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Genetics

Site-specific insertion of DNA into a pseudorabies virus vector

(DNA recombination/herpesviruses/glycoprotein/phage P1/Cre–*lox*)

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ABSTRACT **A simple, efficient method for introducing recombinant DNA into a herpesvirus vector and retrieving it at a later time has been developed. By using the Cre–*lox* site-specific recombination system of coliphage P1, DNA can be readily inserted *in vitro* into a pseudorabies virus (PRV) vector containing the *lox* recombination site. The vector PRV42 contains a *lox* site within the nonessential gIII gene, which encodes a virion envelope glycoprotein. Incubation *in vitro* of PRV42 DNA with Cre protein and a circular plasmid containing a *lox* site generates approximately 5% recombinant molecules having the plasmid integrated into the PRV genome at the *lox* site. Transfection of the reaction mixture into cultured cells allows recovery of the infectious recombinant virus, which is readily identified by a nondestructive "black-plaque assay" using a gIII-specific monoclonal antibody. PRV42 plaques stain black when treated with the gIII monoclonal antibody and a peroxidase-linked second anti-antibody because the *lox* site placed within the gIII gene of PRV42 does not destroy the gIII epitope. However, Cre-mediated integration of heterologous DNA at the *lox* site disrupts the gIII epitope so that the resulting recombinant virus produces white plaques. The recombinant virus is infectious, stable, and grows as well as the parental PRV42 vector. The inserted plasmid can be efficiently excised (>50%) from viral DNA by Cre and recovered by transformation of *Escherichia coli*.**

Herpesviruses are large DNA-containing animal viruses that typically have genomes of 120–250 kilobase pairs (kbp). Because of their size, the analysis and molecular manipulation of these large genomes has proceeded more slowly than for smaller viruses. For example, mutations can be made on small *Escherichia coli* plasmids but then must be transferred to the viral genome by special gene replacement techniques. Most commonly, *in vivo* homologous recombination in cultured cells is used to transfer mutations or heterologous DNA to the viral genome (1). Such techniques require that the inserted DNA be flanked by DNA homologous to the target region of the viral genome.

The introduction of heterologous DNA into large DNA genomes could be facilitated by the use of an *in vitro* site-specific recombination system. By requiring only that both the viral genome and the insert-containing plasmid have the recombination site, the need to flank insert DNA with viral homology is obviated. Moreover, site-specific recombination events *in vitro* may well be both more efficient and precise than homologous recombination events that occur after DNA transformation of cultured cells (1, 2). The Cre–*lox* site-specific recombination system of coliphage P1 is an attractive choice on which to base a technique for insertion of recombinant DNA molecules into viral genomes.

Phage P1 encodes an efficient site-specific recombination system consisting of a short DNA sequence called *loxP* and the 38-kDa Cre recombinase protein (3–5). The *loxP* site is a 34-base pair (bp) sequence composed of two 13-bp inverted repeats separated by an asymmetric 8-bp core sequence. Recombination between *loxP* sites (*i*) can occur either intermolecularly or intramolecularly, (*ii*) can occur when the sites are present on either supercoiled or linear DNA, and (*iii*) is independent of the relative orientation of the *loxP* sites on the DNA molecule. Recombination between two directly repeated sites on the same DNA molecule excises the DNA segment lying between the sites. Conversely, Cre can integrate a circular DNA molecule into a second DNA molecule if each contains a *lox* site. Recombination between *lox* sites proceeds efficiently *in vitro* and requires only the Cre recombinase, which is easily purified from appropriate strains of *E. coli* (6).

We have used the Cre–*lox* system to insert heterologous DNA into pseudorabies virus (PRV), an alphaherpesvirus and an important pathogen of swine (7, 8). The genome is a linear 150-kilobase (kb) infectious double-stranded DNA molecule (7). Homologous recombination has been used previously to introduce DNA at the nonessential locus encoding gIII (a virion envelope glycoprotein), which allows the easy detection of recombinant virus by means of a monoclonal antibody and the black-plaque assay (9). We have availed ourselves of this convenient and rapid detection system in conjunction with the Cre–*lox* system to generate a useful PRV vector and a simple, efficient technique for producing recombinant virus containing insertions of heterologous DNA.

