoresis.

RESULTS

Construction of recombinant transfer vector ploxZ

An oligonucleotide pair corresponding to the sequence of *lox* (figure 1) was synthesised and cloned into the *Kpn*I site of the Bluescript+ vector (Stratagene). This construction was designated pKSlox. A 4.6kbp *Sal*1-*Hin*dIII fragment from pJVP10Z, a derivative of pJVNheI (7), containing the β-galactosidase (lacZ) gene under the transcriptional regulation of the very late viral P10 promoter and the very late viral polyhedrin promoter immediately upstream of a unique *Nhe*I cloning site, was cloned between the *Sal*I and *Hin*dII sites of pKSlox. The resulting construct was designated ploxZ (figure 2a).

Construction of the recombinant virus vAclox

An oligonucleotide pair corresponding to the sequence of *lox* (figure 1) was synthesised and cloned between the *Eco*RV and *Kpn*I sites of the baculovirus transfer vecter p36C (5). The resulting construct was designated p36Clox (figure 2b).To obtain the recombinant virus (vAclox), Sf cells were irradiated with short wave ultra-violet light as described (9) and transfected with 4μg p36Clox and 1μg wild type viral DNA using the lipofectin reagent (10μg/ml−GIBCO-BRL) in 5ml serum free medium for 6 hours. The transfection medium was replaced with 5ml of complete medium and the incubation continued at 27°C. After sevety two hours, the supernatant was harvested and the progeny virus were screened in a standard agarose overlay assay (2). Plaques were screened visually for the absence of polyhedra. Potential polyhedrin negative recombinant plaques were purified to homogeneity by successive rounds of agarose overlay assay. The presence of the *lox* insertion was confirmed by purifying DNA from 2 million infected cells followed by Southern analysis. High titre recombinant virus (up to 10^9 plaque forming units/ml) was routinely prepared by infecting monolayer cultures at 27°C.

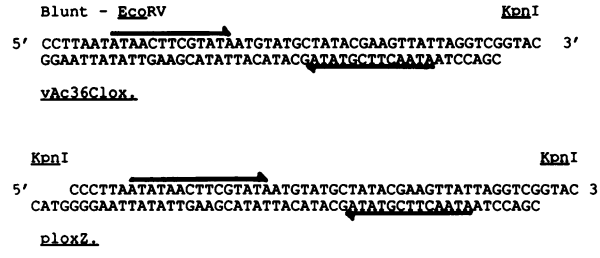


Figure 1. Sequences of the oligonucleotide pairs used to construct p36Clox and vAclox (vAc36Clox) and pKSlox (ploxZ). The *lox* sites, consisting of two 13bp perfect inverted repeats separated by an 8bp spacer are indicated by a half arrow.

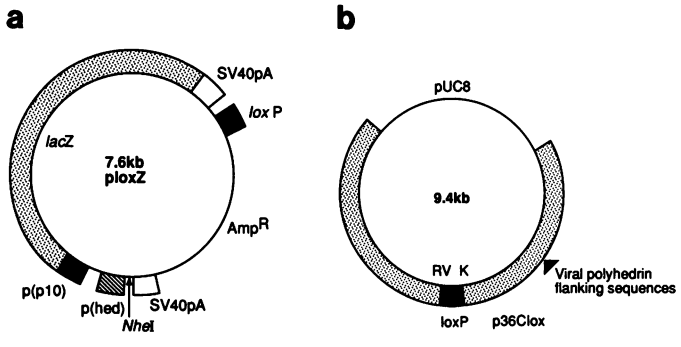


Figure 2a. Structure of plasmid ploxZ. The plasmid backbone is Bluescript+. Expression of the β-galactosidase coding sequence (lacZ) is controlled by the very late viral P10 promoter [p(P10)] and transcribed in a clockwise direction. Genes of interest are cloned into the unique *Nhe*I site and are transcribed in the counter-clockwise direction by the very late viral polyhedrin promoter [p(hed)]. Polyadenylation signals from SV40 (SV40pA) direct processing of both genes. The lox site (loxP) is cloned downstream of the β-galactosidase expression cassette. **2b.** Structure of p36Clox. The polyhedrin sequences between the *Eco*RV (RV) site and the *Kpn*1 (K) site of p36C have been deleted and replaced with a *lox*P site using the oligonucleotides shown in figure 1. Sequences flanking the lox site allow transplacement by homologous recombination with the wild-type viral DNA.