MATERIALS AND METHODS

Animal Cells and Viruses. The swine kidney cells (PK15), the Becker strain of PRV (PRV-Be), PRV2, and their culture have been described (9, 10). The recombinant virus PRV42 carrying a functional *lox* site was constructed by gIII gene replacement after cotransfection of PRV2 DNA and *Nco* I-digested pBS109 DNA into PK15 cells as described (9). PRV DNA was prepared from nucleocapsids (11).

Bacterial Strains. *E. coli* NF1829 is F′ *lacI*^{q} *lacZ*::Tn*5*/MC-1000. DH5 Δ*lac* is a derivative of *E. coli* DH1 (12) and was obtained from M. Berman (Bionetics Research, Rockville, MD). Other *E. coli* strains used were HB101 (13) and JM101 (14).

Bacterial Plasmids. All plasmids were constructed and prepared by standard techniques (10, 15). Plasmid pALM3 contains the PRV *Nco* I fragment containing the gene encoding gIII and produces a Cro-gIII fusion protein in *E. coli* under the control of the *tac* promoter (10). Plasmid pBS64 was constructed by inserting the *loxP* site on the 59-bp *Xho* I–*Bam*HI fragment of pBS30 (16) into the large *Sal* I–*Bam*HI fragment of pSP64 (17). This results in a 3.1-kb plasmid containing the *SP6* promoter, a *loxP* site, and several unique restriction sites: *Hin*dIII, *Pst* I, *Bam*HI, *Sma* I, *Sac* I, and *Eco*RI.

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Abbreviation: PRV, pseudorabies virus.

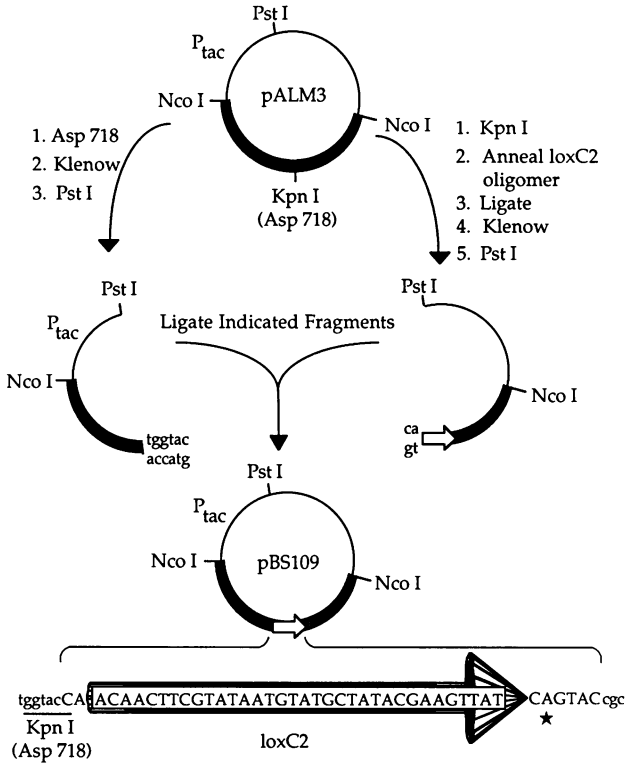

FIG. 1. Construction of the gIII-*loxC2* gene. Details of the construction are given in *Materials and Methods*. The *loxC2* site and its orientation are represented by the large arrow encasing its sequence. The synthetic oligomer is represented by the sequence in uppercase letters. DNA sequencing of the *lox*-containing region of pBS109 revealed that the adenosine indicated by the star in the predicted fusion sequence had been deleted.

The construction of plasmid pBS109 is diagrammed in Fig. 1. A 42-nucleotide single-stranded oligomer of the indicated sequence (synthesized by Ellen Doran, DuPont) was used to insert the *loxC2* site into the gIII gene. The 3′ end of this oligonucleotide hybridizes to a *Kpn* I-generated DNA end but does not reform a *Kpn* I site. Plasmid pALM3 was digested with *Kpn* I, leaving a 3′ DNA overhang. The oligonucleotide was hybridized to the *Kpn* I-digested pALM3 DNA and then treated with the Klenow fragment of DNA polymerase I to create a blunt end. In a parallel experiment, pALM3 was digested with *Asp*718, which recognizes the same DNA sequence as *Kpn* I (GGTACC) but which leaves a 5′ protruding end, and was similarly treated with the Klenow fragment to fill in the ends. Both DNAs were then cut at the unique *Pst* I site in the β-lactamase gene, and the appropriate fragments were purified and ligated to produce pBS109. Proper joining of the blunt ends predicts the reconstruction of a *Kpn* I site 5′ to the inserted oligomer. This was verified by digestion with *Kpn* I and also by sequence analysis after subcloning into phage M13 (14, 18). The *loxC2* oligomer was also inserted into the gIII gene in the opposite orientation at the *Kpn* I site to produce plasmid pBS110. This orientation generates a termination codon in the reading frame of the gIII gene at the *loxC2* site.

Analysis of Cro-gIII Fusion Proteins in *E. coli*. The Cro-gIII fusion protein is produced in *E. coli* under the control of the *tac* promoter from pALM3 and pBS109 as an insoluble aggregate or inclusion body (9). Its production was induced with isopropyl β-D-thiogalactopyranoside, and the aggregate protein was purified (9) prior to analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (19).

Cre-Mediated Recombination *in Vitro*. Conditions for Cre-mediated recombination *in vitro* were similar to those described in ref. 6. Briefly, 2 μg of DNA was incubated at 30°C for 30 min in the presence of 50 mM Tris·HCl, pH 7.5/33 mM NaCl/10 mM $MgCl_2$ and 40 ng of purified Cre protein (a gift from Ken Abremski, DuPont). In the case of intermolecular recombination, the complete reaction also included 1.7% polyvinyl alcohol to facilitate the recombination, presumably by an excluded volume effect (20). The reactions were stopped by heating the samples at 70°C for 5 min to inactivate Cre. The DNA was then used for transfection of PK15 cells or was digested with the appropriate restriction enzyme. DNA samples were extracted with 1 vol of chloroform/isoamyl alcohol, 24:1 (vol/vol), before electrophoresis. The extent of recombination *in vitro* was determined by electrophoresis of a 10-fold range of dilutions of the reaction mixture and comparison of the intensity of the indicated ethidium bromide-stained recombinant band to that of the molar, nonrecombinant 13.2-kb PRV *Bgl* II F fragment. Values obtained after densitometry were corrected for differences in molecular mass between the two fragments. Topoisomerase I (Bethesda Research Laboratories) was used to generate a relaxed pBS64 marker for gel electrophoresis.

Detection of Recombinant Virus. The black-plaque assay was used to detect virus having an altered gIII protein (9, 21, 22). This technique uses the M1 monoclonal antibody to gIII (23) and a horseradish peroxidase-linked second antibody to detect the M1 antigen. M1 monoclonal antibody was a gift of Harmut Hampl (Free University of Berlin) and Tamar Ben-Porat (Vanderbilt University).