Assay of *in vitro* Cre mediated recombination between pKSlox and p36Clox

In order to test the functionality of the lox sequences in the vectors pKSlox and p36Clox *in vitro* Cre mediated recombination assays were initially attempted. Plasmids pKSlox and p36Clox were digested with *Sal*I and *Pvu*II respectively. The linear DNAs were mixed in the absence or presence of varying amounts of Cre recombinase and incubated for 30min. at 37°C. After termination of the reaction, the products were electrophoresed on an agarose gel and analysed by Southern blotting (figure 3) using a 445bp *Pvu*II probe corresponding to the *lox* containing fragment of pKSlox. In the presence of Cre recombinase, recombination events lead to the appearance of hybridizing bands larger than the substrate 445bp species. In the reactions containing higher levels of Cre, the bands corresponding to recombinant products are more intense and, in addition, a number of higher molecular weight species are visible (lanes 5 and 6). It is difficult to assign structures to these recombinant species since each new recombinant becomes a fresh substrate for Cre recombinase. However, it is clear that the recombination is dependent on both the presence and amount of Cre recombinase and also that the lox sequences in this context are an efficient substrate for the Cre protein.

Stoichiometry and kinetics of Cre mediated recombination

Because the Cre mediated recombination is an enzymic reaction (figure 4) we investigated the relationship between efficiency of recombination and both the ratio of the viral and plasmid DNAs and the time of incubation with the Cre recombinase prior to transfection.

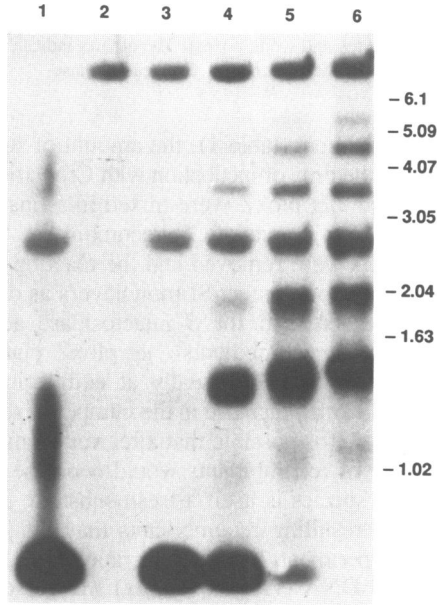


Figure 3. *In vitro* Cre mediated recombination of pKSlox and p36Clox. Plasmids pKSlox (2μg) and p36Clox (2μg) were linearised with *Pvu*II and *Sal*1 respectively and incubated together in the presence or absence of Cre recombinase. The products of the reaction were analysed by agarose gel electrophoresis and Southern blotting using the 445bp *Pvu*II fragment from pKSlox as a probe. Lane1; pKSlox, lane 2; p36Clox, lane3; pKSlox + p36Clox, lanes 4−6; pKSlox + p36Clox in the presence of 1,2,or 4 units of Cre recombinase respectively. The positions of molecular weight markers (in kbp) are shown on the right.