RESULTS

Insertion of a Functional *lox* Site into the PRV gIII Gene. A *lox* site was inserted into the PRV gIII gene present on the *E. coli* expression plasmid pALM3 and then transferred to the PRV genome by gene replacement at the gIII locus. A 42-nucleotide oligomer was synthesized containing the 34-nucleotide *loxC2* site shown in Fig. 1. This site, enclosed by the large arrow to show orientation, differs from wild-type *loxP* at a single position: the second base of the *lox* sequence has been changed from a thymidine to a cystidine. This alteration changes a potential TAA ochre termination signal to a glutamine codon. The *loxC2* site was shown to be functional by a recombination assay performed *in vitro* (data not shown), as expected from previous work with various mutants of *loxP* (24). The resulting *loxC2*-containing plasmid was designated pBS109 (Fig. 1).

pBS109 Expresses a Fusion Protein in *E. coli*. The in-frame insertion of *loxC2* into the gIII gene predicts the production of a protein similar in size to the 479-amino-acid primary translation product (Fig. 1). Support for this prediction is presented in Fig. 2. After induction, the parental pALM3 made a gIII protein with an apparent molecular mass of 57 kDa (lanes 1 and 2) as shown previously (9). The protein expressed by pBS109 after induction (lane 3) was similar in size to that expressed from pALM3. Insertion of a nonsense codon at the *Kpn* I site (plasmid pBS110) produced a truncated protein with an apparent molecular mass of 46 kDa, thus confirming our identification of these aggregate proteins as gIII. However, subsequent sequencing of the insert in pBS109 revealed that the adenosine indicated by a star in Fig. 1 had been deleted, causing a −1 frameshift. Fortuitously, this frameshift resulted in the production of an open reading frame of 456 amino acids that terminates near the authentic gIII termination codon. Thus, the gIII-*loxC2* fusion produces a gIII-sized protein containing the first 343 amino acids of gIII.

Transfer of the gIII-*loxC2* Gene to PRV. Transfer of the gIII-*loxC2* gene from pBS109 to PRV was facilitated by knowledge that the product of the wild-type (PRV-Be) gIII gene is a major constituent of the viral envelope and is also

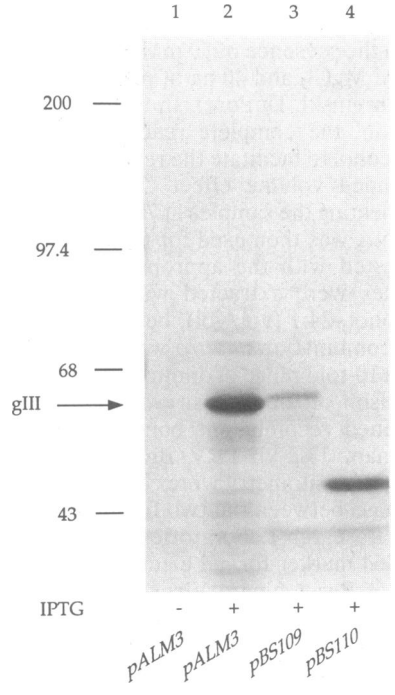

FIG. 2. Synthesis of the Cro-gIII fusion protein in *E. coli*. Aggregate protein preparations were electrophoresed on an 8% polyacrylamide gel containing sodium dodecyl sulfate. The arrow indicates the position of the Cro-gIII fusion protein from pALM3. Protein molecular mass markers (in kilodaltons) are shown. Lanes: 1, uninduced pALM3; 2, induced pALM3, 3, induced pBS109; 4, induced pBS110.

present on the surface of infected cells. Consequently, PRV-Be makes a black plaque when stained first with the M1 monoclonal antibody and then with a horseradish peroxidase-linked second antibody. The mutant virus PRV2 contains a 402-bp deletion within the coding sequence of the gIII gene (5′ to but not including the *Kpn* I site). In the black-plaque assay, PRV2 plaques remained white (9) because the M1 antibody did not react with the mutant PRV2 gIII protein, although the protein was synthesized. Because virus carrying an insertional fusion of an *Eco*RI linker at the *Kpn* I site made a black plaque with the M1 monoclonal antibody (unpublished data), we anticipated that PRV containing the gIII-*loxC2* gene would also make a black plaque.

PRV2 DNA and an *Nco* I digest of pBS109 were cotransfected into PK15 cells and a black-plaque assay was performed with the resulting plate stock. Should the *loxC2* insertion not destroy the M1 epitope, then PRV gIII-*locC2* recombinants arising by homologous recombination with PRV2 would exhibit a black-plaque phenotype. This was exactly the result obtained. Of 1000 plaques screened, 5 were black. No black plaques were obtained after screening 1000 plaques when pBS109 DNA was omitted. The presence of a *lox* site was verified in each of three plaque-purified isolates by Cre-mediated recombination of viral DNA *in vitro* (as is shown for one such isolate in the next section). One isolate was selected for further study and was named PRV42.

Site-Specific Insertion *in Vitro* of Plasmid DNA into PRV42 at the *loxC2* Site. Cre-mediated recombination between the *lox* site on the linear PRV42 genome and the *loxP* site on a circular plasmid, pBS64, should result in the integration of the plasmid into the PRV genome (Fig. 3). Cre reactions with PRV42 DNA and pBS64 were performed, and the products of the reaction were digested with *Eco*RI, which cuts pBS64 once but does not cleave the PRV genome, and with *Bgl* II, which does not cut pBS64 but does cleave the PRV genome five times (7). Digests were then analyzed by gel electrophoresis (Fig. 3 *Upper A*). The PRV gIII gene lies within the 33.9-kb *Bgl* II B fragment: integration of pBS64 and its accompanying *Eco*RI site predicts the formation of two *Bgl* II–*Eco*RI fragments of 20.6 kb and 16.5 kb. The amount produced was directly proportional to the amount of integrative recombination. These fragments were only produced after incubation with the Cre protein (Fig. 3 *Upper A*, lane 1). When polyvinylalcohol, which promotes intermolecular recombination by an excluded volume effect (20), was omitted from the reaction, there was a slight decrease in recombination (lane 2); omission of either the Cre protein (lane 3) or pBS64 (lane 4) prevented formation of the two *Bgl* II–*Eco* RI fragments, as did the substitution of PRV-Be DNA for PRV42 (lane 5). Thus, PRV42 contains a functional *lox* site, and Cre can insert pBS64 into the PRV42 genome.