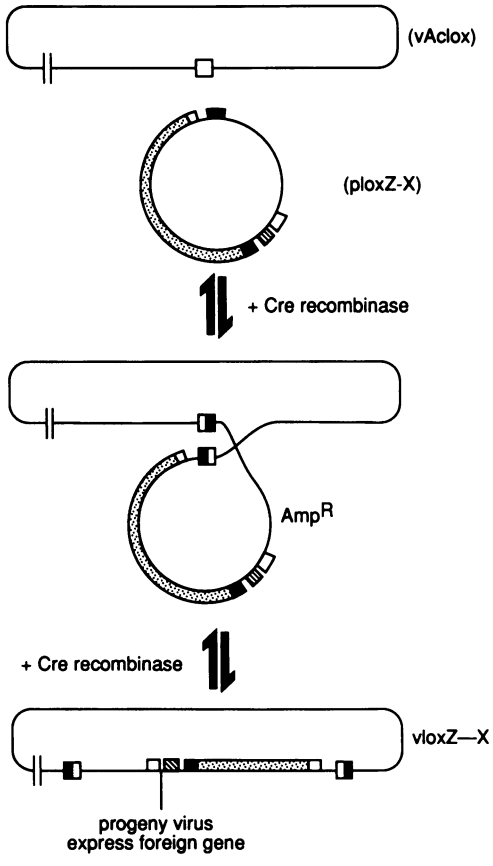


Figure 4. Diagrammatic representation of Cre mediated recombination at *lox*P sites. The viral genome (vAclox) is shown as a broken circle containing a *lox*P site (open box). The key for ploxZ is as for figure 2a with 'X' indicating the presence of a foreign gene. The recombinant virus is shown containing two hybrid *lox*P sites (shown as black and white boxes). The arrows indicate the reversable nature of the reaction in the presence of Cre recombinase.

Table 1.

Time (min)	Number of recombinants ($\times 10^6$)/μg plasmid DNA
0	0
10	5.6
20	6.8
40	6.6
80	7.8

Assay of number of recombinant virus with time of incubation with Cre recombinase. Plasmid ploxZ and viral vAclox were incubated with Cre recombinase and then used to transfect Sf cell monolayers. The progeny virus were screened in a standard agarose overlay assay. The chromogenic indicator X-gal was added 72hpi and recombinants identified as blue plaques.

Table 2.

Viral DNA (μg)	ploxZ (μg)	No. recombinants ($\times 10^6$)/μg plasmid DNA	% viral progeny expressing β-gal.
	0	0	0
	3	2.1	23
vAclox	0.3	27	49
(0.5)	0.03	27	2
	0.003	54	0.6
wild type	0	0	0
(0.5)	3	0	0

Titration of vAclox with ploxZ. 0.5μg vAclox was incubated with 10-fold dilutions of ploxZ for 20 minutes at 37°C. After standard transfection and agarose overlay assay, the chromogenic indicator, X-gal, was added and recombinants identified as blue plaques. Wild type *Autographa californica* viral DNA was included as a negative control.

Table 3.

Viral DNA	Cre	No. Amp^R clones.
Wild type (0.05μg)	−	0
Wild type (0.05μg)	+	0
vloxZ (0.05μg)	−	0
vloxZ (0.05μg)	+	7
(0.1μg)	+	5
(0.5μg)	+	14
(1.0μg)	+	17
(5.0μg)	+	24

Recovery of ploxZ DNA from vloxZ. Total DNA from infected Sf cells was incubated with Cre for 30 minutes at 37°C and then used directly to transform *Escherichia coli* DH5 and plated onto L-agar plates containing 100μg/ml ampicillin. The plates were incubated overnight at 37°C and the colonies counted.

In the first experiment (table 1), the amount of substrate was kept constant and the time of incubation with Cre varied. Initially, 5μg of vAclox and 1μg ploxZ were mixed in a final volume of 60μl in the presence of 2 units of Cre recombinase. At each time point, 10μl aliquots were removed and the reaction terminated. The DNA was used to transfect Sf monolayers as descibed and the viral progeny screened for β-galactosidase activity. The results, expressed as recombinants / μg ploxZ plasmid DNA, indicate that the reaction is virtually at equilibrium after 10 minutes with only a small increase in the number of recombinants at later time points. It is possible that after very long incubation times the number of recombinants would decrease, since each new recombinant species is itself a fresh substrate for Cre (see figure 4) and the resulting recombinants may not be viable.

In the second experiment, (table 2), the ratio of vAclox to ploxZ was varied. Viral DNA (vAclox−0.5μg) was mixed with ten-fold dilutions of ploxZ in a final volume of 10μl and the DNA incubated for 20 minutes at 37°C in the presence of Cre recombinase. After termination, the DNA was used to transfect Sf monolayers and the viral progeny screened for recombinants expressing β-galactosidase. The results, adjusted according to the amount of plasmid DNA used, indicate increased viable recombinants with lower amounts of ploxZ but a lower percentage of recombinant virus to non-recombinant virus. This may be due to unfavourable recombination events in the reactions containing higher concentrations of ploxZ (eg. ploxZ-ploxZ) or secondary and tertiary recombination with products of previous integrations which are non-viable. The observation that the total number of viral progeny was considerably reduced in the reactions containing the higher concentrations of ploxZ supports this possibility. These results suggest that very small amounts of transfer vector DNA are sufficient to generate significant numbers of recombinant viruses. Representative blue plaques were purified to homogeneity and remain stable through successive rounds of agarose overlay assay.

Although it is clear from the coloured plaques that the infected cells are expressing β-galactosidase, Sf monolayers were infected with progeny virus to compare the levels of expression between the various constructs and to ensure that Sf cells do not possess an endogenous enzyme that could produce spurious results. Not

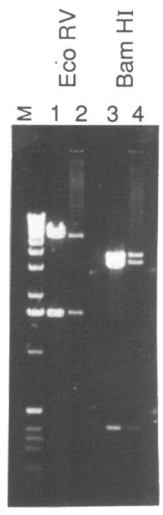
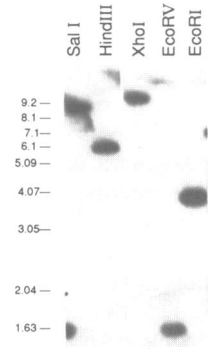


Figure 5. Cre mediated recovery of ploxZ from vloxZ DNA. DNA prepared from cells infected with vloxZ or control virus was incubated with Cre and the reactions used to transform *E.coli*. DNA was prepared from ampicillin resistant colonies and analysed by restriction endonuclease digestion followed by agarose gel electrophoresis; Lanes 1 and 3; control ploxZ, lanes 2 and 4; DNA miniprep from *E coli* transformed with vloxZ DNA incubated with Cre. M: 1kbp ladder molecular weight marker.

surprisingly, cells infected with viral progeny generated from a clear plaque do not express β-galactosidase at detectable levels. Uninfected Sf cells similarly do not possess a detectable endogenous β-galactosidase activity. In contrast, cells infected with vloxZ express greater than 50,000 units of β-galactosidase. This very high level of expression explains why blue plaques are visible so soon after the addition of X-gal. This is an advantageous feature since recombinant plaques can be confidently identified immedeately following the plaque assay rather than the following day after staining and destaining with neutral red.

Recovery of ploxZ DNA from cells infected with vloxZ

If integration of ploxZ is genuinely site specific and mediated by Cre recombinase, the resulting progeny virus, vloxZ, should contain the entire sequence of ploxZ flanked by two *lox* sites (refer to figure 4). We tested the reversibility of the Cre mediated recombination by incubating vloxZ DNA with Cre recombinase to recover the plasmid DNA. Sf monolayers were infected with vloxZ or a control virus. Total DNA (only a fraction of which is viral) from infected cells was incubated with Cre and the incubation mix used to transform *Escherichia coli* DH5 cells (Table 3). The recovery of ampicillin resistant clones from vloxZ, which is absolutely dependent on the presence of Cre, suggests the integration has occured as predicted and that both *lox* sites are intact. As a final confirmation of the integrity of the recovered plasmid, DNA was prepared from the ampicillin resistant bacterial colonies and analysed by gel electrophoresis after digestion with diagnostic restriction enzymes (figure 5). Digestion of the recovered DNA with *Bam*HI and *Eco*RV shows it to be identical to the starting plasmid ploxZ. This is further confirmation that the integration has occured as predicted and that inserts can be conveniently and rapidly recovered from viral DNA. The significance of this is discussed further.



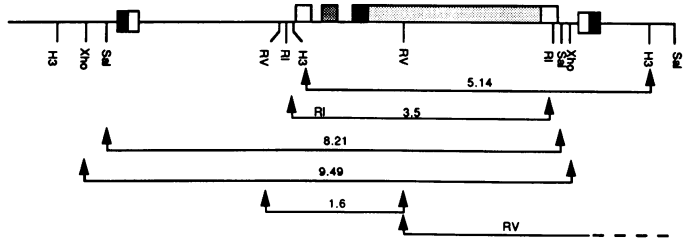


Figure 6. Southern analysis of vloxZ recombinants. DNA from cells infected with vloxZ was digested with a number of restriction endonucleases, transferred to a nylon membrane and probed with a fragment corresponding to the lacZ gene. The top half of the figure shows an autoradiograph of such a blot together with the positions of molecular weight markers (in kbp) and the restriction enzymes used. Below is a representation of the predicted structure of vloxZ with restriction sites and the sizes (in kbp) of cross-hybridizing fragments. The hybrid lox sites (black and white boxes) flank the plasmid sequences (key as for figure 2a) H3; *Hin*dIII, Xho; *Xho*I, Sal; *Sal*I, RV; *Eco*RV, RI; *Eco*RI.