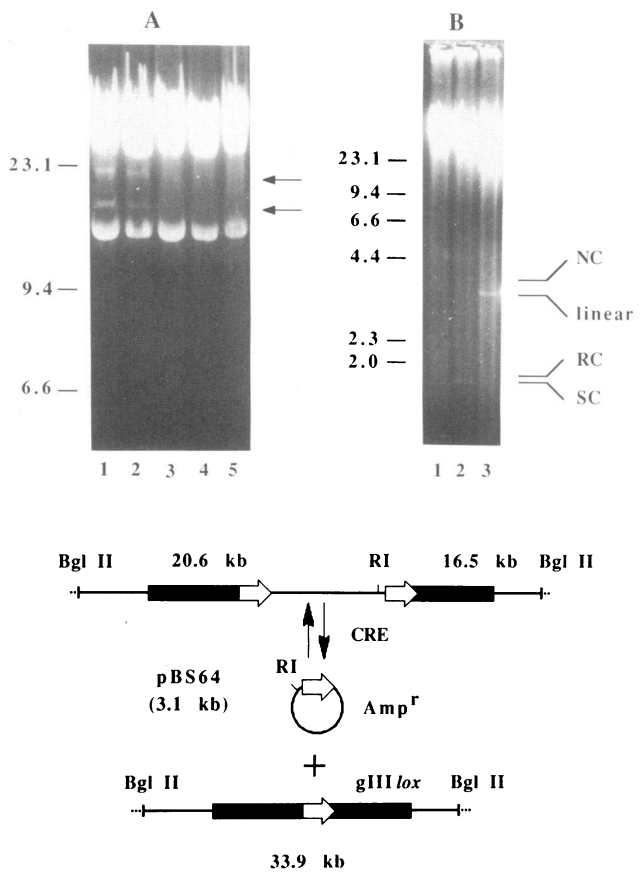

FIG. 3. Cre-mediated recombination *in vitro* with the PRV genome. (*Lower*) The ability of the Cre protein to catalyze the integration of the circular plasmid pBS64 into a linear molecule containing a *lox* site in the gIII gene is diagrammed. Similarly, Cre can catalyze excision of a circular molecule from a *lox*[2] substrate. (*Upper A*) Integration of pBS64 into PRV42. Arrows indicate restriction fragments arising from recombination. The band below them is the 13.2-kb *Bgl* II F fragment of PRV. Cre reactions containing 2.5 μg of PRV DNA and 0.25 μg of pBS64 were analyzed on a 0.7% agarose gel containing 0.5 μg of ethidium bromide per ml (15) after digestion with *Bgl* II and *Eco*RI. Lanes 1, the complete reaction with PRV42; 2, PVA omitted; 3, Cre omitted; 4, pBS64 omitted; 5, PRV-Be DNA substituted for PRV42. (*Upper B*) Excision of pBS64 from PRV42::pBS64. Cre reactions were performed with 1 μg of PRV42::pBS64 DNA and analyzed on a 0.7% agarose gel containing 0.5 μg of ethidium bromide per ml. Lanes: 1, Cre protein omitted; 2, the complete reaction; 3, same as for lane 2 but digested with *Bgl* II and *Eco*RI. Linearized (by *Eco*RI), supercoiled, nicked, and relaxed pBS64 species were run in parallel on the same gel, and the mobilities of these species are indicated by lin, SC, NC and RC, respectively.

The recombinant PRV genomes formed *in vitro* have a disruption of the nonessential gIII gene. To determine both

Table 1. Insertion of plasmid DNA into PRV

Reaction component	Infectious centers, no.		Virus stock, no.		Recombinant virus, %	DNA recombination *in vitro*, %
	White	Black	White	Black		
With PRV-Be	0	7500	0	11,000	0	0
With PRV42	1	960	116	4,312	2.6	8.1
Without PVA	0	4200	61	5,039	1.2	5.0
Without Cre	0	6500	0	4,300	0	0
Without pBS64	0	7700	0	5,300	0	0

Cre-mediated recombination *in vitro* was performed as described in Fig. 3 or with the indicated component of the reaction omitted. Two micrograms of PRV DNA from the indicated reaction was transfected into PK15 cells either for the formation and detection of infective centers or for the preparation of a plate stock. Virus from the plate stock was then plaqued on PK15 cells. Both the infectious centers and plaques derived from plate stocks were subjected to the black-plaque assay. The amount of DNA recombination *in vitro* was determined by measuring the amount of the 16.5-kb *Bgl* II–*Eco*RI fragment in each reaction in comparison to the 13.2-kb *Bgl* II F fragment of PRV as shown in Fig. 3.

the gIII phenotype and the infectivity of these molecules, DNA from the recombination experiments described in Fig. 3 was transfected into PK15 cells. Both infectious centers and progeny virus were screened by the black-plaque assay (Table 1). The site in pBS64 is immediately followed by the amber codon TAG in the gIII reading frame so that integration of pBS64 should result in the production of a truncated gIII protein. Although PRV42 makes a black plaque, the disruption of the gIII gene after Cre recombination would likely destroy the M1 epitope and generate a virus that makes white plaques. Table 1 indicates that this in fact occurred. White-plaque-forming progeny virus were present only in stocks derived from Cre reactions in which recombination had taken place. The percentage of white plaques in the viral stocks was roughly proportional to the amount of recombination detected *in vitro*. The apparently low frequency of white-plaque-forming viruses in the infectious center assay compared to that observed in the progeny virus suggests that during DNA transfection individual cells take up multiple copies of PRV DNA (some white, some black). The presence of white-plaque-forming virus in the resulting mixed plaques was obscured by the preponderance of parental black-plaque-forming virus. Generally, we observed that the production of recombinant virus mirrored the amount of recombination detected *in vitro*, although some variability was seen. This may be due to the process of transfection, as there was little or no demonstrable growth advantage of PRV42 parental virus over the recombinant virus (unpublished observations). Moreover, the experiment presented represents the lowest frequency of recombinant virus that we have observed. More typically recombinant virus were obtained at a frequency of 5–15%.