Southern analysis of vloxZ recombinants

As further confirmation of the structure of the recombinant virus, vloxZ, DNA from purified high titre viral progeny was analysed by Southern blotting (figure 6). DNA from 2 million cells infected with vloxZ was prepared and digested with a number of diagnostic restriction enzymes and Southern blots of these digests probed with a labelled fragment corresponding to the *lac*Z gene present in ploxZ. Clearly the sizes of the bands on the blot are consistent with the structure predicted in figure 6. It is important to verify this fact to confirm that integration of the transfer vector, ploxZ, in the presence of Cre recombinase is site specific and not simply a random integration anywhere in the viral chromosome.

DISCUSSION

Here we describe a new system using an enzyme catalysed *in vitro* reaction for the construction and identification of recombinant baculoviruses. The method utilises the Cre-lox recombination process derived from bacteriophage P1 (11). In the context that we have constructed, recombination is absolutely dependent on the presence of the Cre enzyme and a functional lox sequence. The efficiency of recombination is dependent upon concentration of the enzyme and its substrates (vAclox and ploxZ)

and time of reaction. The recombinant virus produced (vloxZ) contains the entire ploxZ plasmid with its expression cassettes, selectable markers and origins of replication, flanked by two functional hybrid lox sequences allowing rapid and straightforward recovery of the original plasmid. The Cre mediated recombination has several advantages over previously described methods: because recombinant viral DNA molecules are generated by insertion of an entire largely non-homologous plasmid by a single enzyme catalysed cross over event the efficiency of recombination is not affected by the size of the plasmid and can be altered by manipulating the reaction conditions. In the experiments described here, we have found that by selecting appropriate reaction conditions, up to 50% of progeny virus generated by Cre mediated recombination contain an inserted plasmid. Because the non-recombinant virus (vAclox) does not express polyhedrin (the promoter and coding sequences have been deleted in this construction) we have included the positive selection marker β-galactosidase in an expression cassette on the transfer vector to allow rapid and straightforward identification of recombinant plaques. Since only a single crossover event is required to generate recombinants, we assume that all blue plaques represent correct insertions and therefore contain the gene of interest under the transcriptional control of the polyhedrin promoter. The problems we have encountered with the previous dual expression vectors (7), namely a predominance of undesired single crossover recombinants, are not a consideration in this system. However, we cannot exclude the possibility of several sequential Cre mediated recombination events leading to tandem plasmid insertions into the vAclox molecule although this arrangement must be very rare as we have not detected a virus with such a structure on the basis of Southern mapping.

A further, and potentially more powerful, advantage of the system we describe lies in the total number of recombinants that we have been able to detect in the population of progeny virus. We have achieved up to 5×10^7 blue (i.e. recombinant) plaques per microgram of input ploxZ DNA. While such levels are not required simply for making recombinant virus to express a protein of interest, the possibility emerges that this system could be used to generate libraries representative of mixed populations of cDNA molecules. The baculovirus/insect cell system is particularly suitable for this use; infection is lytic (and therefore the expression of toxic products does not compromise a healthy cell) and the insect cells are able to carry out complex post-translational modifications. Because of this, this system would be particularly suitable for expression screening of cDNA libraries from higher eukaryotic species. A modification of ploxZ is currently being made to allow direct cloning of directional cDNA molecules. Having identified a viral plaque of interest, we have shown that the insert can simply be recovered as described on a high copy number plasmid for sequencing and sub-cloning. Further modifications are being made to vAclox to select against the non-recombinant virus to further improve the method for construction of libraries. We have shown that inserts can readily be recovered as a high copy number plasmid when Cre mediated recombination occurs between two *lox* sites in the recombinant virus allowing straightforward 'shuttling' between plasmid and virus. The presence in the vector of a M13 origin of repilication allows sequential rounds of mutation, insertion, excision, further mutation and re-insertion. Whilst we have described here an highly efficient system for the generation of recombinant AcNPV, a similar system could be used for other baculoviruses.

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