Determination of the genomic structure of four plaque-purified isolates showed that the white-plaque-forming viruses are the expected recombinants. Viral DNA derived from each virus was digested with *Eco*RI and *Bgl* II. All four isolates produced the diagnostic restriction fragments described above. One such isolate was selected for further analysis and named PRV42::pBS64.

Precise Excision of Plasmid DNA from PRV42::pBS64. The anticipated structure of the gIII gene region of PRV42::pBS64 is shown in Fig. 3 *Lower*. Plasmid pBS64 sequences in PRV42::pBS64 are flanked by directly repeated *lox* sites. This structure predicts that Cre-mediated recombination *in vitro* should precisely excise the plasmid pBS64 from the linear PRV42::pBS64 genome as a relaxed circle (4). This is shown in Fig. 3 *Upper B*. Treatment of PRV42::pBS64 DNA with Cre *in vitro* produced a new DNA molecule (lane 2) that comigrated with relaxed pBS64 DNA. Production of this new molecule was dependent upon the Cre protein, as omission of Cre prevented its production (lane 1). The identity of this molecule was confirmed by digesting Cre-treated PRV42::pBS64 DNA with *Bgl* II and *Eco*RI (lane 3). The mobility of the new molecule shifted to that of linear pBS64.

Cre-mediated excision of pBS64 from PRV42::pBS64 DNA also generated a linear PRV molecule, which should be identical to the parental PRV42. In particular, such molecules should give rise to virus that makes a black plaque when treated with the M1 monoclonal antibody. Therefore, PRV42::pBS64 DNA was treated with Cre *in vitro* and then transfected into PK15 cells. The resulting virus was subjected to the black-plaque assay (Table 2). Black-plaque-forming (recombinant) virus was produced by Cre at a frequency of >50%, nearly identical to the amount of recombination that had occurred *in vitro*. The recombination event was completely dependent on the Cre protein. In the absence of Cre, PRV42::pBS64 exhibited a stable white-plaque phenotype. This shows that the Cre-mediated insertion and excision of DNA occurred precisely at the *lox* sites. The experiment also shows that homologous recombination between the directly repeated 34-bp *lox* sites (but containing a 1-bp mismatch) did not readily occur in mammalian cells. Indeed, we observed

Table 2. Cre-mediated excision of plasmid DNA from PRV

Reaction component(s)	Virus stock, no.		Recombinant virus, %	DNA recombination *in vitro*, %	*E. coli* transformants, no.*
	Black	White			
PRV42::pBS64	0	1600	0	0	1
PRV42::pBS64/Cre	1794	1468	56	57	434

The indicated PRV DNA was treated *in vitro* as described in Fig. 3 *Upper B*. PK15 cells were transfected with 1 μg of DNA to prepare a virus stock that then was analyzed by the black-plaque assay. The amount of DNA recombination *in vitro* was determined by measuring the amount of the linear pBS64 excised in comparison to the 13.2-kb *Bgl* II F fragment of PRV as shown in Fig. 3 *Upper B*. *E. coli* HB101 was transformed with 100 ng of the indicated PRV42::pBS64 DNA, which corresponds to 1.2 ng of excised DNA sequences containing pBS64. In a parallel experiment 1 ng of purified pBS64 DNA gave 1200 colonies either in the presence or absence of exogenous PRV DNA.
*Number of ampicillin-resistant transformants with 100 ng of indicated DNA.

no black plaques in our stocks of PRV42::pBS64 (frequency of $<10^{-5}$).

Table 2 also shows that the excised pBS64 DNA transformed *E. coli* HB101 with good efficiency. From the experiment shown in Table 2 and from other experiments (not shown), we estimate that the excised plasmid in the reaction mixture transforms at an efficiency of 20–50% of that of purified supercoiled plasmid. The origin of the single colony obtained from non-Cre-reacted PRV DNA is not understood, although it is clear that the plasmid obtained is not pBS64 (data not shown).

DISCUSSION

We have constructed a viral cloning vector using pseudorabies virus, into which heterologous DNA can be efficiently inserted *in vitro* by using the Cre–*lox* site-specific recombination system of phage P1. Approximately 5% recombinant virus is produced after preparation of a viral stock. Because the recombinant virus produces a distinctive white plaque, recombinants are easily distinguished from parental (black) plaques. The procedure is simple and easy to perform, requiring only the Cre protein, which is readily obtained (6). Moreover, efficient recombination *in vitro* allows analysis of the recombinants prior to their introduction into the cell. In contrast, methods using homologous recombination to insert heterologous DNA into herpesvirus genomes vary widely in efficiency because recombination must occur *in vivo*. One apparent requirement for high recombination is that the insert DNA be embedded in long stretches of viral homology. An important feature of Cre-generated recombinants is that they result from a well-characterized site-specific *in vitro* recombination event and not from poorly understood recombinational processes in the cell that can result in accompanying DNA rearrangements.

The method has a number of additional attractive features. The fragment of DNA to be inserted into the viral vector needs only to be circular and to contain a *lox* site. Therefore, it is convenient to insert an entire *E. coli* plasmid into the viral vector. In fact, we have used the small (3.1 kb) plasmid pBS64, which contains a number of unique restriction sites and the *SP6* promoter. DNA fragments of interest can be introduced easily into pBS64 by standard cloning procedures and then transferred to PRV42 by incubation *in vitro* with the Cre protein. The size limit of the insert to be transferred to PRV is likely to be that imposed by viral packaging. Preliminary experiments indicate that PRV42 can accept an insert of 10 kb (B.S. and N. Lomax, unpublished data). By placing *lox* sites at various locations in the PRV genome, in principle the same plasmid DNA cassette could be inserted at any of several PRV locations.

The recombinant virus produced by the method appears to be quite stable. Homologous recombination between the two 34-bp *lox* sites flanking the insert in PRV42::pBS64 occurs at a frequency of $<10^{-5}$ in a virus stock. Such events are easily detected because they would produce a black plaque.

A particularly useful feature of the system is that the insert DNA can be efficiently excised from PRV DNA and retrieved for analysis by simply treating viral DNA with Cre protein *in vitro* to release a circular plasmid capable of transforming *E. coli*. Thus, rare events that may have occurred to the insert DNA during its sojourn in a mammalian cell or a whole animal could be easily monitored. Because PRV-based recombinant viral vaccines may prove to be of some utility, the ability to retrieve and analyze inserted DNA cassettes expressing various antigens in such recombinant viruses would be of considerable advantage in assessing the authenticity of the vaccine preparation.

The *lox* site could also serve as a convenient joint between the gIII gene and another (inserted) coding sequence to generate a novel fusion protein expressed under the control of the PRV gIII promoter. Thus, the use of the *lox* site could be exploited to generate a family of *lox* fusion cassettes, which would facilitate the determination of the relative contributions of each protein component in a series of fusion proteins.

PRV *lox* viruses such as PRV42 may be useful as shuttle vectors for the introduction of DNA into mammalian cells, especially because of the broad host range of the herpesviruses (7). It is clear that the generation and identification of insert-containing recombinant pseudorabies virus in a few days is greatly facilitated by use of the Cre–*lox* site-specific recombination system and the black-plaque assay.

Our results suggest that the adaptation of the Cre–*lox* system, which we have described for PRV, could be used to insert heterologous DNA into any viral DNA that is infectious and, thus, would be of considerable utility in the construction and analysis of a variety of recombinant viral genomes.

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